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Cis-regulation and sporogenic reversion frequency of a new mutable *Anl* allele in *Petunia hybrida*

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A new mutable *Anl* allele is described: $anl^{s/p7}$. The genetic analysis of this allele supports the model of the *Anl* locus as consisting of a structural gene and a cis–acting regulating region. The $anl^{s/p7}$ allele shows a constant number of sporogenic revertants towards an $anl^{+/p7}$ state in subsequent generations. There is a systemic difference in number of sporogenic revertants between male (65 per cent) and female (29 per cent) gametogenesis. All the independently arisen $anl^{+/p7}$ alleles are phenotypically indistinguishable.

INTRODUCTION

The gene *Anl* influences the biosynthesis of anthocyanins in flowers of *Petunia hybrida* in several ways. Mutants homozygous recessive for *Anl* are white flowered and accumulate dihydroflavonols, precursors of anthocyanins (Gerats *et al.*, 1982*b*). A correlation has been demonstrated between *Anl* and four different enzyme activities in the flavonoid-biosynthetic pathway: UDPG: flavonoid 3-*O*-glucosyltransferase (Kho *et al.*, 1978; Gerats *et al.*, 1983), UDPG: anthocyanin 5-*O*-glucosyltransferase (Jonsson *et al.*, 1984), SAM: anthocyanin 3′,5′-*O*-methyltransferase (Gerats *et al.*, 1984) and flavone 3′-5′hydroxylase (G. Forkman personal communication). In all these cases, activity is severely reduced or virtually zero in an *anl/anl* background.

Mutable alleles of *Anl* arose spontaneously in selfed progeny of the red flowered line R27. These mutants show white flowers with red spots, and besides these give rise to descendants bearing self-coloured red flowers with a colour intensity phenotypically indistinguishable from those of R27. Mutants with selfcoloured pale flowers have been observed in some progenies (Bianchi *et al.*, 1978). Selfing of one such pale flowered derivative gave rise to a new mutable allele: $anl^{+/p6s}$ which very frequently mutates towards self-coloured pale derivatives in different colour intensities. These derivative alleles mutate with a frequency of 0·05–0·5 per cent towards new mutable alleles, like $anl^{s/p7}$ (Gerats *et al.*, submitted).

We assume that the *Anl* locus consists of a structural region and a regulating region, comparable to the *Rosy-* locus of *Drosophila melanogaster* (Chovnick *et al.*, 1976; McCarron *et al.*, 1979). On the basis of the two main types of mutation (white flowers with spots or selfcoloured pale flowers), Bianchi *et al.* (1978) hypothesised that the regulatory region in its turn consists of two subunits: one is called the mutator which switches the structural gene on or off (start of transcription?), the second, the expressor, determines the amount of gene-product (termination of transcription?). The *Anl* mutable systems show characteristics, which are described for other mutable systems as well. They resemble closely the phenotypical phenomena of the *Spm* or *En* system in *Zea mays* (McClintock, 1956; Peterson, 1965) and the instability at the *Pallida* and other loci in *Antirrhinum majus* (Fincham and Harrison, 1967).

A new mutant was obtained by selfing an $anl^{+/p6s}/anl$ plant. This mutant had light pale flowers with dark pale spots (fig. 1). In order to characterize the mutation(s) involved, we selfed the mutant and obtained progeny with six different phenotypes. Representatives of these phenotypes

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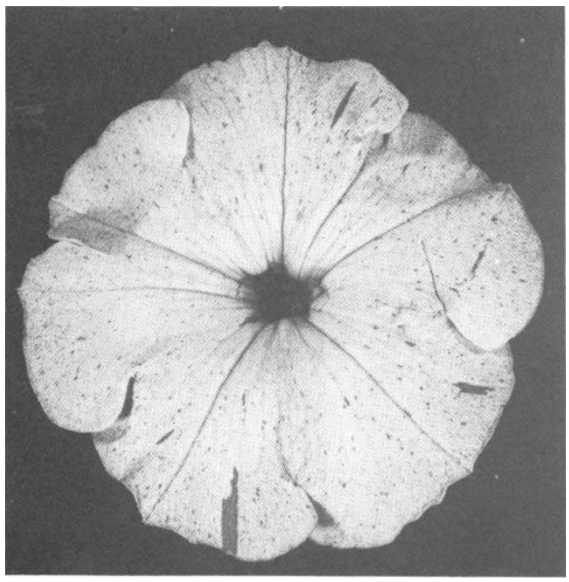

Figure 1 A *P4*, spotted *P8* coloured flower of the $anl^{+/p4}/anl^{s/p7}$ mutant obtained by selfing of an $anl^{+/p6s}/anl$ plant.

were selfed and crossed with white flowered *anl/anl* plants. The double mutant also was used to obtain information on whether the regulatory region acts only in a cis-position or also in a trans-position.

MATERIALS AND METHODS

The plants were grown in a greenhouse, with a daylength of at least 16 hours. The allelic symbols as used in this paper are listed in table 1. Because environmental conditions such as temperature, influence spot frequency and colour intensity of the flower (Bianchi *et al.*, 1978), each group of crosses was scored in as short a period as possible. A colour chart was used as a reference to score colour intensity of pale coloured flowers. This chart has nine pale classes between white and self-coloured red. The number of spots per cm^2 of a corolla was used as a measure of the somatic reversion frequency (Bianchi *et al.*, 1978). Spots were counted only on flowers with a white background. The spot densities of representatives of each family were determined on a single day to avoid the influence of environmental changes.

The percentage of sporogenic revertants (PSR) was taken as a measure of the sporogenic reversion frequency. The PSR of the $anl^{s/p7}$ allele, upon selfing of a white, spotted P7 plant

Table 1 List of different *Anl* alleles and abbreviations for the colour classes mentioned in this paper; the mentioned colour intensities occur when the specific allele is heterozygous with an *anl* allele of the line *W*78

Anl	gene involved in the conversion of dihydroflavonols into anthocyanins; influences the activity of UDPG: flavonoid-3-*O*-glucosyltransferase, UDPG: anthocyanin 5-*O*-glucosultransferase, SAM: anthocyanin 3′,5′-*O*-methyltransferase and flavanone 3′,5′-hydroxylase
$anl^{s/+}$	allele causing white flowers with red spots in a varying frequency; the mutator part of the regulating element is unstable (*s*), the expressor part is not mutated(+)
$anl^{+/+}$	allele derived from the $anl^{s/+}$ allele by reversion, it causes self-coloured red flowers, phenotypically indistinguishable from the dominant *Anl* allele
$anl^{+/p}$	allele causing self-coloured pale flowers (in the colour-class *P*1-*P*9); the mutator part of the regulating element is not mutated (+), the expressor part is mutated (*p*)
$anl^{s/p7}$	allele causing white flowers with spots in the colour-class *P*7; the allele is mutated both in the mutator (*s*) and in the expressor part (*p*7); the $anl^{s1/p7}$ allele causes a low spot frequency
$anl^{+/p7}$	allele derived from the $anl^{s/p7}$ allele by reversion, causes self-coloured pale flowers in the colour-class *P*7
anl	alleles from different origin, causing white flowers; whether the mutation affects the mutator, expressor or the structural part of the *Anl* gene is unknown
W	plant with plain white flowers
P	plant with self-coloured Pale flowers; the colour-class, based on arbitrary pale classes between white and red (R27), is indicated with a number (*P*1-*P*9)
s	plant with spotted flowers, the colour of the spots is indicated behind; For instance *WsP*7 is the abbreviation for plants with white flowers on which Pale 7 spots occur

($anl^{s/p7}/anl^{s/p7}$), was calculated as follows:

$$\mathrm{PSR} = \sqrt{1 - \frac{n\mathit{WsP7}}{n\mathrm{total}}} \times 100\%, \qquad (1)$$

where $n\mathit{WsP7}$ is the number of plants with white, spotted *P*7 flowers and *n*total is the total number of plants in the progeny. Upon selfing of the *P*7, spotted *P*9 flowered plants ($anl^{s/p7}/anl^{+/p7}$), the following formula was used:

$$\mathrm{PSR} = \sqrt{2 \times \frac{n\mathit{P9}}{n\mathrm{total}}} \times 100\%, \qquad (2)$$

where $n\mathit{P9}$ is the number of plants with *P*9 self-coloured flowers. The distinction between classes

P8 and P9 sometimes was difficult to make. Therefore the PSR of an $anl^{s/p7}$ allele upon selfing of a *P*4, spotted *P*8 plant ($anl^{s/p7}/anl^{+/p4}$) was calculated as follows:

$$PSR = \sqrt{\frac{n\text{total} - (nP6 + nP4sP8 + nWsP7)}{n\text{total} - nP6}} \times 100\% \quad (3)$$

where *nP*6 is the number of plants with *P*6 self-coloured flowers and *nP*4*sP*8 is the number of plants with *P*4, spotted *P*8 flowers.

RESULTS AND DISCUSSION

Evidence for a cis-acting regulating region at the Anl locus

When the original *P*4, spotted *P*8 flowered mutant was selfed, six different colour classes were observed in the progeny (table 2). This result can be explained by assuming that the parent plant contains two different *Anl* alleles: an $anl^{+/p4}$ allele and an $anl^{s/p7}$ allele. The $anl^{s/p7}$ allele shows a systematic sporogenic reversion to the $anl^{+/p7}$ allele, leading to a total of six classes in selfed progeny of mutant $anl^{+/p4}/anl^{s/p7}$: three classes from normal combinations between the parental alleles, and three more because of the sporogenic reversion of the $anl^{s/p7}$ allele. In order to verify the assumed genotype, plants from each colour class were selfed and crossed with white flowered *anl*/*anl* plants.

The selfing results are shown in table 3 and the crossing results are shown in table 4. Colour classes in progenies of selfed plants and crosses corresponded to expectations based on the assumed genotype. According to our model, the new mutant contained an $anl^{+/p4}$ allele with an incomplete expressor, and an $anl^{s/p7}$ allele with mutations in both the expressor and the mutator.

The $anl^{+/p4}/anl^{s/p7}$ plant has *P*4, spotted *P*8 flowers which indicated that the complete mutator part of the $anl^{+/p4}$ allele cannot activate the expressor part of the $anl^{s/p7}$ allele in which case selfcoloured *P*8 flowers should have been found. Both alleles retained their own expression, which indicated that both mutations act in a cis-position only.

Table 2 Progeny of selfing of the *P*4, spotted *P*8 mutant: $an^{+/p4}/an^{s/p7}$ (data from six capsules)

	*WsP*7*	*P*4*sP*8	*P*6	*P*7*sP*9	*P*8	*P*9	Total
number:	49	145	154	85	133	18	548
percentage:	8·4	24·8	26·4	14·6	22·8	3·1	100

$\chi^2_{\text{homogeneity between the capsules}} = 8{\cdot}31$, df = 10, $p = 0{\cdot}6$.
$\chi^2_{2:1:1}$† = 1·36, $df = 2$, $p = 0{\cdot}51$.
PSR of the $anl^{s/p7}$ allele: 47%‡

* For abbreviations, see table 1.
† For the calculation of the homogeneity and the 2 : 1 : 1 segregation, three classes were formed: *P*6, *P*4*sP*8 + *P*8 and *WsP*7 + *P*7*sP*9 + *P*9. This was done to avoid negative influence of differences in percentage of sporogenic revertants per capsule on homogeneity and segregation.
‡ Calculated using formula 3 (see material and methods).

Reversion frequency of the $anl^{s/p7}$ allele towards the $anl^{+/p7}$ allelic state

The $anl^{s/p7}$ allele reverts with a very high frequency towards an $anl^{+/p7}$ allelic state. When the *P*4 spotted *P*8 mutant was selfed, a PSR of 47 per cent was obtained (table 2). All $anl^{+/p7}$ alleles were phenotypically indistinsuishable. The $anl^{+/p4}$

Table 3 Observed numbers of progeny obtained on selfing the progeny classes of the *P*4, spotted *P*8 mutant (see table 2)

Parental phenotype	Number of families	Number of capsules	Progeny								Homogeneity			mean PSR**
			*WsP*7*	*P*4*sP*8	*P*6	*P*7*sP*9	*P*8	*P*9	other	Total	χ^2	dF	*P*	
*WsP*7	2	4	29			58		25		112	1·1	2	0·58	48%
	1†	3	121			93		9		223				23%
*P*4*sP*8	5	14	48	192	180	94	171	46		731	12·9	20	0·61	48%
*P*7*sP*9	3	7	30			160		236		426	6·3	4	0·18	49%
*P*6	7	18			1209				3‡	1212				
*P*8	3	7			77		119	62	1§	259	14·9	4	0·02	
*P*9	2	6						152		152				

* For abbreviations, see table 1.
† In this family, C5219 one of the parental $anl^{s/p7}$ alleles apparently caused a reduced reversion frequency.
‡ Three plants with *P*4, spotted *P*6 flowers occurred, all in one capsule.
§ One plant with *P*4 spotted pale flowers occurred.
** Calculated using formula 1, 2 or 3 (see material and methods).

Table 4 Observed numbers of progeny, obtained on crossing the progeny of the *P*4, spotted *P*8 mutant, with white flowered *anl*/*anl* plants, in which the latter were used as pollen donors

Parental phenotype	Number of families	Number of capsules	Progeny					Homogeneity			Mean PFR
			*WsP7**	*P4*	*P7*	other	total	χ^2	df	*P*	
WsP7 × white	2	7	117		45	2†	164	1·8	1	0·63	29%
	1‡	3	49		12		61				20%
P4sP8 × white	1	1	12	6	4		22				25%
P7sP9 × white	3	5	137		265	1§	395	2·5	2	0·28	30%
P6 × white	3	8		215			215				
P8 × white	3	7		62	55		117	2·9	2	0·23	
P9 × white	2	5			129		129				

* For abbreviations, see table 1.
† One plant with *P*8 self-coloured flowers and one plant with *P*6, spotted flowers.
‡ One of the parental $anl^{s/p7}$ alleles apparently caused a reduced reversion frequency.
§ A plant with *P*3 spotted pale flowers.

allele and the new arisen $anl^{+/p}$ alleles were relatively stable; *i.e.*, only a few mutations were observed (tables 3 and 4).

Spotted flowers of plants with mutable *Anl* alleles often show a sector in which the spot density is clearly different from the rest of the corolla. This phenomenon is assumed to be the result of a change in the reversion frequency of the mutable

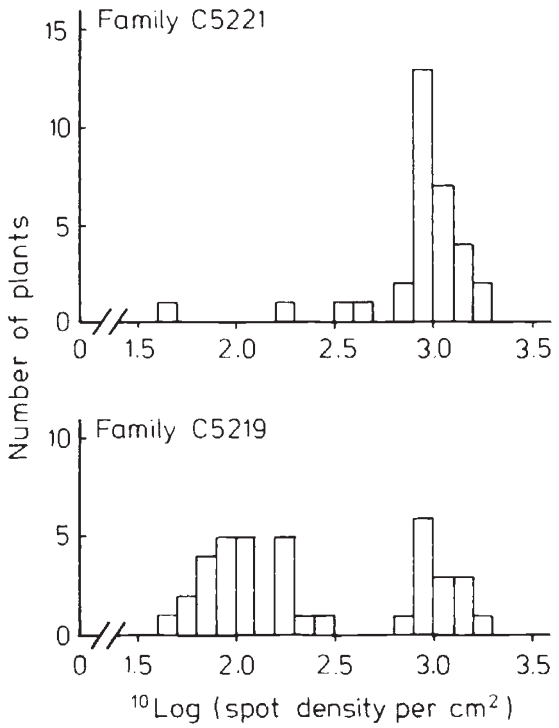

Figure 2 Spot density on the corolla's of white, spotted *P*7 coloured flowers of plants from families C5221 and C5219, derived from the cross $anl^{s/p7}/anl^{s/p7} \times anl/anl$. Family C5221 had four capsules, C5219 had three capsules.

$anl^{s/p7}$ allele and also occurs in the sporogenous tissues (Bianchi *et al.*, 1978). Thus, one has to be sure that the sporogenous tissues of the parent plant contain the original $anl^{s/p7}$ allele, when calculating the PSR from the progenies. The spot density was determined on white, spotted *P*7 flowers from plants from the crosses given in table 3. Fig. 2 shows the results of these determinations for two representative families. In family C5221, two of the 33 screened plants showed a significant decrease in somatic reversion frequency. On the other hand, more than half of the plants in family C5219 showed such a decrease, which was observed for all three capsules. The white, spotted *P*7 parent plant of family C5219 apparently contained two different alleles: an original $anl^{s/p7}$ allele and an allele with a reduced somatic reversion frequency ($anl^{s1/p7}$). In family C5219, there were twice as many plants with an $anl^{s1/p7}$ allele as with an $anl^{s/p7}$ allele; this indicates that the $anl^{s1/p7}$ allele also reverts sporogenically with a lower frequency than the $anl^{s/p7}$ allele.

Determinations of spot density in other progenies of the crosses gave results similar to those of family C5221. Therefore, we conclude that these parent plants contained the original $anl^{s/p7}$ allele.

The number of sporogenic revertants

The PSR closely resembled each other (with the exception of the family discussed earlier), when the white, spotted *P*7 and the *P*4, spotted *P*8 flowered plants were selfed. The PSR values were 47 per cent and 48 per cent respectively. Table 5 shows that the PSR was stable in subsequent generations. However, the data obtained from test crosses indicated a significantly lower PSR of about 30 per cent (table 4). The lower PSR found

Table 5 The percentage of sporogenic revertants in selfed progeny of *P*4, spotted *P*8 plants $anl^{+/P4}/anl^{+/P7}$ for four subsequent generations

Generation	Number of plants	Number of capsules	PSR*
1	584	6	46·7 ± 5·2
2	731	14	45·7 ± 15·5
3	263	6	44·7 ± 13·4
4	103	3	49·8 ± 8·7

* Calculated using formula 3 (see material and methods).

in the test crosses can be explained by two mechanisms. First, there could be a difference in sporogenic reversion frequency between male and female gametogenesis. The consequence of this hypothesis would be a PSR via the male gametogenesis of some 70 per cent. Second, the difference in apparent sporogenic reversion frequency could be caused by selection against the $anl^{s/P7}$ allele when transmitted via the microspores. This would result in a higher percentage of male $anl^{+/P7}$ gametes contributing to fertilization.

It should be noted that Farcy and Cornu (1979) described a reduced transmission frequency for different stable alleles derived from the unstable *an2-n* system in *Petunia hybrida*. Demerec (1932) noted that the rate of instability of a *miniature-gamma* allele of *Drosophila virilis* was about twice as high in males as in females. Harrison (1967) reported a difference in mean percentage of mutants in progenies of reciprocal crosses with the mutable *pal-rec* allele of *Antirrhinum majus*; although he concluded that there was a difference in mutability of the *pal-rec* allele in the male and female germline, certation cannot be ruled out with the information available. Nelson and Klein (1984) reported a significant difference in the frequency of transposition of either *Rs* or *Spm* to *Bz* when a certain stock was used as a male rather than a female parent.

In order to determine the correct hypothesis concerning the $anl^{s/P7}$ allele, the following crossing schedule was carried out:

group:	phenotype	genotype	cross
I	*P*7	$anl^{+/P7}/anl$	reciprocal with *anl*/*anl*
II	white, spotted *P*7	$anl^{s/P7}/anl$	reciprocal with *anl*/*anl*
III	*P*7, spotted *P*9	$anl^{+/P7}/anl^{s/P7}$	reciprocal with *anl*/*anl*

The group I crosses were expected to give an indication of the relative viability of the $anl^{+/P7}$ allele versus the *anl* allele. If both were equally viable, a 1 : 1 segregation and no differences between the reciprocals were expected. The group II crosses were expected to show a difference in the percentage of sporogenic revertants in the progenies of the reciprocal crosses. In case of selection against the $anl^{s/P7}$ allele the number of *anl*/*anl* plants would exceed 50 per cent of the progeny of the cross *anl*/*anl* (female) × $anl/anl^{s/P7}$ (male) assuming an equal viability of the *anl* and the $anl^{+/P7}$ alleles. A progeny with more than 50 per cent white flowered plants also theoretically could be the result of mutation of the $anl^{s/P7}$ or the $anl^{+/P7}$ allele towards *anl*. The group III crosses were carried out to test this possibility. If there was a difference in PSR between male and female gametogenesis, a 1 : 1 segregation would be expected in group II for the *anl*/*anl* progeny versus the $anl^{s/P7}/anl$ plus $anl^{+/P7}/anl$ progeny. Group III crosses also would be expected to show a difference between the reciprocal crosses in percentage of sporogenic revertants.

The results of these crosses are given in table 6. For group I, a convincing 1 : 1 segregation was obtained and there were no significant differences between the reciprocals. Group II crosses also gave a 1 : 1 segregation (*anl*/*anl* versus $anl^{s/P7}/anl$ plus $anl^{+/P7}/anl$); furthermore, there were significant differences between reciprocal crosses for the segregation of $anl^{s/P7}/anl$ versus $anl^{+/P7}/anl$ in group II. The percentage of mutations towards *anl* in group III crosses was far too low to explain the difference in PSR; these crosses also show a difference in number of sporogenic revertants between reciprocal crosses.

We conclude from these results that the difference in percentage of sporogenic revertants in progenies from selfed plants and back-cross progenies is caused by a difference in sporogenic reversion frequency in male and female gametogenesis. The PSR on selfing was 48 per cent; via the male gametogenesis a PSR of about 65 per cent was obtained. The female gametogenesis gave a PSR of about 29 per cent. With the 65 per cent and 29 per cent values, expected values for progenies obtained by selfing can be calculated; table 7 shows that the calculated expectations were in good accordance with the actual values.

Differences in reversion frequency of the mutable *Anl* system have been reported previously. Gerats *et al.* (1982*a*) described the gene *Inl*, which influences anthocyanin synthesis and the reversion frequency of mutable *Anl* alleles.

Bianchi *et al.* (1978) found that temperature and nutrition influenced the somatic reversion

Table 6 Compiled results of the reciprocal crosses

Group	Parental genotype	Progeny				
		*W**	*WsP7*	*P7*	total	PSR
I	$anl^{+/p7}/anl \times anl/anl$	265	—	260	525	—
	$anl/anl \times anl^{+/p7}/anl$	100	—	93	193	—
II	$anl^{s/p7}/anl \times anl/anl$	104	60	38	202	38·8%
	$anl/anl \times anl^{s/p7}/anl$	547	180	332	1094	64·8%
III	$anl^{s/p7}/anl^{+/p7} \times anl/anl$	1	295	329	535	23·0%
	$anl/anl \times anl^{s/p7}/anl^{+/p7}$	1	72	335	408	64·2%

χ^2 homogeneity between reciprocals:
I: $\chi 2 = 0{\cdot}1$, df = 1, $p = 0{\cdot}75$
II: $\chi^2 = 22{\cdot}2$, df = 2, $p = 0{\cdot}001$
III: $\chi^2 = 116$, df = 2, $p < 0{\cdot}001$

* For abbreviations, see table 1.

Table 7 Observed (obs.) and expected (exp.) numbers of progeny from selfed plants with the mutable $anl^{s/p7}$ allele; expectations are based on the per cent of sporogenic revertants calculated from the progeny of reciprocal crosses with *anl/anl* plants (table 6)

Parent plant		*WsP7*	*P4sP8*	*P6*	*P7sP9*	*P8*	*P9*	Total
WsP7	obs.	29			58		25	112
	exp.	27·8			63·1		21·1	112
$\chi^2 = 1{\cdot}18$, df = 2, $p = 0{\cdot}55$								
P4sP8	obs	97	337	334	179	304	64	1315
	exp.	81·7	348·5	328·7	185·1	309·1	62·0	1315
$\chi^2 = 3{\cdot}68$, df = 5, $p = 0{\cdot}60$								
P7sP9	obs.	30			160		236	426
	exp.	26·5			172·8		226·7	426
$\chi^2 = 1{\cdot}79$, df = 2, $p = 0{\cdot}41$								

frequency. They also noticed that the frequency of gametes with a reverted allele was much higher than the frequency of reverted cells in the epidermis of the corolla. Thus, the opportunity for mutation is far greater during meiosis than during mitosis. The results presented in this paper showed a difference in reversion frequency between male and female gametogenesis. This might be due to differences in micro-environmental conditions during ontogenesis of the male and female gametes. We conclude that the process of reversion starts earlier in micro- than in macrosporogenesis.

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