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A Gene Controlling Rate of Anthocyanin Synthesis and Mutation Frequency of the Gene *An1* in *Petunia hybrida*

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Summary. The difference in colour intensity between flowers of sporogenic revertants of the white flowering lines W17 and W28 is caused by an incompletely dominant gene *In1*. This gene is not linked to the anthocyanin gene *An1*. In the dominant state *In1* causes a 50% decrease in colour intensity of selfcoloured red flowers.

Chromatographic analysis of anthocyanins of plants homozygous recessive or dominant for *In1* showed that the same anthocyanins are produced in both genotypes (cyanidin-3-glucoside and cyanidin-3-diglucoside). Anthocyanin synthesis starts at the same stage of development of the flower in both genotypes. When the bud reaches a length of approximately 45 mm, however, anthocyanin synthesis in the *In1 In1* line slows down.

No influence of the gene *In1* on the concentration of dihydroquercetin-7-glucoside in buds and flowers could be observed, which indicates that the influence of *In1* on flower colour development is restricted to the last part of the biosynthesis of anthocyanins, i.e. the conversion of dihydroflavonols into anthocyanins.

In addition to *In1* having a decreasing effect on flower colour intensity, evidence is produced that the gene *In1* also influences the reversion frequency of unstable alleles of the gene *An1*.

Key words: Anthocyanin synthesis – Unstable genes – *Petunia hybrida*

Introduction

A number of genes involved in the occurrence of coloured flowers in *Petunia hybrida* is known (Wiering 1974; Wiering et al. 1979). A mutation in one of the basic genes for anthocyanin synthesis in the red flowering cultivar 'Roter Vogel' led to the occurrence of white flowering plants which have red spots on the corolla. Because of the very high reversion frequency (up to 10 percent) to the selfcoloured wildtype, it has been stated that the mutation affected a regulating element rather than a structural gene. Reversions occur with a wide range of frequencies, somatically as well as in the sporogenic tissues; reversions in the sporogenic tissues lead to the occurrence of selfcoloured red flowering progeny, called revertants (Bianchi et al. 1978). The mutation is located at the *An1* locus.

Two of the original mutants produced seed after selfing and from their progenies the lines W17 and W28 were derived by further inbreeding. The line W17 shows light-red spots on an otherwise white corolla, whereas W28 plants show dark-red spots on a white corolla. Likewise, corolla's of sporogenic revertants are respectively light-red or dark-red. Somatic and sporogenic reversion frequency is somewhat lower in W17 as compared to W28.

In this paper it is shown that the difference in colour intensity and reversion frequency is controlled by a gene not linked to the *An1* locus. Dominant alleles of this gene decrease anthocyanin synthesis and the reversion frequency of unstable alleles of the gene *An1*.

Materials and Methods

All plants were cultivated in the greenhouse. In winter, extra light was given (Philips HPI/T400.5E) to a daylength of at least 14 h. The experiments were carried out with red spotted white flowering plants and selfcoloured red flowering revertants of the lines W17 and W28. Furthermore, stable recessive *An1* mutants and mutants with *An1* alleles, giving rise to lower or higher reversion frequencies than those found in lines W17 and W28, were used.

The following symbols for genes and alleles are used in this paper:

gene symbol	*phenotypic effect in a heterozygote with an1*
An1	wildtype allele as found in 'Roter Vogel'; involved in the conversion of dihydroflavonols into anthocyanins.

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anl stable recessive, causing white flowers.
$anl^{s/+}$ unstable allele, causing a red spotted, white corolla; typical for the lines W17 and W28.
$anl^{+/+}$ stable allele, descended from $anl^{s/+}$ by reversion, causing a wildtype colour intensity; stability is lower as in the wildtype allele *Anl* (see Bianchi et al. 1978).
$anl^{+/p}$ stable allele, causing a selfcoloured pale corolla.
$anl^{sl/+}$ allele, causing a red spotted, white corolla; spot frequency is lower than in the lines W17 and W28.
$anl^{sh/+}$ spot frequency, on the red spotted, white corolla is higher as compared to W17 and W28.

In crosses, the lines W17 and W28 were used, besides derivatives of them:
W17 $anl^{s/+}anl^{s/+}$, light-red spotted, white.
W28 $anl^{s/+}anl^{s/+}$, dark-red spotted, white.

Somatic reversion frequency was estimated by counting the spots on a certain surface of a corolla. The counts were made microscopically with transmitted light using a magnification of 10×10 (for details see Bianchi et al. 1978). Anthocyanins and dihydroflavonols were extracted using methanol-HCl (0,1% v/v). The identity of the anthocyanins and the concentration of dihydroquercetin-7-glucoside (DHQ-7-g) was determined using High Performance Liquid Chromatography. To 1 ml of the methanol extract, 0.75 ml H_2O and 2 ml chloroform were added, resulting in a Folch partition (Folch et al. 1957). The upper phase of this partition contains the dihydroflavonols and/or the anthocyanins. Part of this upper phase was injected into the liquid chromatograph, which was equipped with a Lichrosorb 10RP18 column. Elution was performed at a flow rate of 3 ml/min at 30 °C using 12.5% methanol and 5% acetic acid in water as a solvent for the detection of DHQ-7-g. Detection occurred at 290 nm. The retention time of DHQ-7-g using these conditions is 1.8 min, as determined with an appropriate standard. Concentrations were calculated from peak area. Anthocyanins were eluted using a 20 min gradient of 0% to 20% methanol in 10% formic acid at a flow rate of 4 ml/min and at a temperature of 45 °C. The retention times of cyanidin-3-glucoside and cyanidin-3-diglucoside were respectively 4.8 and 3.5 min, as determined with appropriate standards. Detection occurred at 530 nm.

The anthocyanin concentration was calculated from the absorbance at 530 nm, using a millimolar coefficient of 34.

Results

A. Genetical Experiments on the Difference in Colour Intensity

A light-red flowering revertant ($anl^{+/+}anl^{s/+}$) of the line W17 was reciprocally crossed with a dark-red flowering revertant ($anl^{+/+}anl^{s/+}$) of the line W28. The results of these crosses are presented in Table 1. As might be expected, both parent plants appeared to be heterozygous for $anl^{+/+}$.

The colour of the red flowering descendants of the crosses, described in Table 1, although intermediate between that of both parent plants, was closer to that of the light-red flowering parent plant (W17). This result suggests the existence of a gene, that, in the dominant state, causes a lower anthocyanin production. The gene *Inl* (for intensity) is not completely dominant. The line W17 is homozygous dominant, the line W28 is homozygous recessive, for *Inl*. The surplus of red flowering plants in the progeny can be explained by sporogenic reversion of the $anl^{s/+}$ allele towards $anl^{+/+}$, selfcoloured red. To obtain more information, 8 medium-red flowering F1 plants were reciprocally backcrossed with red spotted white flowering W28 plants. From these plants four were heterozygous and four homozygous for $anl^{+/+}$. The results are presented in Table 2. In the selfcoloured red flowering progeny a clear 1 : 1 segregation for medium-red (*Inlinl*) against dark-red (*inlinl*) was observed. The significant shortage of spotted red white flowering plants is ascribed to the occurrence of the sporogenic reversion of $anl^{s/+}$ towards $anl^{+/+}$. These results demonstrate the existence of a gene (*Inl*), influencing colour intensity in the flowers of plants from the W17 and W28 lines. This gene is not linked to *Anl*.

Table 1. Progenies of the reciprocal cross light-red × dark-red
$\frac{anl^{+/+}Inl}{anl^{s/+}Inl} \times \frac{anl^{+/+}inl}{anl^{s/+}inl}$

Parent types	Progeny		
	Medium-red	Red spotted, white	Total
Light-red × dark-red	35 (89.7%)	4 (10.3%)	39
Dark-red × light-red	26 (86.7%)	4 (13.3%)	30
Total	61 (88.4%)	8 (11.6%)	69[a]

[a] Ratio medium-red: red spotted, white $x^2_{3:1} = 6.614$ dF = 1 $p < 0.01$

B. Physiological Experiments on the Difference in Colour Intensity

Anthocyanins, synthesized in revertants, homozygous dominant or recessive for *Inl*, were identified using High Performance Liquid Chromatography. In flowers of both genotypes, the same anthocyanins are synthesized (cyanidin-3-glucoside; cyanidin-3-diglucoside), as is illustrated in Fig. 1. This indicates that *Inl* influences only the anthocyanin concentration and not the nature of the anthocyanins present in flowers of both genotypes.

From a series of small flower buds (10 mm) to ripe flowers of selfcoloured red plants, homozygous dominant for *Anl* and homozygous dominant or recessive for *Inl*, anthocyanin concentration was measured in the limb (Fig. 2).

Table 2. Progenies of reciprocal backcrosses of medium-red F1 plants to red spotted, white W28 plants

Parent plant	No.	Progeny			
		Medium-red	Dark-red	Red spotted, white	Total
W28[a] × med. red[b]	4	462 (29.1%)	438 (27.6%)	688 (43.3%)	1588
Med. red × W28		84 (28.7%)	89 (30.3%)	120 (41.0%)	293
Total		546 (29.0%)	527 (28.0%)	808 (43.0%)	1881[d, e]
W28 × med. red[c]	4	200 (48.7%)	211 (51.3%)	– –	411
Med. red × W28		276 (50.2%)	274 (49.8%)	– –	550
Total		476 (49.5%)	485 (50.5%)	– –	961[f]

[a] W28 $\frac{an1^{s/+}}{an1^{s/+}}$ [b] med-red: $\frac{an1^{+/+}}{an1^{s/+}}$ [c] med-red: $\frac{an1^{+/+}}{an1^{+/+}}$

[d] Ratio red : red spotted: $X^2_{1:1} = 37.334$ dF = 1 $p < 0.001$
[e] Ratio med-red : dark-red: $X^2_{1:1} = 0.336$ dF = 1 $p = 0.56$
[f] Ratio med-red : dark-red: $X^2_{1:1} = 0.084$ dF = 1 $p = 0.77$

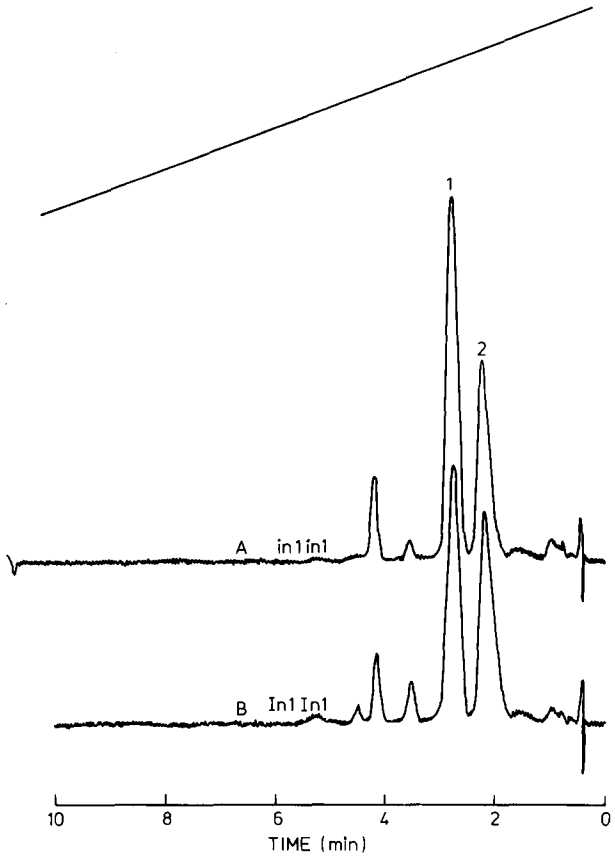

Fig. 1. HPLC analysis of anthocyanins extracted from mutants homozygous recessive (A) or dominant (B) for *In1*.
peak 1: cyanidin-3-glucoside
peak 2: cyanidin-3-diglucoside

Anthocyanin synthesis is the same in both genotypes until the flower bud is approximately 45 mm in length. From that stage of development, however, anthocyanin synthesis in *In1In1* buds slows down.

Preliminary experiments (Schram et al. 1981) have shown that anthocyanins are not in turnover in *Petunia hybrida*. We therefore conclude that *In1* interferes in the biosynthesis of anthocyanins during the last phase of flowerbud development.

The cultivar 'Roter Vogel' accumulates some dihydroquercetin-7-glucoside. An effect of *In1* on the concentration of this compound could indicate that *In1* besides the synthesis of anthocyanins, influences

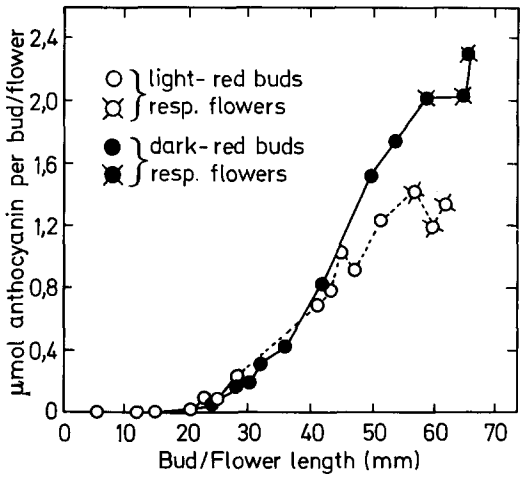

Fig. 2. Anthocyanin concentration in buds and ripe flowers of dark-red (*in1in1*) and light-red (*In1In1*) flowering genotypes during development

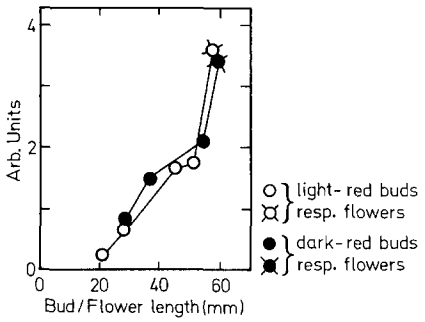

Fig. 3. Dihydroquercetin-7-glucoside concentration in dark-red (*in1in1*) and light-red (*In1In1*) flowering genotypes during flower development

the biosynthesis of other end products of flavonoid biosynthesis. However, in buds and flowers homozygous recessive or dominant for *In1*, no difference in the concentration of dihydroquercetin-7-glucoside was observed (Fig. 3). This indicates that the gene *In1* interferes in the conversion of dihydroquercetin into anthocyanin and not in the synthesis of dihydroquercetin, the precursor for dihydroquercetin-7-glucoside and cyanidin (derivatives).

C. Influence of In1 on the reversion Frequency of Unstable An1 Alleles

By further mutation of the original $an1^{s/+}$ allele, selfcoloured pale flowering ($an1^{+/p}$) and white flowering (*an1*) mutants were also obtained, in addition to mutants with a higher ($an1^{sh/+}$) or a lower ($an1^{sl/+}$) reversion frequency. These were used to investigate the influence of the gene *In1* on the reversion frequency of unstable *An1* alleles.

Selfcoloured pale flowering plants were crossed with spotted red, white flowering plants (see also Bianchi et al. 1978). Both parent plants were homozygous recessive for *In1*. The progeny of the reciprocal crosses is presented in the upper half of Table 3. On crossing the same spotted red, white flowering parent plant with a selfcoloured pale flowering phenotype, heterozygous for *In1*, two new classes of spot frequency appeared in the progeny (compare upper and lower half of Table 3). These two classes are designated "medium" and "very low" spotted. It is assumed that the occurrence of these two classes with decreased spot frequency is the result of *In1* being heterozygous. The selfcoloured red descendants of the *in1in1* crosses were all dark-red flowering, whereas those of the *In1in1* crosses were dark- or medium-red flowering (not shown in Table 3). We assume that sporogenic reversions of the $an1^{sh/+}$ allele towards $an1^{+/+}$ occur with a higher frequency than those of the $an1^{sl/+}$ allele. This explains the shortage in the "high spotted red, white" flowering progeny in the *in1in1* crosses. We had difficulties in discriminating the medium spotted phenotypes from the low and high spotted phenotypes. This explains the unequal number of individuals in the four classes.

As is illustrated in Table 3 four putative classes of spotted red, white flowering plants can be discriminated:

1) VL: $\frac{an1^{sl/+}}{an1}; \frac{in1}{In1}$ 2) L: $\frac{an1^{sl/+}}{an1}; \frac{in1}{in1}$

3) M: $\frac{an1^{sh/+}}{an1}; \frac{in1}{In1}$ 4) H: $\frac{an1^{sh/+}}{an1}; \frac{in1}{in1}$

The spotted red, white flowering plants of a representative family (C5172, Table 3) were selected and, on the

Table 3. Progenies of reciprocal crosses between selfcoloured pale, homozygous recessive and heterozygous for *In1* and red spotted, white

Parental genotypes	Progenies						
	Red spotted, white[b]				Red spotted, pale[a]	Selfcoloured red	Total
	VL	L	M	H			
$\frac{an1^{+/p}}{an1}; \frac{in1}{in1} \times \frac{an1^{sl/+}}{an1^{sh/+}}; \frac{in1}{in1}$		53		28	77	66	224
$\frac{an1^{sl/p}}{an1^{sh/+}}; \frac{in1}{in1} \times \frac{an1^{+/p}}{an1}; \frac{in1}{in1}$		11		5	9	5	30
$\frac{an1^{+/p}}{an1}; \frac{In1}{in1} \times \frac{an1^{sl/+}}{an1^{sh/+}}; \frac{in1}{in1}$[d]	84[c]	107	57	27	280	284	859
$\frac{an1^{sl/+}}{an1^{sh/+}}; \frac{in1}{in1} \times \frac{an1^{+/p}}{an1}; \frac{In1}{in1}$	13	37	15	35	118	31	249

[a] Classification of spot frequency on a pale background is difficult; results of these classifications are not presented
[b] Spot frequency classes Very Low, Medium and High, with a mean spot density/cm² of resp. 4, 21, 71 and 375 spots/cm²
[c] Including 7 plants which, on the eye, were unspotted
[d] Including family C5172

Table 4. Crosses between dark red and red spotted, white from the spot density classes "very low", "low", "medium" and "high" (see table 3)

Spot density class	Descendants			
	Selfcoloured red		Red spotted	Total
	Medium *In1in1*	Dark *in1in1*		
VL (*In1in1*)[a]	129	148	235	512
L (*in1in1*)	–	171	148	319
M (*In1in1*)	114	139	233	486
H (*in1in1*)	–	329	312	641

[a] In each class three plants were tested

basis of three counts, classified as belonging to one of the four classes mentioned above. On crossing with dark-red flowering revertants (*in1in1*), which were heterozygous for *An1*, all groups were expected to give at least 50% red flowering descendants. On crossing with mutants belonging to group 2 and 4 these red descendants should be dark red. On crossing with mutants belonging to group 1 and 3, half of the red descendants should be dark red whereas the other half should be medium red flowering. The results of these crosses are presented in Table 4. All 12 plants crossed as described above showed the expected progenies. These results indicate that dominant alleles of *In1* decrease the reversion frequency of *An1*.

Discussion

The gene *In1* clearly decreases anthocyanin synthesis in the growing flower bud of plants, producing cyanidin-3-glucoside as the main pigment. At this moment it remains unknown whether or not *In1* exhibits a comparable effect on the synthesis of other anthocyanidins, but most probably it is in this way comparable to the gene *In* (intensifier) in maize (Reddy and Peterson 1978). The stabilizer gene in *Antirrhinum majus* (Harrison and Fincham 1968) shows a completely comparable influence on the reversion frequency of the unstable pal^{rec} alleles as *In1* does on unstable *An1* alleles. *In1* does not influence the synthesis of direct precursors for anthocyanins, nor the kind of anthocyanins produced. Furthermore, turnover of anthocyanins is not likely (Schram et al. 1981). Therefore, the decrease in anthocyanin synthesis is most likely due to an effect of *In1* on the expression of one or more genes involved in anthocyanin synthesis. The effect of *In1* on reversion frequency of unstable *An1* alleles suggests that *In1* affects the transcription of the *An1* locus. This effect of *In1* is a trans-regulation effect; *In1* is not linked to the *An1* locus. Kho et al. (1978) showed that the gene *An1* controls the expression of glucosyltransferase activity in the flowers of *Petunia*. We are currently working on the effect of *In1* on glucosyltransferase activity.

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