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On the origin of anthocyanin methyltransferase isozymes of *Petunia hybrida* and their role in regulation of anthocyanin methylation

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Summary. Four genes controlling anthocyanin methylation in flowers of *Petunia hybrida* have been described. Three of them, *Mt2*, *Mf1* and *Mf2*, caused a dosage effect on anthocyanin methyltransferase activity and degree of methylation of anthocyanins. Antiserum raised against partially purified Mf2-enzyme precipitated three of the four anthocyanin methyltransferases. In two subspecies of one of the ancestral species of *P. hybrida*: *Petunia integrifolia*, different anthocyanin methyltransferases were found as determined by immunoprecipitation. The methyltransferase isozymes showed no differences in subcellular or tissue location, and had no physiologically important difference in time course of activity during bud development. The methylation-system in *Petunia* is discussed with regard to anthocyanin methylation in other plant species.

Key words: *Petunia hybrida* – Anthocyanin methyltransferases – Gene-dosage effect – Isozymes

Introduction

Plant isozymes are defined as multiple forms of one enzyme, arising from genetically determined differences in primary structure [1].

In studies of the flavonoid biosynthetic pathway, one system of isozymes has become known: that of the anthocyanin methyltransferases, as yet only demonstrated in flowers of *Petunia hybrida*. The methylation of the B-ring is the very last step of anthocyanin modification in *Petunia hybrida* (Wiering 1974; Jonsson et al. 1982). On the basis of genetic studies the existence of four genes related to anthocyanin methylation has been postulated: *Mt1* and *Mf1*, located on chromosome III and *Mt2* and *Mf2*, located on chromosome V (Wiering 1974; Wiering and de Vlaming 1977; Maizonnier and Moessner 1979; Cornu et al. 1980). Flowers of plants which are dominant for either of the genes *Mf1* or *Mf2* accumulate malvidin-pigment, carrying two methyl groups at the 3′,5′-positions. When these genes are homozygous recessive and either of the genes *Mt1* or *Mt2* is dominant, petunidin with one methyl group at position 3′ is the major pigment. Some *Petunias* are unable to synthesize delphinidin and thus have only one hydroxyl group in the B-ring available for methylation. In these plants the cyanidin-pigment occurs in the methylated peonidin-form if any one of the methylation genes is present with a dominant allele (Jonsson et al. 1983a). This is illustrated in Fig. 1. Biochemical studies have shown that at least four different methyltransferases occur in *Petunia*. These enzymes are all able to methylate the 3-(p-coumaroyl)-rutinoside-5-glucoside derivatives of anthocyanidins and each one appeared to be controlled by one of the methylation genes (Jonsson et al. 1984a).

The present paper deals with the question of the origin of the isozymes and their role in the regulation of anthocyanin methylation. In a previous report we showed indirect evidence that the methylation genes are structural genes (Jonsson et al. 1984a). The present study adds substantial evidence to this assumption by giving data on the relation between gene-dosage and enzyme activity. As a first step to study the divergence between the methylation genes, we investigated structural similarities between the four isozymes using an immunological approach. Since there might exist a relation between the methyltransferase system and the hybrid character of *Petunia hybrida* we also studied the methyltransferases in two subspecies of an ancestor to *P. hybrida*. On the basis of our results and data from the literature we discuss the possible existence of anthocyanin methyltransferase isozymes in other plant species.

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[1] See IUPAC-IUB Recommendations. Nomenclature of multiple forms of enzymes. Eur J Biochem 82:1–3 (1978)

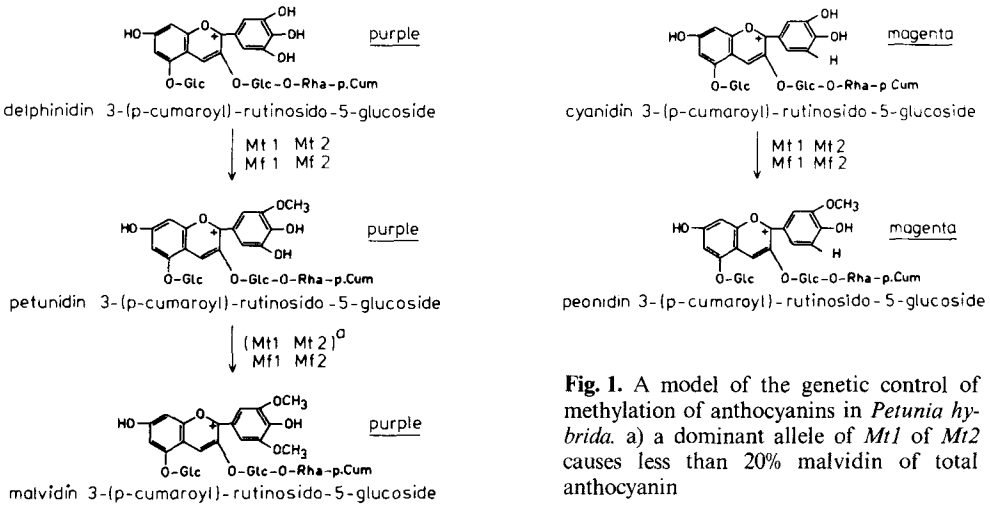

Fig. 1. A model of the genetic control of methylation of anthocyanins in *Petunia hybrida*. a) a dominant allele of *Mt1* of *Mt2* causes less than 20% malvidin of total anthocyanin

Materials and methods

Plant material

Several inbred lines of the *Petunia* collection of the Institute of Genetics, University of Amsterdam were used. S6 (*P. integrifolia* ssp. *inflata* from Dr. K. C. Sink), S12 (*P. integrifolia* s.s.) and S2 (*P. axillaris* s.s.) were used as representatives of ancestral species of *P. hybrida* hort. The genotypes of the inbred lines with regard to the methylation genes are shown in Table 1. The plants were grown in a greenhouse.

Enzyme extraction and chromatofocusing of enzyme

Standard enzyme extracts were prepared as described before (Jonsson et al. 1983a). In chromatofocusing experiments, proteins were precipitated from the supernatant obtained after centrifugation of the crude homogenate by ammonium sulphate fractionation at 0–4 °C. The supernatant was brought to 80% saturation by the addition of solid $(NH_4)_2SO_4$ over a period of 20 min, during which the pH of the solution was maintained at pH 7.3 to 7.6 by adding drops of 1 M KOH. The suspension was centrifuged at 38,000 g for 20 min and the resulting pellet was resuspended in 25 mM histidine-HCl pH 6.2. After recentrifugation under identical conditions to precipitate undissolved material, the final supernatant was applied to a Sephadex G-25 column (pre-equilibrated and eluted with 25 mM histidine-HCl pH 6.2) to remove residual $(NH_4)_2SO_4$. The protein fractions were then applied to a PBE 94 (Pharmacia, Uppsala, Sweden) chromatofocusing column (0.55 × 17 cm), which had been pre-equilibrated with 25 mM histidine-HCl pH 6.2. The column was eluted with Polybuffer 74 (Pharmacia) pH 4.0 at a flow rate of 35 ml/h. In view of the instability of the methyltransferases in acidic media, fractions (2.0 ml) were collected in tubes containing 0.1 ml 2 M potassium phosphate pH 8.1.

Partial purification of Mf2-anthocyanin methyltransferase

The M48-mutant, which contains Mf2- and Mt2-methyltransferases, was used as a source of enzyme. All steps were performed at 0–4 °C. For one isolation 20 to 30 g (fresh weight) petals of flowers and flower buds were homogenized in a mortar with some quartz sand and 40 to 70 ml buffer (100 mM potassium phosphate pH 8.5, containing 1.4 mM β-mercaptoethanol). During homogenization Dowex 1X2-200 (Sigma, St. Louis, USA) was added at half the amount of the fresh weight of the tissue. The homogenate was centrifuged at 38,000 g for 20 min. A second extraction of the pellet was carried out using 20 ml buffer and the homogenate was re-centrifuged (38,000 g, 20 min). The supernatants were combined and brought to 80% saturation by adding solid $(NH_4)_2SO_4$ as described in the preceding section. The suspension was centrifuged at 38,000 g for 20 min and the resulting pellet was resuspended in 10 mM potassium phosphate pH 7.5. After re-centrifugation under identical conditions, the final supernatant was dialyzed against 10 mM potassium phosphate pH 7.5 for 16 h. The protein sample was subsequently applied to a DEAE-cellulose column (2.4 × 6.5 cm), pre-equilibrated with 10 mM potassium phos-

Table 1. Genotypes of inbred lines of *Petunia hybrida hort.*, *P. axillaris*[1] and *P. integrifolia*[2]

Line	Genes			
	Mt1	*Mt2*	*Mf1*	*Mf2*
R27	0		X	
R78	–	–	–	–
M30	–	–	–	–
M43	–	+	–	–
M48	–	+	–	+
M73	+	–	+	–
V2	0		–	+
V10	0		+	+
V12	0		+	+
V14	0		+	–
V32	–	–	–	–
V33	–	+	–	–
S2[1]	X		–	–
S6[2]	0		X	
S12[2]	0		X	

+ = dominant, – = homozygous recessive, X = dominant for one or both genes, 0 = unknown

phate pH 7.5. After washing off all unbound protein, the bound protein was eluted with 300 ml of a gradient of 0 to 250 mM KCl in 10 mM potassium phosphate pH 7.5. Only one peak containing methyltransferase activity was obtained. This peak was pooled and kept at −20 °C. The procedure was repeated and enzyme preparations from three separate isolations were pooled and dialyzed against 25 mM histidine-HCl pH 6.2 and thereafter subjected to chromatofocusing on a PBE 94 column as described in the preceding section. Again, one peak of methyltransferase activity was obtained, which was collected and concentrated using an ultrafiltration cell (Amicon, Lexington, USA), provided with a PM-10 membrane. Finally, the enzyme was lyophilized.

Preparation of antiserum

Mf2-methyltransferase was partially purified as described above. Five hundred µg lyophilized protein was dissolved in 1 ml phosphate-buffered saline (pH 7.4), mixed with 1 ml complete Freund's adjuvant and injected intramuscularly in a rabbit at four different sites. Booster injections with an identical enzyme preparation, mixed with 1 ml incomplete Freund's adjuvant were given two, four, six and ten weeks later. After one more week the rabbit was bled (50 ml). Complement was inactivated by incubation of the antiserum at 56 °C for 30 min.

Incubations of enzyme with anti-Mf2 serum immobilized to Protein A-Sepharose CL-4B

The coupling of anti-Mf2 to Protein A-Sepharose CL-4B (Pharmacia) was carried out at 20 °C for 2 h, with gentle rotation in Eppendorf tubes. To each tube, containing 7.5 mg Protein A-Sepharose CL-4B, 1.0, 2.5, 5.0 or 10 mg antiserum protein in 250 µl phosphate-buffered saline (pH 7.4) were added. A parallel series of incubations contained corresponding amounts of normal rabbit serum protein. The control contained Protein A-Sepharose CL-4B incubated with phosphate-buffered saline pH 7.4. The gel was washed three times with phosphate-buffered saline (pH 7.4) by mixing on a Vortex and centrifugation (10 s, 10,000 g), and thereafter incubated with enzyme sample. After 1 h incubation at 20 °C with gentle rotation, the gel was centrifuged (10 s, 10,000 g) and the enzyme activity in the supernatant was determined. In some experiments pre-incubations with enzyme-extracts were carried out. These were performed as routine enzyme incubations, followed by three washes as described above.

Methyltransferase assay

Routine methyltransferase assays were performed as described before with cyanidin 3-(p-coumaryl)-rutinoside-5-glucoside as anthocyanin substrate (Jonsson et al. 1983a).

Protein was determined according to Bradford (1976) with bovine serum albumin as standard protein.

Analyses of anthocyanin

Anthocyanins were extracted from one piece (diameter 12 mm) of each of the five petals of mature flowers. For determination of anthocyanin composition in upper and lower epidermis, epidermal layers were peeled off. The tissue was extracted during 16 h at −20 °C in methanol-0.5% HCl (v/v). Water and chloroform was added to obtain a Folch-partition (Folch et al. 1957). Part of the upper phase was injected into the high performance liquid chromatograph and analyzed as described before (Jonsson et al. 1982).

Results and discussion

Gene-dosage effect on methyltransferase activity

A gene that causes a dosage effect on an enzyme activity is usually considered to be the structural gene for the given enzyme. Such a relationship has been demonstrated with two genes of the flavonoid biosynthetic pathway in maize: *Bz* and UDP-glucose:flavonoid 3-O-glucosyltransferase (Larson and Coe 1977) and *C2* and chalcone synthase (Dooner 1983). In spite of the dosage effect of these genes on enzyme activity no effect on product level has been reported. However, a gene-dosage effect on product level appeared to exist in *Petunia* (Jonsson et al. 1983a). It seemed that the relative amount of malvidin-pigment was higher in flowers of plants which were homozygous dominant for any one of the *Mf*-genes than plants heterozygous for the same gene. These observations called for an investigation of enzyme activity. If our assumption is right that the methylation genes are structural genes for methyltransferases, we would expect a dosage effect on enzyme activity.

Four crosses were set up. Each cross consisted of a combination with one parent homozygous dominant and the other homozygous recessive for one of the methylation genes. Anthocyanin composition and methyltransferase activity were determined in samples of flowers and flower buds, respectively, of the parents and the F1's of these crosses. The results are shown in Table 2. In earlier studies of *Petunia* anthocyanidin composition was analyzed after hydrolysis of anthocyanins, subsequent concentration of anthocyanidins

Table 2. Gene-dosage effect on methyltransferase activity and degree of anthocyanin methylation. Each figure represents one determination using 25 flowers (anthocyanins), or flower buds (enzyme activity) from different plants with the indicated genotype

Genotype[a]		Specific activity (pkat/mg protein)	Degree of methylation ratio *cyanidin:peonidin or delphinidin:petunidin:malvidin
M30	*mt2mt2*	< 0.5	*100:0
M43	*Mt2Mt2*	50	*28:72
M43×M30:F1	*Mt2mt2*	33	*38:62
V32	*mt2mt2*	< 0.5	100:0:0
V33	*Mt2Mt2*	38	21:61:18
V33×V32:F1	*Mt2mt2*	18	40:60:0
V14	*Mf1Mf1*	195	4:39:57
V14×V33:F1	*Mf1mf1*	125	6:44:50
V2	*Mf2Mf2*	93	9:46:45
V2×V33:F1	*Mf2mf2*	55	13:49:38

[a] Only the segregating gene is shown. See Table 1 for a complete description of the genotype with regard to the methylating genes

using iso-amyl alcohol and separation by means of paper chromatography (Wiering and de Vlaming 1977; Jonsson et al. 1983a). In the present investigation, the anthocyanin composition was directly analyzed using high performance liquid chromatography. The methods did not give identical results because of differences in iso-amyl alcohol solubility between the anthocyanidins. Nevertheless, earlier findings were confirmed inasmuch as a gene-dosage effect was observed on product level. Moreover, a dominant allele of *Mf1* was again correlated with relatively more malvidin-pigment than a dominant allele of *Mf2* (Jonsson et al. 1983a).

In all crosses a doubled gene-dosage resulted in increased methyltransferase activity (Table 2), supporting the assumption that the genes *Mt2*, *Mf1* and *Mf2* are structural genes for methyltransferases. Similar data with reference to *Mt1* are as yet not available, because of lack of a suitable genotype (*Mt1Mt1mt2mt2mf1mf1mf2mf2*). Only in one of the crosses did we find an arithmetic relation between the doubled gene-dosage and the increase in enzyme activity. In the first place, the methyltransferase activity varied during bud development. Although buds of the same length (25 to 35 mm from the basis of the flower receptacle to the top of the corolla) were used as source of enzyme, the samples were not completely identical. Secondly, in the crosses involving the *Mf*-genes, one of the parents was V33. This line is dominant for one *Mt*-gene and consequently gave a contribution of Mt-activity in the F1's. The lines V2 and V14 themselves have not been characterized with regard to the *Mt*-genes (see Table 1).

The analyses of anthocyanins showed that increased methyltransferase activity caused a higher degree of methylation (Table 2). This indicates that the amount of enzyme might be a limiting factor in the methylation process.

Structural similarities between methyltransferase isozymes

Partial purification of Mf2-methyltransferase. In order to study the structural relationship between the four different anthocyanin methyltransferases by way of immunoprecipitation, partial purification of the Mf2-enzyme was carried out and the resulting preparation was used to immunize a rabbit. In Table 3 a representative summary of the purification procedure is given. Because of the lability of the methyltransferase we did not attempt to purify the enzyme to homogeneity. Although the pooled fractions contained all detectable methyltransferase activity, at each purification step a considerable loss of activity was observed. The final recovery of activity was only 3% and a 3-fold overall increase in specific activity was achieved. An important aim of the purification was to obtain a separation between the Mt2-enzyme and the Mf2-enzyme, which are both present in a crude extract of M48-flowers.

Table 3. Partial purification of Mf2-methyltransferase

Fraction	Protein (mg)	Activity (pkat)	Specific activity (pkat/mg protein)
Crude extract	206	16,480	80
$(NH_4)_2SO_4$-precipitate	176	3,344	19
DEAE-cellulose	12	1,164	97
PBE 94	2	458	229

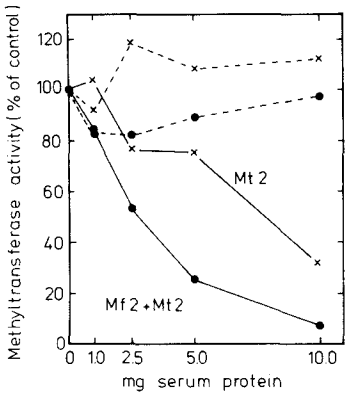

Fig. 2. Effect of incubation with anti-Mf2 serum (——) and normal rabbit serum (- - -) on methyltransferase activity. (●–●–●) = M48-extract (Mf2-enzyme and Mt2-enzyme); (×-×-×) = M43-extract (Mt2-enzyme). In both cases, the samples contained 15 pkat activity

In an earlier report we showed that these isozymes have different iso-electric points and can be separated by means of chromatofocusing (Jonsson et al. 1984a). The Mt2-enzyme is even more labile than the Mf2-enzyme and during this purification procedure it was not observed as a distinct peak in the fractions from the DEAE-cellulose column or the PBE 94-column. We assume that the activity was lost during the first step of purification and that the two enzymes were separated. Nevertheless, the possibility that the Mf2-preparation was contaminated with inactive (and thus undetectable) Mt2-enzyme has to be considered.

Structural relationship between Mf2- and Mt2-enzyme. The anti-Mf2 antiserum was immobilized to Protein A-Sepharose CL-4B and increasing amounts were tested for the capacity to precipitate methyltransferase activity from crude extracts and partially purified enzyme preparations. From a crude preparation of M48 (158 µg protein, 15 pkat methyltransferase activity) up to 94% of the activity was precipitated by 10 mg antiserum protein (Fig. 2). The line M48 contains both

Mf2- and Mt2-enzyme and the precipitation of as much as 94% of the total activity indicated that the antiserum reacted with both enzymes. This was confirmed when 15 pkat methyltransferase activity in a crude enzyme preparation from a line (M43) with only Mt2-enzyme was titrated with antiserum. The results are also illustrated in Fig. 2 and show cross-reactivity between Mf2-antiserum and Mt2-enzyme.

As mentioned above, we had no positive evidence that the preparation used for immunization did not contain inactive Mt2-enzyme. Such a contamination might be the cause of the precipitation of Mt2-enzyme. In order to test this assumption, the following experiment was carried out. A crude M48-extract was directly chromatofocused. Under these conditions the Mt2-enzyme remained active and was detectable as a distinct peak. Thus, it was possible to isolate the Mf2-peak separately. This Mf2-enzyme preparation was used for pre-incubation with antiserum (67 pkat enzyme activity in each incubation). Subsequently, a M43-extract was incubated with antiserum as described above. No precipitation of Mt2-activity was found. We conclude that Mt2- and Mf2-enzyme share antigenic determinants. The slope of the titration-curve obtained with a M43-extract suggested that more antiserum was needed to precipitate the same amount of Mt2-enzyme as compared to Mf2-enzyme. Therefore we suggest that Mt2- and Mf2-enzyme are structurally related but not identical.

Pre-incubations were also carried out in order to test whether mutants which are homozygous recessive for the methylation genes contain inactive methyltransferases. A protein extract (2 mg) from flower buds of R78 and V32 was used in a pre-incubation preceding titration with a crude extract from M48 and M43, respectively. The pre-incubations did not alter the slopes of the titration curves. This implies that R78 and V32, both homozygous recessive for the methylation genes do not contain any appreciable amounts of cross-reacting methyltransferase protein.

Structural relationship of Mf1- and Mt1-enzyme with Mf2-enzyme. In order to isolate Mf1- and Mt1-enzyme for study of precipitation of these enzymes with the anti-Mf2 serum, an extract from the line M73 was subjected to chromatofocusing. This line is characterized as being dominant for the genes *Mf1* and *Mt1* and gives two uncompletely separated peaks of methyltransferase activity upon chromatofocusing (Jonsson et al. 1984a). Figure 3 shows the titration curves of the two enzyme preparations. The Mt1-methyltransferase exhibited cross-reactivity with the anti-Mf2 serum, whereas the Mf1-enzyme could not be precipitated to any significant extent. When a crude enzyme extract from the M73-line was titrated against antiserum, the amounts of activity precipitated at increasing amounts of antiserum corresponded with those obtained with the purified Mt1-preparation.

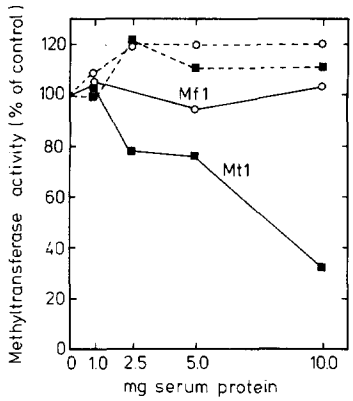

Fig. 3. Effect of incubation with anti-Mf2 serum (——) and normal rabbit serum (- - -) on methyltransferase activity. (○-○-○) = Mf1-enzyme preparation; (■-■-■) = Mt1-enzyme preparation. The samples contained 11 pkat activity (Mf1-enzyme) or 10 pkat activity (Mt1-enzyme)

We conclude that three of the four methyltransferases have antigenic determinants in common and thus show structural relationship.

Do the methylation genes have a common origin?

The immunological studies suggest that *Mf2*-enzyme is related to *Mt2*-enzyme and *Mt1*-enzyme but not to *Mf1*-enzyme. The genes *Mt2* and *Mf2* are located on chromosome V, whereas *Mt1* and *Mf1* are located on chromosome III. The occurrence of two pairs of rather closely linked methylation genes on two chromosomes suggest that the genes may have originated by translocation of a (duplicated) segment. In this view the methylation genes would have a common origin. However, the results of the immunological studies do not give support to this notion. To make the model fit we have to assume that the *Mf1*-gene is an exception and diverged in such a way that its gene-product, the Mf1-enzyme, does not share antigenic determinants with the other methyltransferases. Moreover, the distances between *Mt1/Mf1* on the one hand and *Mt2/Mf2* on the other differ: at least one gene is located between *Mt2* and *Mf2* (Jonsson et al. 1983a), whereas *Mt1* and *Mf1* seem to be very closely linked. Actually, the very existence of the postulated gene *Mt1* is questionable. According to the model presented by Wiering (1974), the *Mf*-genes can only be expressed if a *Mt*-gene is represented by a dominant allele. This model implied that two *Mt*-genes had to be present. However, it has been shown that the expression of *Mf2* is independent of *Mt2* (Jonsson et al. 1983a). Furthermore, the Mf1-enzyme is able to accomplish methylation of delphinidin-pigment to malvidin in vitro, which makes it likely that the expression of *Mf1* also is independent of a *Mt*-gene. In view of these observations the results of genetic studies can be explained in two ways: either *Mf1* and *Mt1* are very strongly linked or *Mt1* does not exist. The fact that we found two methyltransferases in the line M73, homozygous recessive for *Mt2* and *Mf2*, argues against the latter idea. However, a clear-cut answer can only be obtained after finding the cross-over type *Mt1Mt1mt2mt2mf1mf1mf2mf2*. In a previous report we suggested that the line V33 was such a cross-over type

(Jonsson et al. 1984a), but recent linkage studies falsified this suggestion: in V33 the gene *Mt2* is dominant. Since the enzyme in V33 has clearly different properties compared with that in M43, but shows an identical titration-curve with anti-Mf2 serum (results not shown), we are dealing not only with true isozymes, but probably also with multiple forms of one isozyme. In conclusion, there is little evidence that the methylation genes have a common origin.

The next questions arising are the following: Did the methylation genes exist already in the ancestral species of *P. hybrida?* Or can they be related to the hybrid character of *Petunia hybrida*, in a way that, for example, one ancestor contributed the genes *Mt1 Mf1* and the other *Mt2 Mf2*?

Multiple forms of methyltransferases in an ancestral species of Petunia hybrida

In order to investigate a possible relation between the methyltransferase isozymes and the hybrid descent of *P. hybrida* we studied the methyltransferase in flowers of two subspecies of *Petunia integrifolia* by means of immunoprecipitation. The lines S6 (*P. integrifolia* ssp. *inflata*) and S12 (*P. integrifolia* s.s.) were used. *Petunia axillaris*, the second ancestor was not suitable for this study. This species contains some petunidin in the flower tube, but the flower limb is white. One line of *P. axillaris* (S2) was investigated and found not to have any detectable methyltransferase activity in the flower limb. This is explained by the gene *An2* being homozygous recessive in this species. *An2* controls more than one enzyme activity, among others anthocyanin methyltransferase (Gerats et al. 1984) and is believed to be a regulatory gene (Farcy and Cornu 1979).

S6 and S12 both contain the methylated pigments petunidin and malvidin in the flowers, but with different ratios. In flowers of S6 the ratio is about 20:80 and in S12 50:50, as determined by high performance liquid chromatography. It has been shown that a dominant allele of *Mf1* results in relatively more malvidin than a dominant allele of *Mf2* (Table 2). Therefore, the ratios give an indication that the lines contain different methyltransferases. An enzyme extract was prepared from either of the two lines S6 and S12 and 15 pkat methyltransferase activity of each extract were incubated with 5 mg anti-Mf2 serum protein immobilized to Protein-A Sepharose CL-4B. In repeated experiments, the antiserum failed to bind methyltransferase from S6, but precipitated 7 pkat of activity from the line S12. One might speculate then, that the methyltransferase in S6 is of the Mf1-type, which is in accordance with the ratio of petunidin:malvidin in this line. The precipitation of 7 pkat in an extract of S12 suggests that this line contains methyltransferase of the Mf2-type, possibly in combination with Mt-enzyme (see Fig. 2). Again, this is in agreement with the ratio petunidin:malvidin in S12. We conclude that a diversity of methyltransferases exists already in one of the ancestral species of *P. hybrida*, and that the occurrence of isozymes is not related to the hybrid descent.

The question remains whether having anthocyanin methyltransferase isozymes is an unique feature of *Petunia* or common to other plant species.

Is Petunia unique in having several anthocyanin methyltransferases?

Enzymatic studies of anthocyanin methyltransferases in other species have as yet not been reported, but the genetic control of anthocyanin methylation has been studied in a number of species. These studies provide indirect information about methyltransferases. We will consider here only those reported methylation genes which do not have pleiotropic effects. In the case of pleiotropic effects it is likely that the potency of methylation is independent of the gene concerned, but cannot be expressed due to an inhibition earlier in the pathway. It should be noted that enzyme extracts from *Petunia* blocked early in the anthocyanin biosynthetic pathway do contain anthocyanin methyltransferase activity (Jonsson et al. 1982). Thus, the enzyme is formed even if no anthocyanin substrate is available.

Besides *Mt1* and *Mt2* in *Petunia*, a gene called *P2* in alfalfa, *Medicago sativa* is, to our knowledge the only gene ever described that is related to the formation of petunidin (Gupta 1970).

Genes seemingly controlling the formation of malvidin from petunidin have been reported more frequently. Seyffert (1959), working with *Primula malacoides* showed that malvidin was present in the genotype *BBBB* and petunidin in the recessive genotype. Likewise, Harborne (1967) mentioned that there were indications for two genes being related to methylation of delphinidin to malvidin in *Primula obconica*. In potato, *Solanum tuberosum*, production of malvidin pigment was related to a dominant allele of the gene *Ac'*. Other genotypes contained petunidin-pigment (Harborne 1960, 1967). Besides the gene *P2* in flower petals of *Medicago sativa* mentioned above, a second methylation gene was identified (*P3*), which was related to methylation of petunidin to malvidin (Gupta 1970).

The examples show that these species, belonging to different families, contain at least two classes of anthocyanin methyltransferases. They are distinguishable by their effect in vivo: petunidin- or malvidin-derivatives being the main pigments. Clearly, *Petunia* is not unique in having several anthocyanin methyltransferases.

The role of isozymes in the control of anthocyanin methylation

To understand the physiological role of isozymes their properties need to be investigated. Some properties of the anthocyanin methyltransferases have been determined before (Jonsson et al. 1984a). It appeared that all four investigated enzymes methylated derivatives of cyanidin to peonidin and derivatives of delphinidin to petunidin and malvidin. However, when methylation of delphinidin was measured with equal input of activity and at initial velocity, quantitative differences between the Mt-enzymes and the Mf-enzymes were found. The Mf-enzymes were able to form more malvidin than petunidin whereas the Mt-enzymes did not form more than 40% malvidin as a total of methylated product. This reflected the situation in vivo and showed that the relative amount of malvidin-pigment very much depends on the properties of the isozyme that is present.

Furthermore, isozymes may be distinguished by their location in differ subcellular fractions or in different celltypes, or they may show different time course of activity. The anthocyanin methyltransferases were examined in respect of these points.

Subcellular location. It has been shown before that the methyltransferase activity of the line R27 (containing Mf-enzyme and possibly Mt-enzyme) was completely soluble (Jonsson et al. 1983b). The location of methyltransferase in extracts from a line with only Mt-activity (M43) was now studied by means of differential centrifugation. All activity was found in the soluble fraction (results now shown). We conclude that the methyltransferases are not located in different organelles.

Tissue location. Petunia flowers accumulate anthocyanins in the epidermal cell-layers. Possibly different isozymes control the methylation in upper and lower epidermis. In this case one would expect to find different mixtures of unmethylated and methylated pigments in the two epidermal layers. In order to investigate this possibility, anthocyanin composition was determined in upper and lower epidermis from flowers of V14 (*Mf1Mf1mf2mf2*), V12 (*Mf1Mf1Mf2Mf2*) and V33 (*Mt2Mt2mf1mf1mf2mf2*) (see Table 1). The ratio's of delphinidin:petunidin:malvidin in upper and lower epidermis are shown in Table 4. Each line had relatively more methylated anthocyanin in the upper epidermis. The differences were, however, not big enough to support the idea that the epidermal layers contain different isozymes. They can be explained as a consequence of lower enzyme amounts or higher local substrate concentrations in the lower epidermis, or both (see below). Therefore, the results allow the conclusion that Mt2-, Mf1- and Mf2-enzyme are all active in both tissues.

Different isozymes in limb and tube of the flower? Different parts of the same flower may show variations in the degree of anthocyanin methylation. In *Petunia,* the relative amounts of malvidin found in the flower tube are generally lower than those of the flower limb. Thus, plants of the *Mf*-genotype contain predominantly petunidin in the tube. It has been suggested therefore, that the action of the genes *Mf1* and *Mf2* mainly is restricted to the flower limb (Wiering 1974; Wiering and de Vlaming 1977; Wiering et al. 1979). However, the studies of the methyltransferases in vitro mentioned above, revealed that, regardless of which enzyme was studied, the amount of malvidin formed, relative to petunidin, varied with the substrate (delphinidin 3-(p-coumaroyl)-rutinoside-5-glucoside) concentration. At a substrate concentration above 12 µM, decreasing amounts of malvidin were formed. In view of this information it seems plausible that the difference in methylation degree between limb and tube is not the consequence of a tissue-specific gene expression, but rather a physiological effect. The anthocyanins present in the tube are mostly restricted to the veins. The substrate concentration in the veins might locally be higher than in the cells of the flower limb, thereby inhibiting the synthesis of malvidin. Moreover, differences in methylation degree might be caused by different amounts of enzyme in different cell-types (see Table 2).

Table 4. Anthocyanin composition in upper and lower epidermis of flowers of different genotype. Each sample contained epidermal layers from flower limbs of three different flowers of the indicated line

Line	Dominant methylation genes	Ratio delphinidin:petunidin:malvidin	
		Upper epidermis	Lower epidermis
V33	*Mt2*	17:67:16	24:60:16
V12	*Mf1, Mf2*	0:23:77	0:32:68
V14	*Mf1*	3:33:64	8:42:50

Table 5. Relation between methyltransferase activity and anthocyanin composition in 25 to 35 mm buds and mature flowers. Values of anthocyanin composition are averages of determinations in three to five flower buds or flowers of the indicated line

Line	Dominant methylation genes	Specific activity (pkat/mg protein		Ratio delphinidin:petunidin:malvidin	
		Buds	Flowers	Buds	Flowers
V33	*Mt2*	38	19	18:65:17	22:61:17
V10	*Mf1, Mf2*	543	500	0:13:87	0:15:85

Time course of activity. Plants with different genotypes with regard to the methylation genes were used to study the methyltransferase activity at three stages of flower bud development: 15 to 25 mm buds, 25 to 35 mm buds and mature flowers. No differences were found among the lines with regard to the start of development of enzyme activity. However, in *mfmf*-plants the methyltransferase activity was generally reduced to about half in the mature flower as compared to 25 to 35 mm buds, whereas in *MfMf*-plants the activity in the mature flower was equal to that of the 25 to 35 mm buds (results not shown). To study whether the prolonged high Mf-enzyme activity has an influence on anthocyanin composition, we determined methyltransferase activity and the relative amount of malvidin during bud development in the lines V33 (*Mt2Mt2mf1mf1mf2mf2*) and V10 (*Mf1Mf1Mf2Mf2*). The results are shown in Table 5. These lines followed the general pattern: V10, containing dominant alleles of *Mf*-genes kept a high level of methyltransferase activity in the mature flower, whereas that of V33 (homo-

zygous recessive for both *Mf*-genes) was reduced to half. However, when comparing the ratio between delphinidin:petunidin:malvidin in 25 mm buds with that of the mature flower, very small changes were found not only in V10 but also in V33. Obviously, the difference in time course of activity between Mt-enzyme and Mf-enzyme in these lines does not influence the methylation pattern. This is in agreement with earlier studies, which showed that there is no active anthocyanin biosynthesis in mature flowers in vitro (Jonsson et al. 1984b).

In conclusion, three factors affecting the degree of anthocyanin methylation have been found: catalytic properties of methyltransferase isozymes, amount of enzyme and concentration of anthocyanin substrate during biosynthesis. Based on the reports in the literature one might state that this complex genetic and physiological control is not restricted to our object of study *Petunia hybrida*, but seems to be general among plants containing methylated anthocyanins. Interestingly, there is suggestive evidence that the hydroxylation pattern of anthocyanins is regulated in a similar way. Genetic studies have shown the occurrence of genes correlated with either one or two hydroxyl groups in the B-ring of the anthocyanidin in various species. Different patterns of hydroxylation in different parts of the same plant have also been described (see Harborne 1967, for a review). This indirect evidence of agreement between methylating and hydroxylating enzymes makes a careful study of the properties of the latter worthwhile.

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References

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Cornu A, Maizonnier D, Wiering H, de Vlaming P (1980) Petunia genetics. 3. The linkage group of chromosome V. Ann Amelior Plant 30:443–454

Dooner HK (1983) Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol Gen Genet 189:136–141

Farcy E, Cornu A (1979) Isolation and characterization of anthocyanin variants originating from the unstable system an2-1 in *Petunia hybrida* (Hort.). Theor Appl Genet 55:273–278

Folch J, Less M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509

Gerats AGM, Farcy E, Wallroth M, Groot SPC, Schram A (1984) Control of anthocyanin synthesis in *Petunia hybrida* by multiple allelic series of the genes *An1* and *An2*. Genetics 106:501–508

Gupta SB (1970) Biochemical aspects of the inheritance of floral anthocyanins in diploid alfalfa. Genetics 65:267–278

Harborne JB (1960) Anthocyanin production in the cultivated potato. Biochem J 74:262–269

Harborne JB (1967) Inheritance and biosynthesis of flavonoids in plants. In: Harborne JB (ed) Comparative biochemistry of flavonoids. Academic Press, London New York, pp 250–280

Jonsson LMV, Aarsman MEG, Schram AW, Bennink GJH (1982) Methylation of anthocyanins by cell-free extracts of flower buds of *Petunia hybrida*. Phytochemistry 21:2457–2459

Jonsson LMV, de Vlaming P, Wiering H, Aarsman MEG, Schram AW (1983a) Genetic control of anthocyanin-O-methyltransferase activity in flowers of *Petunia hybrida*. Theor Appl Genet 66:349–355

Jonsson LMV, Donker-Koopman WE, Uitslager P, Schram AW (1983b) Subcellular localization of anthocyanin methyltransferase in flowers of *Petunia hybrida*. Plant Physiol 72:287–290

Jonsson LMV, Aarsman MEG, Poulton JE, Schram AW (1984a) Properties and genetic control of four methyltransferases involved in methylation of anthocyanins in flowers of *Petunia hybrida*. Planta 160:174–179

Jonsson LMV, Donker-Koopman WE, Schram AW (1984b) Turnover of anthocyanins and tissue compartmentation of anthocyanin biosynthesis in flowers of *Petunia hybrida*. Z Pflanzenphysiol (in press)

Larson RL, Coe EH Jr (1977) Gene-dependent flavonoid glucosyl transferase in maize. Biochem Genet 15:153–156

Maizonnier D, Moessner A (1979) Localization of linkage groups on the seven chromosomes of the *Petunia hybrida* genome. Genetica 51:143–148

Seyffert W (1959) Untersuchungen über interallele Wechselwirkungen. 3. Der Dosiseffekt eines die Methylierung von Anthocyanen kontrollierenden Gens. Naturwissenschaften 46:271

Wiering H (1974) Genetics of flower colour in *Petunia hybrida* Hort. Genen Phaenen 17:117–134

Wiering H, de Vlaming P (1977) Glycosylation and methylation patterns of anthocyanins in *Petunia hybrida*. 2. The genes *Mf1* and *Mf2*. Z Pflanzenzücht 78:113–123

Wijsman HJW, Berg BM van den (1982) Location of structural genes for glucose phosphate isomerase and for leucyl aminopeptidase on chromosome VII of *Petunia*. Theor Appl Genet 63:283–287