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Genetic control of anthocyanin-O-methyltransferase activity in flowers of *Petunia hybrida*

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Summary. The relation between four methylation genes (*Mt1, Mt2, Mf1* and *Mf2*) in flowers of *Petunia hybrida* and anthocyanin-methyltransferase activity was investigated in vitro. All genes controlled methyltransferase activity. This activity was measured with cyanidin- and petunidin-derivatives as substrates. A cross provided evidence that the *Mf*-genes regulate methyltransferases which are distinct from those controlled by the *Mt*-genes. Different effects of the two *Mf*-genes in vivo are shown. The results suggest that the four methylation-genes control four different methyltransferases.

Key words: *Petunia hybrida* – Anthocyanin biosynthesis – Hydroxylases – Methyltransferases

Introduction

Many plants contain a multitude of methylated flavonoid compounds. In flowers of *Petunia hybrida,* derivatives of the methylated anthocyanidins petunidin, peonidin (methylated at the 3′-position) or malvidin (methylated at the 3′,5′-positions) accumulate (Wiering 1974). Anthocyanin methylation in *Petunia* is related to two pairs of genes: *Mt1/Mf1* and *Mt2/Mf2* (Wiering 1974; Wiering and de Vlaming 1977). When a dominant allele of one or both of the genes *Mt1* or *Mt2* is present, mainly derivatives of petunidin or peonidin accumulate, whereas a dominant allele of one or both of the genes *Mf1* or *Mf2* is required for malvidin to be the major pigment (Fig. 1).

Abbreviations: KPi: potassium phosphate; OMT: O-methyltransferase; SAM: S-adenosyl-L-methionine; 3RGac5G: 3(p-coumaroyl)-rutinoside-5-glucoside

A possible explanation of the effects of these genes is that they control different, specialized methylating enzymes. A number of flavonoid-O-methyltransferases (OMT's) have been described and there are reports of several OMT's within one plant, differing in substrate or position specificity (reviewed in Poulton 1981; de Luca and Ibrahim 1982). Recently we demonstrated a SAM: Anthocyanin-3′,5′-OMT activity in cell-free extracts of *Petunia* flower-buds (Jonsson et al. 1982). The enzyme methylates only the 3(p-coumaroyl)-rutinosido-5-glucoside derivatives of anthocyanidins, thus confirming that methylation is the last modification step of anthocyanin biosynthesis in *Petunia hybrida.* This paper reports on the relation between the four methylation-genes and anthocyanin-OMT activity. The results support the assumption that *Petunia hybrida* contains four different anthocyanin-OMT's, which are directly controlled by the methylation genes.

Materials and methods

Plant material

All plants were grown in a greenhouse. The genotypes and phenotypes of inbred lines and F1's of the crosses are given in Table 1, and the genotypes of other inbred lines used in Table 2.

Short description of the segregation genes

Un-	flower limb undulate, sepals broad
unun	flower limb flat (plana), sepals narrow
Po-	pollen white
popo	pollen yellow

Methylation genes; main anthocyanidin in the flower-limb:

Mt1-	3′-methylated: petunidin or peonidin
mt1mt1	unmethylated: delphinidin or cyanidin
Mt2-	3′-methylated: petunidin or peonidin

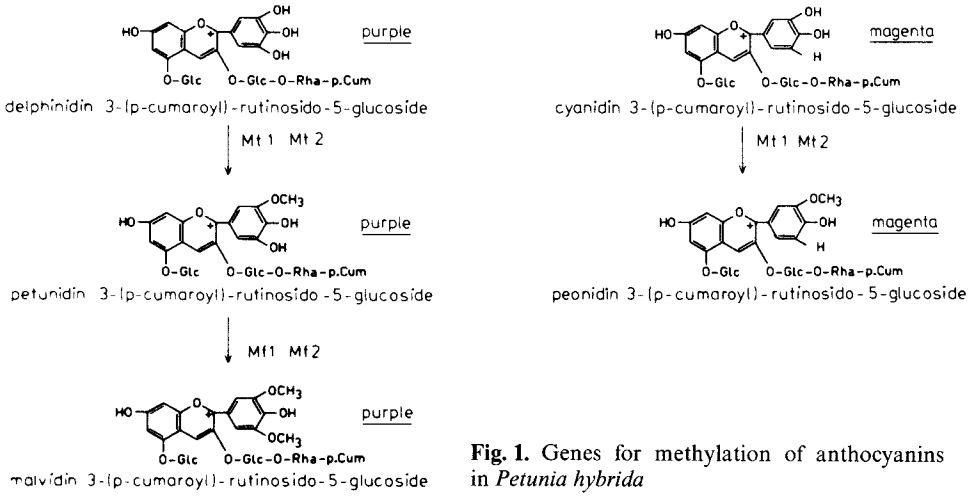

Fig. 1. Genes for methylation of anthocyanins in *Petunia hybrida*

mt2mt2	unmethylated: delphinidin or cyanidin
Mf1-	3′,5′-methylated: malvidin (accounts for more than 20% of total)
mf1mf1	3′-methylated: petunidin or peonidin (accounts for more than 80% of total)
Mf2-	3′,5′-methylated: malvidin (accounts for more than 20% of total)
mf2mf2	3′-methylated: petunidin or peonidin (accounts for more than 80% of total).

The genes *Un, Po, Mt2* and *Mf2* are located on chromocome V. Cross-over values *Mt2-Un*: 2–9%, *Mt2-Mf2*: 1–13%, *Mf2-Po*: 0–1% (Cornu et al. 1980).

Genes *Mt1* and *Mf1* are located on chromosome III (Maizonnier and Moessner 1979). Cross-over value *Mt1-Mf1*: 3% (unpublished data).

Analysis of anthocyanidins

Malvidin was distinguished from delphinidin/petunidin by the ammonium sulphate test (Wiering and de Vlaming 1973). Delphinidin 3RGac5G could be visually distinguished from petunidin 3RGac5G as flowers with the former pigment are more grey.

Extracts of flowers from all cross-over phenotypes, a sample (12%) of the non cross-over and all doubtful types were after hydrolysis chromatographed on paper or cellulose TLC, as described in Wiering and de Vlaming (1973). The relative amounts of anthocyanidins were visually estimated from papers or plates after chromatography.

Preparation of enzyme-extracts

All steps were performed at 4 °C. In each experiment five to ten flower-limbs were homogenized with pestle and mortar in 1.5–2 ml 100 mM KPi pH 8.5, containing 1.4 mM β-mercaptoethanol, some quartz sand, and Dowex 1X2 in an amount equal to half the wet weight of the limb. The homogenate was centrifuged (20 min, 38,000 g) in a MSE 18 centrifuge. The supernatant was poured on a Sephadex G-25 column (PD-10, 9.1 ml) and protein was eluted with 100 mM KPi pH 7.7. Fractions containing the bulk of protein were used in methyltransferase assays. Protein was determined according to Bradford (1976) with Bovine Serum Albumin as standard.

Methyltransferase assay

The isolation of the anthocyanin substrates, the incubation temperature and the quantitative HPLC analysis of the methylated products were as described before (Jonsson et al. 1982). The standard assay mixture consisted of 30 µM anthocyanin substrate dissolved in 5 mM HCl; 200 mM KPi pH 7.7, 20 mM $MgCl_2$, 0.8 mM SAM and 25 to 50 µl enzyme extract (20 to 70 µg protein) in a reaction volume of 100 µl. Under

Table 1. Genotype and phenotype of inbred lines and the F1's. Genetic background *RtRtGfGf*

Line code	Flower-limb colour	Main anthocyanin	HCC[a]	Pollen colour	Flower form	Genotype
M48	very pale magenta	malvidin 3RGac5G	633/1	white	undulate	*UnUnmt1mt1Mt2Mt2mf1mf1Mf2Mf2PoPo*
V2	purple	malvidin 3RGac5G	733	green	plana	*unun* × [b] *mf1mf1Mf2Mf2popo*
V11	purple	petunidin 3RGac5G	34/1	yellow	plana	*unun* × *mf1mf1mf2mf2popo*
V14	purple	malvidin 3RGac5G	33	yellow	plana	*unun* × *Mf1Mf1mf2mf2popo*
V32	purple	delphinidin 3RGac5G	35/1	yellow	plana	*ununmt1mt1mt2mt2mf1mf1mf2mf2popo*
V11 × V2	purple	malvidin 3RGac5G	733	green	plana	*unun* × *mf1mf1Mf2mf2popo*
V14 × V11	purple	malvidin 3RGac5G	33	yellow	plana	*unun* × *Mf1mf1mf2mf2popo*
M48 × V32	purple	malvidin 3RGac5G	32/1	white	undulate	*Ununmt1mt1Mt2mt2mf1mf1Mf2mf2Popo*

[a] Colour according to the Horticultural Colour Chart of the British Colour Council
[b] One or both the *Mt*-genes homozygous dominant

these conditions methyl-transferase activity was optimal for all lines tested. The reaction was terminated by adding 400 µl of a mixture containing two parts of $CHCl_3$ and one part of methanol-HCl (2%, v/v). Control incubations contained no enzyme. Activities were determined as mU/mg protein from initial velocities of the reaction (2 to 20 min of incubation).

Results and discussion

The relation between the methylation genes and OMT-activity in vitro

The effects of the methylation (M-)-genes can be explained by assuming that *Petunia hybrida* contains two groups of anthocyanin-OMT's. The *Mt*-genes could be expected to control one or two methyltransferases which carry out the 3'-methylation steps (Fig. 1):

cyanidin 3RGac5G → peonidin 3RGac5G

or

delphinidin 3RGac5G → petunidin 3RGac5G.

Accordingly, the *Mf*-genes would control one or two OMT's capable of methylating petunidin at the 5'-position to malvidin 3RGac5G. To test these propositions we measured anthocyanin-methyltransferase activities in flower-limb extracts of *Petunia* lines differing in genotypes with regard to the *M*-genes. Cyanidin 3RGac5G and petunidin 3RGac5G were used as substrates. The results are given in Table 2. Activities were determined at three stages of development of the flower-bud: 15 to 25 mm buds, 25 to 35 mm buds and open flowers (bud length was defined as the distance between the flower-receptacle and the top of the corolla). The ratio between the cyanidin- and the petunidin-methylation showed no significant variation during bud development (results not shown). The highest specific activity was found in 25 to 35 mm buds and the results of the determinations of this stage are given. A line with all the *M*-genes homozygous recessive (R 78) had no detectable OMT-activity. This confirms the earlier conclusion that the *M*-genes control this activity (Jonsson et al. 1982).

The Mt-genes

Three lines with one or both *Mt*-genes dominant and the *Mf*-genes homozygous recessive were tested: M3, M43 and W37. All showed methyl-transferase activity with cyanidin 3RGac5G but also with petunidin 3RGac5G as substrate. The *Mt*-enzymes preferred cyanidin 3RGac5G; the methylation of this anthocyanin was 2 to 6 times faster than that of petunidin 3RGac5G. The ratio's of cyanidin/petunidin methylation showed differences among the lines tested. Whether these are due to the presence of different *Mt*-enzymes is unclear, since the genotypes of the lines M3 and W37 with regard to the *Mt*-genes are not completely known.

The degree of substrate specificity of the *Mt*-enzymes corresponds quite well with the accumulation of anthocyanins in vivo. When one or both of the *Mt*-genes are dominant and both the *Mf*-genes homozygous recessive, derivatives of peonidin or petunidin are the main pigments, but malvidin can also be present, accounting for up to 20% of the total anthocyanin content.

The Mf-genes

At the beginning of this study there were no plants available which were dominant for one or both *Mf*-genes and homozygous recessive for both *Mt*-genes. When studying enzyme activities in the lines with dominant *Mf*-genes (M48, M73, V10, V12) we therefore had to keep in mind the contribution of the *Mt*-enzymes.

Table 2. Anthocyanin methyltransferase activities in different lines of *Petunia hybrida*[a]

Line-code	Genotype				Spec.Act. (mU/mg protein)		Ratio
	Mt1	*Mt2*	*Mf1*	*Mf2*	Cyanidin[b]	Petunidin[b]	Cya/pet-activities
R78	–	–	–	–	<0.03	<0.03	–
M3		×	–	–	1.7	1.0	1.7
M43	–	+	–	–	2.3	0.4	5.8
W37		×	–	–	1.7	0.5	3.4
M48	–	+	–	+	6.0	2.2	2.7
M73	+	–	+	–	12.9	3.3	3.9
V10		×	+	+	32.6	10.3	3.2
V12		×	+	+	45.2	14.7	3.1

+ = homozygous dominant, – = homozygous recessive, × = one or both of the genes is homozygous dominant

[a] 25–35 mm buds were used for the determinations

[b] The 3RGac5G derivatives of the anthocyanidins were used as substrate

It appears that both methyltransferase activities tested are considerably higher in the *Mf*-dominant lines than in M3, M43 and W37. This is illustrated by a comparison of the activities in M48 with those in M43. Both lines are dominant for the *Mt2*-gene, and M48 is also dominant for the *Mf2*-gene.

Methylation activities of both cyanidin and of petunidin are higher in the M48-extract than in M43, suggesting that the *Mf2*-gene contributes to 3′ – as well as 5′-methylation activity.

The line M73, which has the *Mt1* and *Mf1* genes dominant, had higher OMT-activities than M48, but the ratio between the cyanidin/petunidin-activities were about the same as in M48.

In V10 and V12 three or four methylation genes are dominant, and these lines gave still higher activities, suggesting an additive effect of different gene-products.

The ratio between the cyanidin- and petunidin-methylation did not differ significantly between the lines with the *Mf*-genes dominant and those with only the *Mt*-genes dominant. All this is inconsistent with the idea that enzymes controlled by the *Mf*-genes preferentially methylate a petunidin-derivative.

The results can be explained by assuming that the ultimate effect of the *Mf*-genes is a contribution to both the 3′- and the 5′-methylation. Even then one is faced with two possible explanations for the effect of the *Mf*-genes. They might control separate OMT's, capable of methylating cyanidin, delphinidin or petunidin, respectively. These enzymes might differ from the *Mt*-enzymes in affinity for their substrates or V_{max} and thereby cause the accumulation of malvidin-derivatives in the flowers.

However, we cannot exclude the possibility that the effect of the *Mf*-genes is caused by an indirect control of the *Mt*-enzymes. The *Mf*-genes may be regulatory genes, stimulating the expression of the *Mt*-genes, thus leading to more methyltransferase enzyme. Alternatively, the *Mf*-gene-products may be activators of the *Mt*-methyltransferase. To establish the effect of the *Mf*-genes in vivo, we needed an *mtmtMf-* recombinant. A cross to obtain such a recombinant was therefore set up.

A cross to obtain an mtmtMf- recombinant

The selection of an *mtmtMf-* recombinant was complicated by the rather strong linkage between the *Mt* and *Mf*-genes. Moreover, analysis of the segregation of the methylation genes had to be performed keeping in mind two mutually exclusiv hypotheses:

I. *Mf* is only expressed in the presence of a dominant allele of *Mt.*

II. The expression of *Mf* is independent of *Mt. Mf* controls a separate methyltransferase, which causes accumulation of malvidin pigment.

If hypothesis I were correct, a recombinant with the genotype *mtmtMf-* would have to be a plant with delphinidin 3RGac5G in the flower limb. This recombinant has not yet been found, although the reciprocal recombinant *Mt-mfmf* with mainly petunidin 3RGac5G in the flower limb is present in our collection. According to hypothesis II, the genotypes *mtmtMf-* and *Mt-Mf-* cannot be distinguished directly, since they would all be plants with malvidin. In the cross designed to create the *mtmtMf-* recombinant we therefore made use of two marker genes: *Un,* situated on one side of the block *Mt2Mf2* and *Po* located on the other side of this block, or between *Mt2* and *Mf2*. The genotypes and phenotypes of parents and F1 are shown in Table 1. The cross confirms the order of the genes *Un* and *Mt2* with regard to *Po* and *Mf2*, as given in Cornu et al. (1980). In this paper two alternatives were given as to the order of *Po* and *Mf2: Un Mt2 Po Mf2* and *Un Mt2 Mf2 Po.* The segregations of the back-cross (M48 × V32) × V32 are given in Table 3. With the gene-order *Un Mt2 Mf2 Po* we get at least 17 double cross-overs according to both hypotheses. The gene-order *Un Mt2 Po Mf2* gives at least 2 double cross-overs and 13 triple cross-overs according to hypothesis I, but only 2 double cross-overs according to hypothesis II.

According to hypothesis I, the cross-over *ununmt2mt2PopoMf2mf2* would give phenotype plana, white pollen, delphinidin. This phenotype was not found, whereas of the reciprocal recombinant *UnunMt2mt2popomf2mf2,* phenotype undulate, yellow pollen, petunidin, 14 plants were found. The results agree altogether better with hypothesis II. According to this idea, most of the 20 cross-overs marked** could be the reciprocals of the 14 *UnunMt2mt2popomf2mf2* types and thus be the sought for type. Moreover, the 13 plants with the phenotype plana, yellow pollen, malvidin can be single cross-overs with the genotype *mt2mt2Mf2mf2.*

The results strongly support hypothesis II and make *Un Mt2 Po Mf2* the most probable gene-order. Only the relatively large number of cross-overs between *Po* and *Mf2* (2.6% as compared to the expected 0–1%) remains then to be explained.

In a sample of the plants expected to have the genotype *mt2mt2Mf2mf2* the methyltransferase activity was determined with cyanidin 3RGac5G as substrate. All plants showed 3′-methylation activity. The plants with the phenotype plana, yellow pollen, malvidin, showed a specific activity of 1.6 ± 1.2 mU/mg protein ($n = 8$). The types marked** showed 2.2 ± 1.1 mU/mg protein ($n = 9$) or 6.0 ± 0.8 mU/mg protein ($n = 3$).

The differences found in the latter group might reflect the different genotypes expected among these plants (*mt2mt2Mf2mf2* and *Mt2mt2Mf2mf2* respectively). Part of the variation in enzyme activity was due

Table 3. Cross (M48 × V32) × V32 F1: A: *Un Mt2 Po Mf2* B: *Un Mt2 Mf2 Po*
Genetic background: *mt1mt1mf1mf1* *un mt2 po mf2* *un mt2 mf2 po*

Genotype	Phenotype[a], expected according to hypothesis		Cross-over[b]		Results, no of plants according to hypothesis	
	I	II[c]	A	B	I	II
Un Mt2 Po Mf2	und w mal		non	non	346	346
un mt2 po mf2	pl y del		non	non	321	321
Un mt2 po mf2	und y del		s	s	3*	3
un Mt2 Po Mf2	pl w mal		s	s	20	20**
Un Mt2 po mf2	und y pet		s	s	14	14
un mt2 Po Mf2	pl w del	pl w mal	s	s	0	20**
Un Mt2 Po mf2	und w pet		s	d	4	4
un mt2 po Mf2	pl y del	pl y mal	s	d	–[d]	13****
Un mt2 Po Mf2	und w del	und w mal	d	d	0	–[d]
un Mt2 po mf2	pl y pet		d	d	0	0
Un Mt2 po Mf2	und y mal		d	s	2	2***
un mt2 Po mf2	pl w del		d	s	0	0
Un mt2 po Mf2	und y del	und y mal	d	t	3*	2***
un Mt2 Po mf2	pl w pet		d	t	0	0
Un mt2 Po mf2	und w del		t	d	0	0
un Mt2 po Mf2	pl y mal		t	d	13	13****

[a] und = undulate, pl = plana, w = white, y = yellow, mal = malvidin, pet = petunidin, del = delphinidin
[b] s = single, d = double, t = triple
[c] only types different from hypothesis I are given
[d] not different from non cross-overs
*, **, ***, **** = each phenotype has two alternative genotypes
□ = phenotype and genotype of recombinant in request

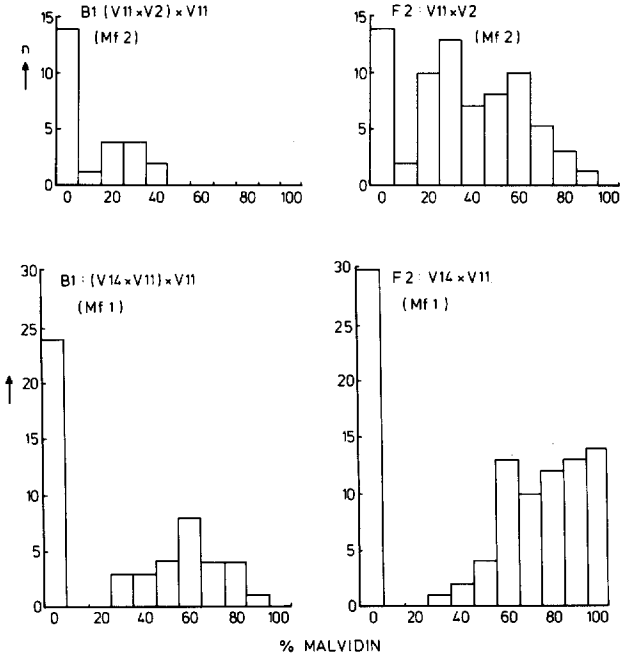

Fig. 2. The effect of the two *Mf*-genes on accumulation of malvidin-pigment in plants from B1's and F2's with segregation of *Mf2* and *Mf1*, respectively. Percentage malvidin of total anthocyanin content

to the limited number of flower buds, which confined us to use mixtures of flowers and flower-buds in different developmental stages as source of enzyme. We conclude that the gene *Mf2* controls a separate methyltransferase. This enzyme seems not to differ from the *Mt2*-enzyme in substrate specificity (Table 2). Nevertheless, unlike the *Mt2*-enzyme, the *Mf2*-activity can accomplish the accumulation of malvidin in vivo (Table 3).

The results in Table 2, the correlation of *Mf1* with accumulation of malvidin, and of *Mt1* with accumulation of petunidin are evidence that the *Mt1-Mf1* pair controls two separate OMT's, just as the *Mt2-Mf2* pair.

Different effects of Mf1 and Mf2 in vivo

Beside the similarities between the effects of the two pairs of genes, there are also differences. This is illustrated in Fig. 2. In the back-cross (V11 × V2) × V11 the gene *Mf2* segregates, giving plants with petunidin and others with malvidin. The plants with malvidin contained 10–40% malvidin. An analysis of the effect of the *Mf1*-gene gave in the back cross (V14 × V11) × V11 an average of 60% malvidin in the plants containing this pigment. It seems that a dominant allele of *Mf1*

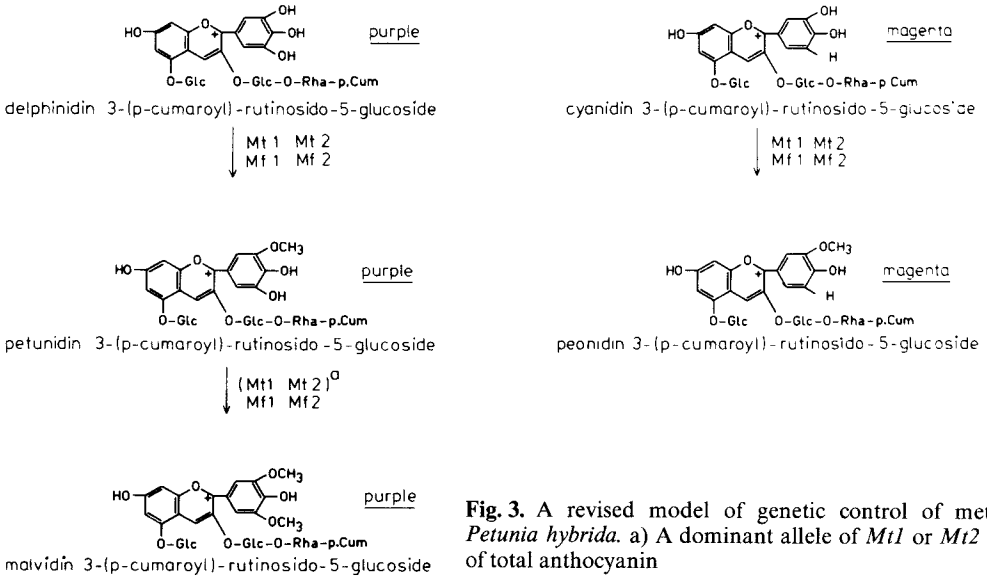

Fig. 3. A revised model of genetic control of methylation of anthocyanins in *Petunia hybrida*. a) A dominant allele of *Mt1* or *Mt2* causes less than 20% malvidin of total anthocyanin

will result in relatively more malvidin than a dominant allele of *Mf2*. An analysis of the malvidin content in the F2's V11×V2 and V14×V11 confirmed this observation. Most of the malvidin plants in the F2 V11×V2 contained less than 60% malvidin, whereas in the F2 V14×V11 a majority of the malvidin-plants had more than 60% malvidin.

The malvidin-plants of the F2's showed more variation in relative amount of malvidin and contained more malvidin than those of the back-crosses. This could be explained by a dosage-effect of the *Mf*-genes, expected to be homozygous dominant in one third of the malvidin plants of the F2's and heterozygous in the other malvidin plants of the F2's and the back-crosses. Whether there also exists a dosage effect of the enzymes controlled by the *Mf*-genes remains to be investigated. Such a gene-enzyme dosage relationship has been demonstrated in *Zea mays* with a flavonoid 3-O-glucosyltransferase and the gene *Bz* (Larson and Coe 1977). To establish the gene-enzyme relationships of the methyltransferases a further understanding of the methyltransferase system is needed. Work is now in progress to separate the different OMT's and to study their properties.

Conclusions

The results presented in this paper demonstrate that flowers of *Petunia hybrida* contain at least two different anthocyanin-methyltransferases, controlled by the genes *Mt1/Mt2* and *Mf1/Mf2*, respectively. Both the *Mt*-enzymes and the *Mf*-enzymes are capable of methylating the 3′- and the 5′-position of the anthocyanin molecule. Different effects of the *Mf*-genes in vivo suggest a methylating system with three or four different enzymes.

The results call for a revision of the model of genetic control of anthocyanin methylation as given in Fig. 1. The new model is illustrated in Fig. 3. All methylation steps can occur if one of the *M*-genes is dominant. The *Mt*-regulated methylation of petunidin 3Rgac5G to malvidin 3RGac5G is peculiar in so far that it never leads to more than 20% malvidin in the flower limb.

Similarities between the methylation and hydroxylation systems

A remarkable aspect of the methylation system in *Petunia hybrida* is the resemblance to the hydroxylation system. Hydroxylation of the B-ring of the anthocyanin-molecule takes place at the flavanone-level, and the genetic control of this process has been described at length (Wiering 1974; Tabak et al. 1978, Tabak et al. 1981; Doodeman et al. 1982). The hydroxylation gene *Ht1* controls hydroxylation at the 3′-position and can be compared with the methylation genes *Mt1* and *Mt2*. Hydroxylation at both the 3′- and 5′-positions is controlled by the hydroxylation genes *Hf1* and *Hf2*. It appears now, that the methylation genes *Mf1* and *Mf2* are similar in their effect to these genes.

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