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Genetic analysis of instability in *Petunia hybrida*

2. Unstable mutations at different loci as the result of transpositions of the genetic element inserted at the *An1* locus

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Summary. In crossing experiments with *Petunia hybrida*, new mutations, some unstable, have been found in descendants of plants having an unstable allele of the anthocyanin gene *An1*. One of the unstable mutations affecting the new anthocyanin gene *An11* was genetically analyzed, and it was subsequently established in which step of anthocyanin synthesis that *An11* is involved. The discovery of new, unstable mutations at other loci indicates that in *Petunia* also a relation exists between unstable mutations and the presence of transposable elements in the genome. It was demonstrated that reverted alleles ($an1^{+/+}$) originating from unstable *An1* alleles are less stable than the original wild-type allele *An1*, and that reversions do not increase the chances of occurrence of new, stable or unstable mutations at other loci. These results provide additional arguments in favour of the hypothesis posed in an earlier paper that reversions of unstable *An1* alleles are not the result of excision of the inserted transposable element, but are due to the repair of secondary mutations induced by the insert in the regulatory region of the locus. Consequently, a reverted allele still contains the inserted element that may again induce mutations leading to inactivation of *An1*.

Key words: Unstable mutations – Transposition – Anthocyanin – Repair – *Petunia hybrida*

1 Introduction

One of the genes responsible for flower pigmentation in *Petunia hybrida* is *An1* (Wiering 1974). This gene controls the expression of glucosyltransferase (Kho et al. 1978). A number of unstable alleles of this gene is known. Genetic analysis of different types of mutants led to the conclusion that the *An1* locus must consist of a structural gene with an adjoining regulatory region. The unstable alleles have originated from mutations within this regulatory region. These mutations therefore only show a cis-activity, i.e., they only have an influence on the structural gene that is part of the same locus (Bianchi et al. 1978). One of the unstable alleles that has been found is the allele $an1^{s/p-+}$. Reversions of this allele occur frequently both in the epidermal layer where it becomes visible as red and pink spots on white flowers and in the sporogenous tissue, resulting in descendants with self-coloured red or pink flowers.

It is assumed that the mutation from which the allele $an1^{s/p-+}$ originated has been induced by a transposable, genetic element inserted in the regulatory region of the *An1* locus (Doodeman et al. 1983). According to McClintock and others, maize instability is also caused by the presence of a transposable element within the locus suppressing gene activity. Reactivation of the gene would then be the result of excision of the inserted element followed by transposition to another position in the genome. If it is inserted at the locus of another gene, this could give rise to a new mutation (McClintock 1965; Peterson 1970; Nevers and Saedler 1977; Starlinger 1980). If this theory that is generally accepted for higher plants, also applies to *Petunia*, reactivation of unstable *An1* alleles would be the result of excision and transposition of inserted elements.

In procaryotes, reversions associated with loss of an inserted transposable element can be detected at rates varying between 10^{-9} and 10^{-6} (Calos and Miller 1980). Unstable *An1* alleles in *Petunia*, however, show reversion frequencies up to 10^{-1}. As this is a complete different order of magnitude, it seems unlikely that each reversion of an unstable *An1* allele is associated with the loss of the inserted element. This consideration, in conjunction with conclusions from earlier experiments, has led to the theory that inactivation of *An1* is not caused by the insertion of a transposable element as such but by a mutation within the regulatory region of the locus induced by the inserted element. According to the possibility of repair of these mutations, stable or unstable mutants will

arise. Reversions of the different unstable *An1* alleles could then be the result of frequently occurring repair of the mutations. Such a reverted allele distinguishes itself from the original wild-type allele *An1* only by the persistence of the transposable element in the locus. Such an allele, indicated by $an1^{+/+}$, would be less stable than the wild-type allele since during cell division the presence of the transposable element could again lead to the occurrence of new, stable or unstable mutations.

We examined which of the two above-mentioned explanations for the occurrence of instability applies best to the phenomena observed in *Petunia*. For this purpose, descendants of unstable mutants were examined for new, unstable mutations of genes at other loci, and the stability of reverted, unstable *An1* alleles ($an1^{+/+}$) was compared to that of the wild-type allele (*An1*). Also investigated was whether reversions increase the chances of incidence of new, stable or unstable mutations somewhere else in the genome.

2 Materials and methods

2.1 Plant material

The unstable mutants used are descendants of mutant PZ 5158 L-1, that bears white flowers with red and pink spots and is heterozygous for the unstable allele $an1^{s/p-+}$ and homozygously dominant for the genes *An2, An3, An6* and *An9* (Doodeman et al. 1983). These last-mentioned 4 genes are, together with *An1*, responsible for the synthesis of anthocyanins in the flower limb (Wiering 1974). The stable white-flowering lines used are homozygously recessive for one of these anthocyanin genes. Tables 1 and 2 show the genotypes of the different lines used in the investigations described in this paper. In Table 3 the phenotypical effects of the genes are listed.

2.2 Feeding experiments

Feeding experiments were carried out according to the complementation technique developed by Kho et al. (1975). Limbs of flower buds were cultured in petri dishes with 3 ml sterile B_2 medium. Buds of donor plants were extracted with acetone. The extracts were evaporated and purified in a Folch partition (Folch et al. 1957). The upper phase was evaporated and taken up in B_2 medium (10 buds/ml). The solution was sterilized over a millipore filter (SLGS 025 OS, 0.22 µm) and 1 ml was added to the acceptor material. After incubation for 72 h at 24 °C, the petals were washed in demineralized water and extracted in 3 ml Methanol-HCl 0.5%. The anthocyanin contents were determined spectrophotometrically at 530 nm.

Table 1. Genotypes of mutant lines used in linkage experiments

Mutant line	Genes[a] (linkage group)							
	An11	*Hf1* I	*Fl* II	*Ht1* III	*Bl* IV	*Po* V	*Rt* VI	*An4* VII
W134	–	–	–	+	+	–	–	–
R51	+	–	–	+	–	+	–	–
V23	+	+	+	–	+	–	+	+

[a] genes *An1, An2, An3, An6* and *An9* are homozygously dominant in these lines
+ = homozygously dominant
– = homozygously recessive

Table 2. Genotypes of mutant lines used in feeding experiments

Mutant line	Genotype[a]					
	An1	*An2*	*An3*	*An6*	*An9*	*An11*
W39	+	+	–	+	+	+
W60	+	–	+	+	+	+
W78	–	+	+	+	+	+
W90	+	+	+	–	+	+
W99	+	+	+	+	–	+
W134	+	+	+	+	+	–

[a] + = homozygously dominant; – = homozygously recessive

2.3 Identification of intermediates

Limbs of flower buds of the stable white-flowering line W134 (*an11an11*) were extracted as described above. From the upper phase of a Folch partition 0.5 ml was hydrolyzed under N_2 by heating it with 0.5 ml 2N HCl for 10 min at 100 °C. Accumulated glucosides and aglycones were identified by means of a high performance liquid chromatograph (HPLC, Perkin Elmer) equipped with a lichrosorb 10 RP 18 column (25 cm × 4.6 mm), using 12.5% methanol, 5% acetic acid and 82.5% water as a solvent. Detection occurred at 290 nm.

3 Experimental results

3.1 An unstable mutation in a newly detected anthocyanin gene

3.1.1 Genetic analysis. In order to establish whether there are positive indications that transposable elements are involved in the occurrence of unstable alleles of *An1* in *Petunia*, experiments were carried out to search for new, unstable mutations of genes at other loci among the descendants of unstable *An1* mutants. Two mutations were found that clearly showed an unstable character. One of these mutations, described in an earlier paper, affected gene *Yg3* and resulted in small plants with yellowish green leaves bearing normally green spots (Doodeman et al. 1983). The unstable allele is indicated by $yg3^r$. It was demonstrated that *Yg3* is linked to *Ht1* situated on chromosome III (Gerats, personal communication).

The second, unstable mutation occurred in a red-flowered descendant (C 5373 B-151) obtained from a cross of a plant bearing white flowers with red and pink spots ($an1^{s/p-+}an1^{s/p-+}$) with a stable white-flowered one (*an1an1*). This plant must have originated from a reversion of $an1^{s/p-+}$ towards $an1^{+/+}$ in the sporogenous tissue of the spotted-flowering parent plant. Accordingly, the revertant C 5373 B-151 would be expected to have the genotype $an1^{+/+}an1$ and, after selfing, to yield a progeny consisting of red-flowering and white-flowering plants in a ratio of 3 : 1. However,

Table 3. List of genes and their phenotypical effect

An1 –	synthesis of anthocyanins and flavonols in flower limbs
an1an1	no synthesis of anthocyanins
An2 –	synthesis of anthocyanins and flavonols in flower limbs
an2an2	no or little synthesis of anthocyanins
An3 –	synthesis of anthocyanins and flavonols in flower limbs
an3an3	no or little synthesis of anthocyanins
An4 –	synthesis of anthocyanins in pollen (in *Hf1* – plants only)
an4an4	no synthesis of anthocyanins in pollen
An6 –	synthesis of anthocyanins and flavonols in flower limbs
an6an6	no synthesis of anthocyanins
An9 –	synthesis of anthocyanins and flavonols in flower limbs
an9an9	no or little synthesis of anthocyanins
Hf1 –	synthesis of myricetin and delphinidin (derivatives)
hf1hf1	no synthesis of myricetin and little of delphinidin (derivatives)
Fl –	synthesis of flavonols
flfl	little synthesis of flavonols
Ht1 –	synthesis of quercetin and cyanidin (derivatives)
ht1ht1	synthesis of kaempferol and little of cyanidin (derivatives)
Bl –	flower limb normal
blbl	flower limb very short, blocking the entrance of the tube (blind)
Po –	pollen without yellow pigment
popo	pollen with yellow pigment
Rt –	synthesis of anthocyanin-3-rutinosides
rtrt	synthesis of anthocyanin-3-glucosides

in the progeny obtained from a selfing (D 5518), in addition to 24 red-flowered and 11 stable white-flowered descendants, 5 plants were encountered bearing white flowers with large numbers of red spots and, only very rarely, pink spots (Fig. 1). Since the original unstable allele $an1^{s/p-+}$ leads to white flowers with red and pink spots in an average ratio of 3 : 1, it was assumed that these 5 plants resulted from a new mutation of a quite different nature in the reverted allele $an1^{+/+}$.

Selfing of one of the 5 plants, D 5518-1, yielded a progeny which consisted for the larger part of plants phenotypically similar to the parent plant. Crossing of this plant with a white-flowering plant homozygously recessive for *An1* resulted in self-coloured red-flowering descendants exclusively. From these results it could be concluded that the mutation in question did not affect *An1*. In order to ascertain whether this concerns a mutation either in one of the other genes known to be involved in anthocyanin synthesis in the limbs of the corolla (viz., *An2, An3, An6* or *An9*), or in a gene that was unknown until now because no mutation had previously been recorded of it, the unstable mutant D 5518-1 was also crossed with lines homozygously recessive for one of the above-mentioned anthocyanin genes. Each progeny obtained from these crosses consisted exclusively of descendants bearing self-coloured flowers. Evidently, complementation occurred in each case, which justifies the conclusion that the unstable mutation under discussion affects a new anthocyanin gene. This gene will be denoted *An11*.

Accordingly, the genotype of the red-flowered revertant C 5373 B-151 must have been:

$an1^{+/+}\ an1\ An11\ an11^{s/+}$; $an11^{s/+}$

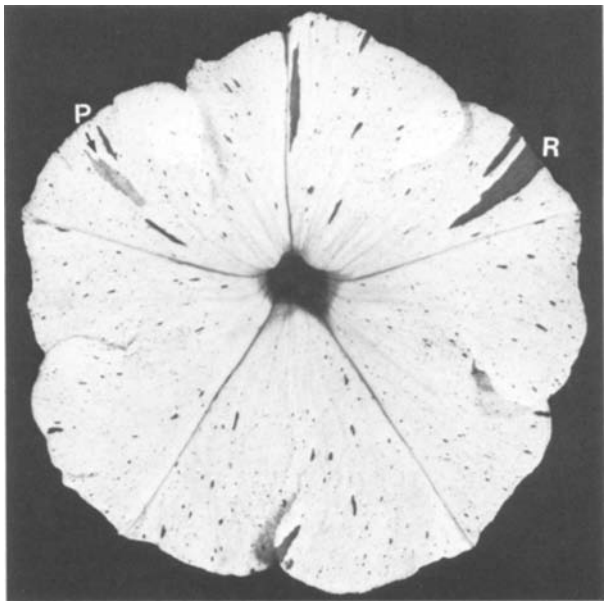

Fig. 1. White flower with red spots of plant no. D 5518-1 (genotype: $an11^{s/+}an11^{s/+}$). *R* = red spot, *P* = one of the very rare pink spots

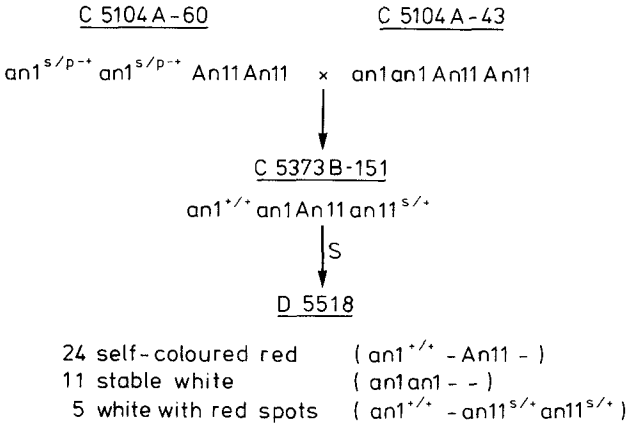

Fig. 2. Origin of revertant C 5373 B-151 and its descendants

indicates the unstable allels originating from *An11* by mutation and is responsible for white flowers with spots (s) that are as red as the wild-type (+). The origin of C 5373 B-151 and its descendants is given in Fig. 2. The segregation found in family D 5518 does not significantly differ from the ratio 9:3:4 that is expected if *An1* and *An11* inherit independently (Table 4).

After selfing the 5 spotted-flowering plants from D 5518, the mutants could be divided into two distinct groups. Two plants yielded only a small percentage of stable, white-flowering descendants and the other three plants produced a progeny that contained over 25% of stable, white-flowering plants (Table 5). These results point to the genotype $an1^{+/+}an1^{+/+}an11^{s/+}an11^{s/+}$ for plants D 5518-1 and -2, and to the genotype $an1^{+/+}an1an11^{s/+}an11^{s/+}$ for plants D 5518-3, -4 and -5.

The stable white-flowering descendants of D 5518-1 and -2 then would be the result of both $an11^{s/+}$ alleles being mutated towards stable white (*an11*) in the sporogenous tissue of the parent plant, and the red-flowering descendants of mutations of $an11^{s/+}$ towards red ($an11^{+/+}$). The pink-flowered descendant might be the result of a rarely occurring, secondary mutation of $an11^{s/+}$ towards pink ($an11^{+/p}$). The possibility of such mutations is also manifest from the pink spots that are occasionally observed in the flowers of the parent plants.

From the segregation in the progenies of D 5518-1 and -2 it can be calculated that mutations from spotted ($an11^{s/+}$) towards white (*an11*), to pink ($an11^{+/p}$) and to red ($an11^{+/+}$) occurred in 12.20%, 0.03% and 0.78% of the gametes, respectively, under the assumption that the frequency of mutation was the same in the male gametes and in the female gametes.

The larger part of the stable white-flowering descendants of D 5518-3, -4 and -5 must be the result of segregation of the recessive allele *an1*. A small percentage of these white-flowering plants, together with the red-flowering and pink-flowering descendants must have originated from mutations of $an11^{s/+}$ in the sporogenous tissue of the parent plant.

The plants D 5518-1, -3, -4 and -5 were crossed with white-flowering plants homozygously recessive for *An1* (Table 6). The results are fully in agreement with the assumed genotypes of these plants.

Table 4. Segregation in family D 5518 obtained from selfing a red-flowering revertant, C 5373 B-151

Parent		Progeny (D 5518)						
Plant no.	Genotype	Self-coloured red		White with red spots		Unspotted white		Total
		No.	%	No.	%	No.	%	No.
C 5373 B-151	$an1^{+/+}an1An11an11^{s/+}$	24	*60.0*	5	*12.5*	11	*27.5*	40

$\chi^2_{9:3:4} = 1.033$; df=2; $P = 0.60$

Table 5. Progenies obtained from selfing 5 white-flowered plants with red spots

Parents		Progeny								
Plant no.	Genotype	White with red spots		Unspotted white		Self-coloured red		Pink with red spots		Total
		No.	%	No.	%	No.	%	No.	%	No.
D 5518-1 and -2	$an1^{+/+}an1^{+/+}an11^{s/+}an11^{s/+}$	1,238	*96.9*	19	*1.5*	20	*1.6*	1	*0.1*	1,278
D 5518-3, -4, -5	$an1^{+/+}an1an11^{s/+}an11^{s/+}$	776	*64.7*	417	*34.8*	6	*0.5*	1	*0.1*	1,200

Table 6. Progenies obtained from crosses of white-flowering plants with red spots with stable white-flowering plants (*an1an1*)

Parents		Progeny		
Plant no.	Genotype	Red	White	Total
D 5518-1	$an1^{+/+}an1^{+/+}an11^{s/+}an11^{s/+}$ × white (*an1an1*)	60	–	60
D 5518-3, -4, -5	$an1^{+/+}an1an11^{s/+}an11^{s/+}$ × white (*an1an1*)	121	123	243

Table 7. Progenies obtained from crosses of white-flowering plants with red spots with stable white-flowering plants (*an11an11*)

Parents		Progeny							
White	White with red spots	White with red spots		Unspotted white		Self-coloured red		Pink	Total
		No.	%	No.	%	No.	%	No. %	No.
an11an11 × $an11^{s/+}an11^{s/+}$		778	*88.3*	97	*11.0*	6	*0.7*	– –	881

Table 8. F_2 of cross R51 × W134 $\left(F_1: \frac{bl}{Bl}\frac{Po}{po}\frac{An11}{an11}\right)$

Gene	Segregation	$\chi^2_{3:1}$	*P*	Linkage		$\chi^2_{2\times2}$	*P*
An11	147 : 51	0.061	0.80				
Bl	146 : 52	0.168	0.68	*An11-Bl*	107 : 40 : 39 : 12	0.265	0.61
Po[a]	119 : 34	0.630	0.43	*An11-Po*	92 : 25 : 27 : 9	0.210	0.65
Fertile : sterile pollen	153 : 45	0.546	0.46	*An11*-pollen	117 : 30 : 36 : 15	1.748	0.19

[a] In fertile pollen only

Some stable white-flowering descendants of D 5518-1 and -2 were likewise crossed with lines homozygously recessive for one of the genes *An1, An2, An3* or *An6*. Complementation occurred in these cases as well. The results of crosses of these white flowered descendants with spotted-flowered plants of genotype $an11^{s/+}$-$an11^{s/+}$ are given in Table 7. These results support the assumption that the stable white-flowered descendants obtained from selfings of D 5518-1 and -2 originated from mutations of $an11^{s/+}$ towards *an11*. The white-flowered and red-flowered descendants obtained from the crosses shown in Table 7 originated from mutations of $an11^{s/+}$ towards *an11* and $an11^{+/+}$, respectively. The mutation frequencies found are comparable to those given above. That no pink-flowered descendants were found must be attributed to the very small mutation frequency towards pink.

3.1.2 Chromosomal localization. Each of the seven linkage groups found in *Petunia hybrida* is located on one of its seven chromosomes (Maizonnier and Moessner 1979). In order to determine in which group gene *An11* is located, several crosses were made. The results of these linkage experiments are listed in Tables 8 and 9.

In the F_2 of cross R51 × W134, plants with pollen of poor quality were found, probably due to a mutation in some gene affecting pollen fertility since the ratio of fertile pollen to sterile pollen was found to be 3 : 1 (Table 8). Segregation of gene *Po* could only be scored in plants with fertile pollen. Segregation of genes *Hf1* and *Rt* was scored in plants with self-coloured flowers only, and segregation of gene *An4* in plants with genotype *An11-Hf1-Rt* only (Table 9). Since there is no significant deviation of a 3 : 1 ratio for the genes *Hf1, Rt* or *An4* among the plants scored, there is no reason to assume linkage between *An11* and one of these genes.

Since no white-flowering plants accumulating kaempferol (genotype *an11an11ht1ht1*) were found, it can be concluded that gene *An11* is linked to *Ht1*

Table 9. F_2 of cross V23 × W134 $\left(F_1: \frac{htl}{Htl}\frac{Hfl}{hfl}\frac{Rt}{rt}\frac{Fl}{fl}\frac{An4}{an4}\frac{An11}{an11}\right)$

Gene	Segregation	$\chi^2_{3:1}$	P	Linkage		$\chi^2_{2\times2}$	P
An11	131:52	1.138	0.29				
Htl	149:32	5.173	0.02	*An11-Htl*	97:32:52: 0	15.670	≪ 0.001
Hfl[a]	100:31	0.125	0.72	*An11-Hfl*	100:31: –: –		
Rt[b]	107:24	3.117	0.08	*An11-Rt*	107:24: –: –		
Fl	142:39	1.151	0.28	*An11-Fl*	102:27:40:12	0.101	0.75
An4[b]	60:19	0.038	0.85	*An11-An4*	60:19: –: –		

[a] In *An11*- plants only
[b] In *An11-Hfl-Rt*-plants only

situated on chromosome III. Linkage to other markers was not found.

3.1.3 Biochemical characterization. Intermediates of anthocyanin biosynthesis accumulated in flower limbs of *an11an11* mutants were investigated using HPLC and subsequently compared to the accumulation of intermediates in an *an1an1* mutant with the same genetic background. The main compounds found in *an11an11* mutants were dihydroquercetin-7-glucoside and, in smaller quantities, dihydroquercetin-4′-glucoside. In a hydrolyzed sample, the aglucon of these compounds, dihydroquercetin, was found. The same intermediates were found in the *an1an1* mutant (see also Gerats et al. 1982). These data indicate that the conversion of dihydroflavonols is blocked by the *An11* mutation, as is the case in *an1an1* mutants.

Figure 3 shows the biosynthesis of anthocyanins in flowers of *Petunia hybrida* (Gerats et al. 1982). In order to determine in which biosynthetic step gene *An11* is involved, feeding experiments were carried out. Isolated flower limbs of plants homozygously recessive for one of the known anthocyanin genes were incubated with an extract of flowers of plants homozygously recessive for *An11*. The reverse experiments were also performed. Results are presented in Table 10.

Anthocyanin synthesis is provoked in flower limbs of W39 (*an3an3*) after administration of extracts from flowers of plants homozygously recessive for *An11*. Apparently these extracts contain intermediates that can be converted into anthocyanins by flowers of W39. This indicates that gene *An11* is involved in a later step of anthocyanin synthesis than gene *An3*.

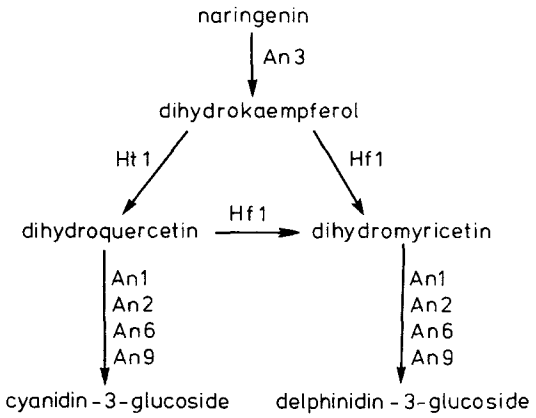

Fig. 3. Biosynthesis of anthocyanins in *Petunia hybrida*

Table 10. Reciprocal feeding experiments with flower limbs of *an11an11* mutants and of lines homozygously recessive for one of the genes *An1, An2, An3, An6* or *An9*

Acceptor	Donor[a]	Synthesis of anthocyanins[b]	No. of flower limbs	Anthocyanin-contents/ flower limb ($A_{530} \pm SD$)
W134 (*an11*)	blanco[c]	–	16	0.05 ± 0.01
	dhQ[d] (1 mM)	–	8	0.05 ± 0.01
	an1an1	–	8	0.06 ± 0.00
	an2an2	–	7	0.07 ± 0.01
	an3an3	–	8	0.06 ± 0.01
	an6an6	–	8	0.06 ± 0.01
	an9an9	–	8	0.08 ± 0.01
W78 (*an1*)	blanco	–	4	0.05 ± 0.00
	an11an11	–	8	0.05 ± 0.01
W60 (*an2*)	blanco	–	4	0.07 ± 0.01
	an11an11	–	7	0.09 ± 0.02
W39 (*an3*)	blanco	–	4	0.06 ± 0.00
	an11an11	+	7	0.35 ± 0.06
W90 (*an6*)	blanco	–	4	0.02 ± 0.00
	an11an11	–	7	0.04 ± 0.01
W99 (*an9*)	blanco	–	9	0.04 ± 0.01
	dhQ (1mM)	±	8	0.13 ± 0.02
	an11an11	±	8	0.08 ± 0.02

[a] 1 ml of extract of flowerbuds was added to 3 ml B_2 medium
[b] + = synthesis of anthocyanins; – = no synthesis of anthocyanins; ± = little synthesis of anthocyanins
[c] 4 ml B_2 medium
[d] dihydroquercetin

Administration of extracts from flowers of *an11an11* mutants to flowers of W99 (*an9an9*) resulted in some anthocyanin synthesis. This can be explained by the fact that in mutants homozygously recessive for *An9*, anthocyanin synthesis is not completely blocked, but can occur to some small extent if dihydroqercetin is present.

No anthocyanin synthesis was induced in experiments with plants homozygously recessive for one of the other anthocyanin genes. In earlier feeding experiments, isolated flower limbs of plants homozygously recessive for one of the genes *An1, An2, An6* or *An9* incubated with extracts of flowers of plants homozygously recessive for one of the other genes never provoked anthocyanin synthesis (Kho et al. 1977; Gerats et al. 1982). In flowers of these mutants dihydroflavonols are always found to have accumulated, which indicates that these plants are not able to convert dihydroflavonols in anthocyanins. From the results presented in this paper it can be deduced that this also applies to mutants homozygously recessive for *An11*. This indicates that gene *An11* is involved in the conversion of dihydroflavonols into anthocyanins, as are the genes *An1, An2, An6* and *An9*.

3.2 Comparison of the stability of reversions of unstable An1 alleles with that of the wild-type

In earlier experiments it was demonstrated that $an1^{+/+}$ alleles originating from reversions of the unstable allele $an1^{s/+}$ in the sporogenous tissue are less stable than the wild-type allele *An1* (Bianchi et al. 1978).

It is manifest from the frequent occurrence of white spots in red flowers of heterozygous plants with genotype $an1^{+/+}an1^{s/+}$ that reverted alleles are also less stable in somatic tissue. Such white spots on the red corolla have not been encountered in plants heterozygous for the original wild-type allele. The number of white spots was taken as a measure of the mutation rate of the reverted allele. In order to be able to inspect a large number of flowers, only spots $\geqq 2$ mm were counted (Table 11). The frequency of occurrence of these spots was expressed in the mean number of spots per 100 flowers. In addition to the mutated $an1^{+/+}$ allele, the cells in the white spots also contain the unstable $an1^{s/+}$ allele. Therefore, as might be expected, smaller red dots were visible in all white spots.

Table 11. Number of white spots $\geqq 2$ mm in red flowers of revertants

Genotype	No. of plants	No. of flowers	No. of white spots $\geqq 2$ mm	Mean no. of white spots per 100 flowers
$an1^{+/+}an1^{s/+}$[a]	400	4,475	285	6.4
$an1^{+/+}an1$[b]	1,785	3,472	1,701	49.0

[a] The allele $an1^{+/+}$ in these plants originated from reversions of $an1^{s/+}$

[b] The allele $an1^{+/+}$ in these plants originated from reversions of $an1^{s/p-+}$

It was investigated if reverted alleles originating from the unstable allele $an1^{s/p-+}$ showed a decreased stability as well. For this purpose plants were used with genotype $an1^{+/+}an1$ (Table 11). The mean number of white spots $\geqq 2$ mm per 100 flowers was considerably larger here, which points to an even smaller stability. In 1,470 of the 1,701 white spots counted, no coloured dots were visible, indicating that mutations of $an1^{+/+}$ towards stable white (*an1*) had occurred. Red dots were observed in the remaining 231 spots, which shows that new mutations of the reverted allele can again be unstable. In 5 cases, red and pink dots could clearly be discerned in the white spots which indicates that the mutations in question had resulted in unstable alleles similar to $an1^{s/p-+}$ from which the reverted allele originated.

3.3 The frequency of occurrence of recessive mutations in unstable mutants and their revertants

It is generally accepted for higher plants that insertion of a transposable element within the locus of a gene may lead to the incidence of an unstable allele characterized by the frequent occurrence of reversions both in somatic and sporogenous tissues. If these reversions would be the result of precise excision followed by reintegration of the released element somewhere else in the genome, these transpositions could give rise to new recessive mutations that might be stable or unstable.

Reverted alleles are dominant. Possible new mutations resulting from insertions of transposable elements at other loci, however, would be recessive with regards to the alleles from which they originated. Accordingly, revertants heterozygous for both the reverted allele and such a mutation will merely exhibit the reversion. The new mutation, however, will only find expression in progenies obtained from selfings.

From the view outlined above a reversional event is a prerequisite for the occurrence of a mutation resulting from insertion at another locus. It follows that aberrant phenotypes resulting from such mutations might be expected to be found at a much higher rate in progenies of self-coloured revertants than in those of variegated-flowering mutants.

In order to investigate this, selfings were made of 40 white-flowering mutants with red and pink spots and of 77 red-flowering revertants, all originated independently of one another from the unstable allele $an1^{s/p-+}$. The progenies of these plants were examined for plants with morphological or other obvious aberrations that

Table 12. Progenies obtained from selfing white-flowering mutants with red and pink spots ($an1^{s/p-+}an1$) and red-flowering revertants ($an1^{+/+}an1$) all originated independently of one another from the unstable allele $an1^{s/p-+}$

Parents		Progeny				$\chi^2_{1:3}$	P
Phenotype	No.	Aberrant phenotypes	No.	Normal	Total		
variegated	32	–	–	3,307	3,307	–	–
variegated	1	aberrant leaves and poorly developed flowers (alf^r)[b]	85	318	403	3.283	0.07
variegated	1	small plants with dark green leaves and small flowers (*dg5*)	28	100	128	0.667	0.41
variegated	6[a]	very small plants that died prematurely	165	499	664	0.008	0.93
self-coloured red	62	–	–	8,465	8,465	–	–
self-coloured red	4[a]	yellowish green leaves	155	429	584	0.740	0.39
self-coloured red	1	yellowish green leaves with normally green spots ($yg3^r$)[b]	39	167	206	4.045	0.04
self-coloured red	1	white flowers with red spots ($an11^{s/+}$)[b]	5	24	29	0.070	0.79
self-coloured red	1	deformed flowers with a twist in the flower tube (*px*)	64	157	221	1.848	0.17
self-coloured red	1	deformed flowers with a split corolla	37	127	164	0.520	0.47
self-coloured red	7[a]	very small plants that died prematurely	147	580	727	8.859	0.003

[a] Progenies in which similar aberrant phenotypes were encountered are combined. The mutants from the separate families, however, exhibited mutually small, but sometimes very manifest differences
[b] These mutations clearly showed an unstable character

could be the result of mutations in major genes controlling disontinuous or qualitative characteristics. The results are listed in Table 12.

The segregations observed indicate that the aberrant phenotypes result from recessive mutations. A significant deviation from the expected 3 : 1 segregation, due to a shortage of the aberrant type, was found in the categories of the slowly growing and very late-flowering plants with yellowish green leaves ($P=0.04$) and of the very small plants strongly retarded in development and never growing larger than a few centimetres and dying within a few weeks ($P=0.003$). Undoubtedly, in both cases a decreased viability led to a selective drop-out of the new mutant.

Table 13. Comparison of progenies obtained from selfing white-flowering mutants with red and pink spots and red-flowering revertants all originated independently of one another from the unstable allele $an1^{s/p-+}$

Parents		Progenies	
Genotype	No.	Normal[a]	Aberrant[b]
$an1^{s/p-+}an1$	40	32	8 (20.0%)
$an1^{+/+}an1$	77	62	15 (19.5%)
Total	117	94	23

[a] No. of families without aberrant phenotypes
[b] No. of families that consisted partly of plants with an aberrant phenotype
$\chi^2_{2\times2}=0.004$; df = 1; $P=0.95$

Three mutations were found that clearly showed an unstable character, viz., two ($yg3^r$ and $an11^{s/+}$, described in the aforegoing) in the 77 progenies of the plants with the reverted *An1* allele, and another one (alf^r) descended from one of the 40 unstable *An1* mutants. In the latter case, the instability is manifest from the development of occasional branches with completely normal leaves and flowers.

Table 13 summarizes the results of the 117 selfings described above. It is evident from this Table that the chance of yielding new, stable or unstable, recessive mutants, is not smaller in a plant with an unstable *An1* allele as compared to a plant with a reverted allele. This conclusion is supported by the recent discovery of two new unstable mutants descended from the unstable $alf^r alf^r$ and $an11^{s/+}an11^{s/+}$ mutants, respectively. These new mutations will be described in a forthcoming paper.

4 Discussion

The discovery of new, unstable mutants among the descendants of plants with an unstable *An1* allele might indicate that whatever has caused instability at the *An1*

locus has been transferred to other loci. These results support the assumption that also in *Petunia,* as generally accepted for other higher plants, a relation exists between the occurrence of instability and the presence of transposable elements in the genome.

In maize, unstable mutations in gene *Sh* induced by the controlling element *Ds* have been examined at the molecular level. These experiments have, in fact, demonstrated the presence of additional DNA at the *Sh* locus (Burr and Burr 1981; Döring et al. 1981).

In *Petunia,* reversion of an unstable *An1* allele is assumed to be the result of frequently occurring repairs of a mutation that was induced by a transposable element inserted in the regulatory region of the *An1* locus. (Doodeman et al. 1983). According to the theory of McClintock (1965) reversion of unstable alleles in maize is the result of the precise excision of the inserted element, leading to complete restoration of the wild-type. If this is also applicable to *Petunia,* reversion of unstable *An1* alleles would result in wild-type stability. The observation that alleles originating from reversions of unstable *An1* alleles are less stable than the wild-type allele *An1* does not agree with this supposition. It supports, to the contrary, the theory postulated for *Petunia* that a reverted allele ($an1^{+/+}$) still contains the transposable element that may again induce a mutation. Also in recent experiments on the molecular level of the *Sh* locus in maize indications were found that reversion of an unstable allele can occur without excision of the inserted element (Burr and Burr 1982).

According to the theory accepted for maize, excision of the inserted element, followed by transposition, might give rise to new, recessive mutations as the result of insertion of the element in other genes leading to a permanent or unstable inactivation of those genes. Consequently, such new, stable or unstable, mutations, might be expected to be found at a much higher rate in revertants than in unstable mutants. The experiments described in this paper, however, yielded no indications that in *Petunia* the frequency of occurrence of recessive mutations at other loci is affected by the incidence of reversions of unstable *An1* alleles, since new mutants were found at the same rate in the progenies of revertants and of plants with an unstable *An1* allele.

The results discussed above might be taken as a clear indication that reversion of unstable *An1* alleles are not the result of excision of the transposable element inserted at the locus. They rather substantiate the hypothesis that these reversions occur as the result of frequently ocurring repair of a mutation that was induced by a transposable element inserted in the regulatory region of the locus. Results of crossing experiments with various unstable *An1* mutants indicated that the mutations induced by the transposable element might be deletions. In procaryotes the occurrence of deletions induced by an inserted transposable element has been demonstrated in several experiments (Reif and Saedler 1975; Wang et al. 1980). In maize experimental results indicate that in a particular mutant a deletion may have occurred extending from the integration site of the controlling element *Ds* in the *Sh* locus (Döring et al. 1981).

The discovery of at least five new, unstable mutations at other loci in *Petunia* supports the assumption that transposition of the element inserted at the *An1* locus did occur. Since reversions of unstable *An1* alleles do not seem to be the result of excisions of the inserted element, the transpositional events that gave rise to these new mutations must have occurred independently of these reversions. For the time being no definite conclusions can be made about the mechanism of transposition in *Petunia.* In red flowers of those $an1^{s/p-+}$ revertants that exhibited in their progenies the unstable mutations $an11^{s/+}$ and $yg3^{r}$, white spots were frequently observed. This might be taken as an indication that even after the transposition leading to the occurrence of those new unstable alleles, the transposable element is still present at the *An1* locus. This might be explained by assuming that the original transposable element inserted at the *An1* locus was duplicated, whereupon the new copy was integrated at the *An11* and *Yg3* locus, respectively.

Grindley and Sherratt (1979) and Shapiro (1979) have proposed similar models for transposition in procaryotes. According to these models, transposition does not require excision of the inserted element. Experimental results that indicate that excision of transposon Tn5 and Tn10 can occur independently of the transposase function of these elements support this theory (Egner and Berg 1981; Foster et al. 1981).

To acount for the unstable character of the mutations in *An1* and other loci it is assumed that these loci consist of a structural gene with an adjoining regulatory region. Insertions of a transposable element in the regulatory region of the loci in question might cause instability by inducing mutations. Frequent repair of such mutations during cell divisions would then lead to reversions. When discussing the *bz-m4* mutant of maize, Gerats et al. (1983) advance arguments in favour of the assumption that the closely linked *Sh* and *Bz* loci also consist of a regulating and a structural part. They point out that the characteristics of this mutant might be explained by assuming that the structural part of the *Sh* locus has been lost by a deletion. As a result, a fusion must have occurred of the regulating part of the *Sh* gene with the structural part of the *Bz* gene, thus causing transcription of *Bz* according to the *Sh* activity pattern.

Two of the genes in which unstable mutations occurred, have now been located. Both genes, viz., *Yg3* and *An11*, are linked to *Ht1*, situated on chromosome III. This could well be a coincidence. However, if more genes, mutated as a result of transpositions away from the *An1* locus, would be found to be linked to *Ht1,* it might indicate a preferential region for integration of the transposable element. Preference for certain regions in the genome has been described for transposons in procaryotes (Calos and Miller 1980).

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