Theor Appl Genet (1984) 67:345–355

Genetic analysis of instability in *Petunia hybrida*

1. A highly unstable mutation induced by a transposable element inserted at the *An1* locus for flower colour

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Received July 13, 1983
Communicated by H. F. Linskens

Summary. A hypothesis is proposed to interpret the results of crossing experiments with unstable mutants of *Petunia hybrida* having variegated flowers and showing variation in the colour intensity and the degree of spotting of the corolla. It is postulated that the *An1* locus, which is involved in anthocyanin synthesis, is composed of a structural gene with an adjoining regulatory region, the latter in turn comprising two components, viz., the 'mutator', responsible for the activation of the structural gene, and the 'expressor', controlling the rate of activity. Unstable *An1* alleles originate from deletions induced by a transposable element inserted within the regulatory region. Such deletions extend from one of the ends of the inserted element across the adjacent DNA and thus may include parts of the 'expressor' and the 'mutator'. Reversions result from repair of the deletions, the inserted element not necessarily becoming lost in the process.

Key words: Unstable mutation – Transposable elements – Deletions – Repair – *Petunia hybrida*

1 Introduction

The red flower colour of *Petunia hybrida* cultivar 'Roter Vogel' is brought about by the synthesis of cyanidin-3-glucoside in the epidermal cells of the corolla (Wiering 1974). Owing to a spontaneous mutation in gene *An1*, selfing of the thus mutated individuals of this cultivar yielded some white-flowered descendants. However, the white flowers were dotted with numerous red spots owing to the frequent incidence of reversions in the dermal layer of the developing flower buds (Bianchi et al. 1978). The reversions were not restricted to epidermal cells but also occurred in the subdermal sporogenous tissue, which resulted in the appearance of self-coloured red-flowering plants in the progeny of mutants with red-spotted white flowers. The spots on the corolla limbs usually were of the same hue as the wild-type red colour, but some of the mutants had spots of a lighter red to pink colour. Mutants with such pink-spotted flowers again differed from one another in the colour intensity of the spots; this difference was also observed in the several self-coloured pink-flowered descendants of these mutants. Some mutants were encountered that differed mutually in the density of spotting, apparently a result of mutations towards higher or lower frequencies of reversion. On average, spot densities varied from below one to over 10,000 spots/cm^2 of the surface of the corolla.

The very high rates of reversion recorded strongly suggest that in such mutants far less specific and, accordingly, much more easily reparable alterations had taken place than in cases of mutation in the nucleotide sequence of a structural gene. This led us to postulate that the *An1* locus is built up from a structural gene coding for some protein involved in anthocyanin synthesis, and a regulatory region controlling the activation of the structural gene at the proper site and at the right time. For the gene *An1* this means that the activity is restricted to the epidermis of the flower limb and to a brief time span during the final stages of development of the flower.

The results from a large number of selfings and crosses between mutants bearing self-coloured flowers of different hues and mutants with spotted corollas exhibiting a wide range of reversion frequencies indicate that both mutations inducing variation in hue and those inducing differences in spot density are active only in the cis-position. It follows that a mutation only influences the activity of the structural gene belonging to the same locus in which this mutation took place.

Since colour intensity and reversion frequency apparently vary independently from one another, it was concluded that the regulatory region of the locus must be compounded of two components to be referred to by the terms of 'mutator' and 'expressor'. Mutations within the 'mutator' prohibit pigment

synthesis, resulting phenotypically in white flowers. Such a mutation may be stable, which yields unspotted white-flowered mutants. If it is unstable, repair may occur all over the corolla limb, which yields coloured spots, the colour intensity of these spots being decided by the state in which the 'expressor' finds itself: is it unmutated, the spots will be wild-type red, but if a mutation took place within the 'expressor' they will be pink. If a mutation took place within the 'expressor' alone this results in a self-coloured pink corolla. The following alleles of the gene *An1* can be distinguished:

An1: self-coloured red, the wild-type allele as present in cultivar 'Roter Vogel'.

an1: stable white owing to such a mutation in the *An1* locus that the potential to synthesize anthocyanins got lost completely.

$an1^{s/+}$: a group of alleles originating from mutations within the 'mutator' and inducing white, red-spotted corollas. These alleles may differ in their reversion frequencies.

$an1^{+/p}$: a group of alleles originating from mutations within the 'expressor' and inducing self-coloured pink flowers whose hue may vary from a very pale pink to almost as red as the wild-type red.

$an1^{+/+}$: the wild-type revertant, whose stability may be lower than that of the original wild-type allele *An1*.

In order to gain some insight into the nature of the mutations inducing the incidence of unstable alleles of genes for flower colour and of other genes, a genetic analysis of an extremely unstable flower colour mutant was carried out.

2 Materials and methods

The experiments were carried out with mutant PZ 5158 L-1 and with a number of descendants obtained by selfing of this mutant. This mutant produced white flowers with a large number of red and a fairly great number of pink spots. White sectors also occurred. The variegation is attributable to a highly unstable allele of the colour gene *An1*.

All plants were reared and grown in a glasshouse. Supplementary lighting was provided during the cold season by means of a Philips HPI (TH 00.5SE) installation adjusted to a day-length of at least 14 h.

The spots on the corollas vary in size: the earlier the mutation, the larger the spots. Each individual colour spot is the result of the effect of a single back-mutation, so that the total number of spots is a measure for the frequency of reversion. The large number of individual plants included in the experiments was prohibitive to a complete count of all spots per flower. It was decided, accordingly, to use the number of red and pink spots that exceeded a certain size as a measure of the mutation frequency. The values thus obtained appeared to yield reproduceable results.

The anthocyanins were identified both on silical gel G plates and spectrophotometrically (for details, see Wiering and De Vlaming 1973).

3 Results

3.1 The origin of the new, unstable An1 allele

In order to assess the stability of reverted alleles, individuals homozygous for the reversion ($an1^{+/+}$ $an1^{+/+}$) were crossed with stable white-flowered plants (*an1an1*). A total progeny of 39,372 individuals was obtained, of which 39,363 were red-flowered and 9 white owing to a mutation within a reverted allele of the red-flowered parent plant (Bianchi et al. 1978). Among these 9 mutants one (viz., PZ 5158 L-1) bore white flowers with a fair number of pink spots in addition to a large number of red ones. The pink colour was not the result of a chemical change of the pigment but of a smaller quantity of the cyanidin-3-glucoside produced. The pink spots differed in hue, and sometimes the flowers exhibited unspotted white sectors (Fig. 1). These white sectors must have originated from mutations of the unstable allele, as the result of which the possibility of reactivation has been lost completely. Although a mutation towards stable white only becomes visible if it results in a large unspotted part of the flower, this does not mean that such a mutation only occurs in an early stage of the development of the flower. Mutations at a later stage, resulting in small stable white spots, will also occur but they will not be recognized as such.

The low frequency of mutation of the reverted allele $an1^{+/+}$ renders the likelihood of the incidence of two separate mutations within the same *An1* locus most improbable. One may, therefore, assume that a single mutational event gave rise to a new, unstable allele of *An1*, to be circumscribed by the symbol $an1^{s/p-+}$. Mutations occurring in the epidermal cells of the flower bud may also occur in the dermatogen of the

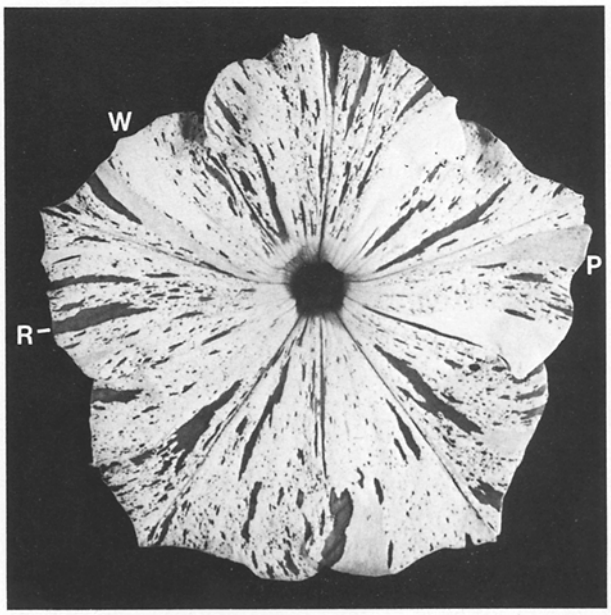

Fig. 1. 'White-red-pink' flower of a plant with the genotype $an1^{s/p-+}an1$. *R* = mutation towards red, *P* = mutation towards pink. Additional mutations towards unspotted white (*W*) also occur, but only mutations in an early stage of flower development, resulting in large white sectors of the flower, are recognizable as such

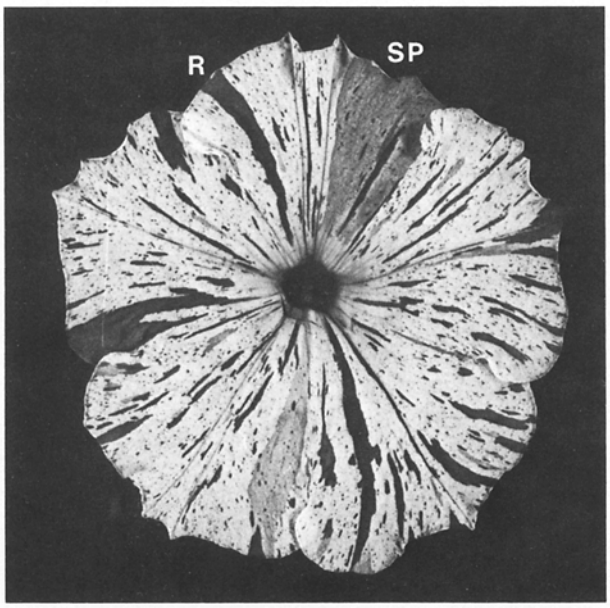

Fig. 2. 'White-red-spotted pink' flower of a plant with the genotype $an1^{s/p-+}an1^{s/p-+}$. *R* = mutation towards red, *SP* = 'spotted pink' = mutation of one of the two unstable alleles towards pink, with smaller and darker pink and red dots as the result of mutations of the second unstable allele

vegetative shoot apex, which may result in the development of lateral branches with self-coloured red, pink or white flowers on nominally spotted-flowered $an1^{s/p-+}an1$ individuals.

3.2 Selfings of mutant PZ 5158 L-1

Since the mutant with the new, unstable *An1* allele originated from a cross between a homozygously red revertant ($an1^{+/+}an1^{+/+}$) and a stable white-flowered plant (*an1an1*), PZ 5158 L-1 must have the genotype $an1^{s/p-+}an1$. Since somatic mutations becoming expressed in the floral epidermis also occur in the subdermal, sporogenous tissue, in addition to gametes with the unstable allele and the allele for stable white, also gametes with alleles expressing themselves in self-coloured pink or red corrollas will originate. In order to find the percentages of mutation towards white, pink and red floral colours occurring in the gametes, a number of selfings were made with the original mutant specimen.

Among the descendants thus obtained, were the 'white-red-pink' parental type and also white-flowered individuals with, likewise, red and pink spots but with smaller and darker pink or red dots within the pink spots (Fig. 2). These 'white-red-spotted pink' plants also differed from the parental phenotype in a clearly greater spot density and in the complete absence of stable white sectors in the corolla limb. These differences with the heterozygous 'white-red-pink' parent point to a homozygosity of the 'white-red-spotted pink' plants for the allele $an1^{s/p-+}$. If, namely, by an early mutation of one of the two unstable alleles a large pink spot originates, later on additional mutations of the second unstabe allele will be responsible for the appearance of smaller and darker pink and red dots within that pink spot. The absence of unspotted white sectors is to be attributed to the fact that a very early mutation towards stable white will not result in the formation of pure white sectors because there is still an unstable allele present. However, an early mutation towards white (*an1*) may result in heterozygous sectors in flowers of an individual homozygous for that factor. These sectors are recognisable by a clearly lower spot density and the absence of darker dots within the pink spots.

In order to establish whether in the homozygote $an1^{s/p-+}an1^{s/p-+}$ the presence of a second, unstable allele has any effect upon the nature and/or the frequency of the mutations of the separate alleles, in hetero- and homozygous individuals the number of red and pink spots exceeding 3 mm were counted (Table 1). Since the number of spots in the homozygotes is nearly twice as large as in the heterozygotes, it may be concluded that these two alleles mutate with the same fre-

Table 1. Number of red and pink spots ≧ 3 mm in flowers of plants heterozygous or homozygous for the unstable allele $an1^{s/p-+}$

Phenotype	Genotype	No. of plants	No. of flowers	Mean no. of red and pink spots ≧ 3 mm			Ratio red/pink
				Red	Pink	Red + pink	
'White-red-pink'[a]	$\frac{an1^{s/p-+}}{an1}$	9	152	22.1	5.9	28.0	3.7
'White-red-spotted pink'[b]	$\frac{an1^{s/p-+}}{an1^{s/p-+}}$	11	166	40.4	10.4	50.8	3.9

[a] 'White-red-pink': white flowers with red and pink spots
[b] 'White-red-spotted pink': white flowers with red and pink spots; within the pink spots small darker pink and red dots

Table 2. Progenies obtained from selfings of mutant PZ 5158 L-1

Family	No. of capsules	Descendants												Total
		White-red-spotted pink[a]		White-red-pink[b]		White		Spotted pink[c]		Pink		Red		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
A 5034	13	241	*18.4*	604	*46.2*	381	*29.1*	10	*0.8*	14	*1.1*	58	*4.4*	1,308
A 5077	19	269	*20.8*	603	*46.5*	340	*26.2*	9	*0.7*	11	*0.9*	64	*4.9*	1,296
Total	32	510	*19.6*	1,207	*46.3*	721	*27.7*	19	*0.7*	25	*1.0*	122	*4.7*	2,604

[a] 'White-red-spotted pink': white flowers with red and pink spots; within the pink spots small darker pink and red dots, genotype: $an1^{s/p-+}an1^{s/p-+}$

[b] 'White-red-pink': white flowers with red and pink spots, genotype: $an1^{s/p-+}an1$

[c] 'Spotted pink': pink flowers with darker pink and red spots, genotype: $an1^{+/p}an1^{s/p-+}$

Test on homogeneity: $\chi^2_{2\times6}=4.521$; df=5; $P=0.48$

quency but independently of one another. That the number of spots is a little less than twice as large must be ascribed to the complete dominance of the allele for wild-type red over all other alleles. This has the effect that when an allele mutates towards red in a homozygous individual all subsequent mutations of the second allele towards white or red or pink will not be observed. That the nature of the mutations does not change either is manifest from the small difference between the ratio of red and pink spots in hetero- and in homozygous individuals. In this case the small difference can also be attributed to the complete dominance of red over pink.

Table 2 shows the results of selfings of the mutant PZ 5158 L-1. The hetero- and homozygous individuals with spotted flowers appearing in the progeny by the segregation of the $an1^{s/p-+}$ allele, together will occur in less than 75% of the total number because a part of the $an1^{s/p-+}$ alleles may also mutate within the sporogenous tissue towards white, pink, or red. The majority of the stable white-flowered descendants originate on account of the segregation of the *an1* allele of the heterozygous parent. Its representation must exceed 25%, however, since mutations of the unstable allele towards white may also occur ($an1^{s/p-+} \rightarrow an1$). The pink-flowered descendants will all have resulted from mutations which yield a partial repair of the activity of the gene *An1* ($an1^{s/p-+} \rightarrow an1^{+/p}$). In combination with the allele *an1* this will cause the incidence of self-coloured pink individuals which exhibit a rate of variation in hue corresponding with that observed in the pink spots of spotted-flowered plants. The combination of the unstable allele $an1^{s/p-+}$ with the allele for pink ($an1^{+/p}$) will produce pink-flowered plants which have corollas dotted with darker pink and red spots. Since wild-type red is completely dominant over spotted, pink and white, the red revertants ($an1^{s/p-+} \rightarrow an1^{+/+}$) may possess any other allele next to the allele for wild-type red without exhibiting any phenotypic differences.

In order to establish to what extent the segregation ratio in the progeny obtained by selfing is reproduceable, six months after the first series of selfings had been carried out (family A 5034) a second series was produced (Table 2, family A 5077). By means of a χ^2 test for homogeneity, the segregations obtained from the first and those from the second series of selfings were mutually compared. It is quite clear from this 2×6 test that the differences observed are not significant ($P=0.48$). Progenies reared from individual capsules of mutant PZ 5158 L-1 were also mutually compared. From the 32 capsules produced after selfing those yielding at least 100 descendants were tested for homogeneity of the segregations (Table 3). The 11×4 test for homogeneity showed that the differences between the 11 progenies were not significant ($P=0.46$).

From the segregation in all 2,604 descendants of PZ 5158 L-1 it can be computed that the mutations from spotted ($an1^{s/p-+}$) to white (*an1*), to pink ($an1^{+/p}$) and to red ($an1^{+/+}$) occurred in 5.2%, 1.8% and 4.8% of the gametes, respectively, under the assumption that the frequency of mutation was the same in the male gametes and in the female gametes.

3.3 Selfings of 12 'white-red-pink' descendants of PZ 5158 L-1

In order to ascertain whether the transmission of the unstable allele $an1^{s/p-+}$ may cause any changes in the mutability of this allele, 12 'white-red-pink' descendants of PZ 5158 L-1 were selfed. A comparison of their segregation (Table 4) with that produced by selfings of

Table 3. Single capsule progenies of at least 100 plants obtained from selfings of mutant PZ 5158 L-1 (phenotype: white-red-pink, genotype: $an1^{s/p-+}an1$)

Family	Descendants												
	White-red-spotted pink		White-red-pink		White		Spotted pink		Pink		Red		Total
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
A 5034 C	56	*21.2*	112	*42.4*	82	*31.0*	2	*0.8*	2	*0.8*	10	*3.8*	264
A 5034 F	31	*19.3*	72	*44.7*	47	*29.2*	–	–	1	*0.6*	10	*6.2*	161
A 5034 G	53	*21.8*	118	*48.6*	62	*25.5*	1	*0.4*	2	*0.8*	7	*2.9*	243
A 5034 I	17	*15.2*	58	*51.8*	33	*29.4*	1	*0.9*	–	–	3	*2.7*	112
A 5034 J	26	*18.4*	64	*45.4*	43	*30.5*	–	–	1	*0.7*	7	*5.0*	141
A 5034 K	20	*19.6*	51	*50.0*	27	*26.5*	1	*1.0*	1	*1.0*	2	*1.9*	102
A 5077 E	31	*24.8*	64	*51.2*	22	*17.6*	–	–	1	*0.8*	7	*5.6*	125
A 5077 F	30	*20.7*	64	*44.1*	40	*27.6*	1	*0.7*	3	*2.1*	7	*4.8*	145
A 5077 G	29	*19.6*	77	*52.0*	36	*24.3*	–	–	2	*1.4*	4	*2.7*	148
A 5077 L	26	*21.7*	45	*37.5*	35	*29.2*	–	–	1	*0.8*	13	*10.8*	120
A 5077 O	25	*20.2*	58	*46.8*	31	*25.0*	2	*1.6*	2	*1.6*	6	*4.8*	124
Total	344	*20.4*	783	*46.5*	458	*27.2*	8	*0.5*	16	*0.9*	76	*4.5*	1,685

Test on homogeneity (the classes 'spotted pink', pink and red combined): $\chi^2_{11\times4}=30.164$; df = 30; $P=0.46$

Table 4. Segregation obtained from selfings of the mutant PZ 5158 L-1 and of 12 'white-red-pink' descendants of this mutant

Parents	No. of capsules	Descendants												
		White-red-spotted pink		White-red-pink		White		Spotted pink		Pink		Red		Total
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
PZ 5158 L-1	32	510	*19.6*	1,207	*46.3*	721	*27.7*	19	*0.7*	25	*1.0*	122	*4.7*	2,604
12 white-red-pink descendants	34	772	*19.8*	1,782	*45.8*	1,045	*26.7*	27	*0.7*	33	*0.9*	231	*5.9*	3,890
Total	66	1,282	*19.8*	2,989	*46.0*	1,766	*27.2*	46	*0.7*	58	*0.9*	353	*5.4*	6,494

Test on homogeneity: $\chi^2_{2\times6}=5.295$; df = 5; $P=0.38$

PZ 5158 L-1 indicates that the two segregations do not differ significantly ($P=0.38$).

In Table 5 the segregation of the 12 individual families are tabulated. A homogeneity test revealed such differences between families that there was no homogeneity at all ($P \ll 0.001$). Since the segregation of the progeny obtained by selfings of the mutant PZ 5158 L-1 and the one of the total progeny of the 12 'white-red-pink' descendants of this mutant were not significantly different it follows that the frequencies of mutation towards white, pink, and red in the 12 individual progenies vary around mean values corresponding with those found in the case of PZ 5158 L-1.

The explanation of the mutual heterogeneity of the separate families is sought in the heterozygosity of the mutant PZ 5158 L-1 for the colour intensity gene *In1*. This gene causes a decrease in intensity of the flower pigment in the dominant form and, in addition, lowers the somatical reversion frequency of unstable $an1^{s/+}$ alleles (Gerats et al. 1982). That the gene *In1* exerts a similar influence on the $an1^{s/p-+}$ allele was demonstrated by counting the number of red and pink spots $\geqq 2$ mm on the corollas of plants heterozygous for $an1^{s/p-+}$ with the genotypes *In1In1*, *In1in1*, and *in1in1*. The results of these counts are shown in Table 6. The hue intensity of the intermediate red flower type

Table 5. Selfings of 12'white-red-pink' descendants obtained from selfings of the mutant PZ 5158 L-1

Family	No. of capsules	Descendants												Total
		White-red-spotted pink		White-red-pink		White		Spotted pink		Pink		Red		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
A 5078	3	49	*21.6*	94	*41.4*	63	*27.7*	2	*0.9*	2	*0.9*	17	*7.5*	227
A 5079	3	67	*20.9*	144	*45.0*	85	*26.6*	6	*1.9*	2	*0.6*	16	*5.0*	320
A 5080	3	49	*15.4*	155	*48.6*	82	*25.7*	3	*0.9*	3	*0.9*	27	*8.5*	319
A 5081	3	49	*18.2*	134	*49.6*	65	*24.1*	–	–	6	*2.2*	16	*5.9*	270
A 5082	3	50	*13.4*	187	*50.3*	117	*31.5*	1	*0.3*	3	*0.8*	14	*3.7*	372
A 5083	3	103	*21.9*	214	*45.5*	140	*29.8*	1	*0.2*	–	–	12	*2.6*	470
A 5084	3	39	*24.2*	70	*43.5*	39	*24.2*	2	*1.3*	1	*0.6*	10	*6.2*	161
A 5085	3	76	*19.3*	176	*44.8*	105	*26.7*	3	*0.8*	2	*0.5*	31	*7.9*	393
A 5086	3	39	*20.8*	82	*43.6*	52	*27.7*	1	*0.5*	4	*2.1*	10	*5.3*	188
A 5087	3	56	*16.9*	145	*43.7*	82	*24.7*	5	*1.5*	7	*2.1*	37	*11.1*	332
A 5100	4	155	*22.1*	322	*45.8*	183	*26.0*	3	*0.3*	3	*0.4*	38	*5.4*	703
A 5118	2	40	*29.6*	59	*43.7*	32	*23.7*	1	*0.8*	–	–	3	*2.2*	135
Total	34	772	*19.9*	1,782	*45.9*	1,045	*26.7*	27	*0.7*	33	*0.9*	231	*5.9*	3,890

Test on homogeneity (the classes 'spotted pink', pink and red combined): $\chi^2_{4\times 12} = 88.994$; df = 33; $P \ll 0.001$

Table 6. Influence of gene *In1* on somatic mutation frequency of the unstable allele $an1^{s/p-+}$

Genotype	No. of flowers	Mean no. of spots ≧ 2 mm per flower						Ratio red/pink
		Red		Pink		Red + pink		
		No.	SEM[a]	No.	SEM	No.	SEM	
$an1^{s/p-+}$*an1In1In1*	88	35.0	*0.96*	10.8	*0.36*	45.8	*1.20*	3.2
$an1^{s/p-+}$*an1In1in1*	88	40.1	*1.07*	12.3	*0.40*	52.4	*1.32*	3.3
$an1^{s/p-+}$*an1in1in1*	88	56.2	*1.17*	18.3	*1.32*	74.6	*1.43*	3.1

[a] SEM = standard error of the mean

(*In1in1*) lies closer to the light red one (*In1In1*) than to the dark red type (*in1in1*). It appears from Table 6 that the same holds true for the spot densities, but the ratio between the numbers of red and of pink spots was not affected by the factor *In1*.

In order to establish whether the gene *In1* does not only have an effect upon mutations in somatic cells but also upon mutations in sporogenous tissue, plants with the genotype $an1^{s/p-+}an1^{s/p-+}In1In1$ and $an1^{s/p-+}an1^{s/p-+}in1in1$ were pollinated with pollen of stable white plants (Table 7). The percentages of white, red, and pink-flowered descendants indicate the rate of incidence of the mutations under discussion in the maternal gametes. A homogeneity test for the segregation ratios found indicated that plants homozygously dominant for *In1* exhibited significantly lower mutation frequencies. It follows that *In1* exerts an influence upon the frequency of mutation of the unstable allele $an1^{s/p-+}$ in both somatic and sporogenous tissue. The heterogeneity of the segregations obtained from selfings of 12 'white-red-pink' descendants of mutant PZ 5158 L-1 (Table 5) can, therefore, to a large extent be ascribed to segregation for *In1* on account of the heterozygosity of this factor in the parent plant.

3.4 Selfings of red revertants descended from plants with the unstable allele $an1^{s/p-+}$

The occurrence of unstable genes in *Zea mays* was explained by Barbara McClintock by postulating the incidence of genetic elements which can transpose

Table 7. Influence of gene *In1* on the percentage of mutated gametes in $an1^{s/p-+}an1^{s/p-+}$ plants

Parents	No. of capsules	Descendants								
		White-red-pink		White		Pink		Red		Total
'White-red-spotted pink' × white		No.	%	No.	%	No.	%	No.	%	No.
$an1^{s/p-+}an1^{s/p-+}In1In1 \times an1an1$	27	3,532	*95.2*	42	*1.1*	30	*0.9*	105	*2.8*	3,709
$an1^{s/p-+}an1^{s/p-+}in1in1 \times an1an1$	15	1,150	*92.7*	19	*1.5*	13	*1.0*	59	*4.8*	1,241

Test on homogeneity: $\chi^2_{2\times4} = 12.835$; df = 3; $P = 0.005$

Table 8. Segregation of flower colour and leaf colour in family A 5140 obtained from selfing of a red-flowering revertant of mutant PZ 5158 L-1

Leaf colour	Flower colour		Total
	Red	White	
Green	124	43	167
Yellowish green with green spots	28	11	39
Total	152	54	206

Red : white $\chi^2_{3:1} = 0.162$; df = 1; $P = 0.69$
Green : yellowish green $\chi^2_{3:1} = 4.045$; df = 1; $P = 0.04$

within the genome. The insertion of such a transposable element at a given locus was supposed to suppress the activity of the gene present within that locus. Excision followed by transposition may result in the repair of the gene activity (McClintock 1965; Starlinger 1980). In order to establish whether there are positive indications that such elements are also involved in the occurrence of unstable alleles of *An1* in *Petunia* a large number of red-flowering revertants among progenies of the mutant PZ 5158 L-1 were selfed. In a number of cases these selfings yielded new recessive mutants showing manifest differences in habit form, habit size, or colour, shape, and size of flowers and leaves. Family A 5140 was particularly interesting in that mutated descendants appeared with yellowish green leaves speckled with sharply delimited spots of the nominal green colour (Fig. 3). These green spots point at the possible frequent repair of normal activity of the mutated gene in somatic tissue, and, indirectly, at the instability of the allele involved. Table 8 shows the segregations of the floral colours and the appearance of the leaves. It appears that the red-flowered parent had the flower pigment controlling genotype $an1^{+/+}an1$ and was also heterozygous for the mutation in the *Yg* gene. The latter factor is involved in the formation of chloroplasts and in the homozygously recessive form causes the incidence of yellowish green leaf blades with spots of the normal green colour. The segregation ratio red : white does not significantly differ from 3 : 1 ($P = 0.69$), whereas the ratio green : yellowish green deviates from 3 : 1 significantly ($P = 0.04$) by an under-representation of the yellowish green leaves. This aberrancy can in all probability be ascribed to the lowered viability of the yellowish green mutants. The segregation ratio clearly indicates the independent heredity of the gene *An1* for floral pigments and of the gene *Yg* for leaf colour.

In order to find out whether the reversions which give rise to the incidence of nominal green spots on

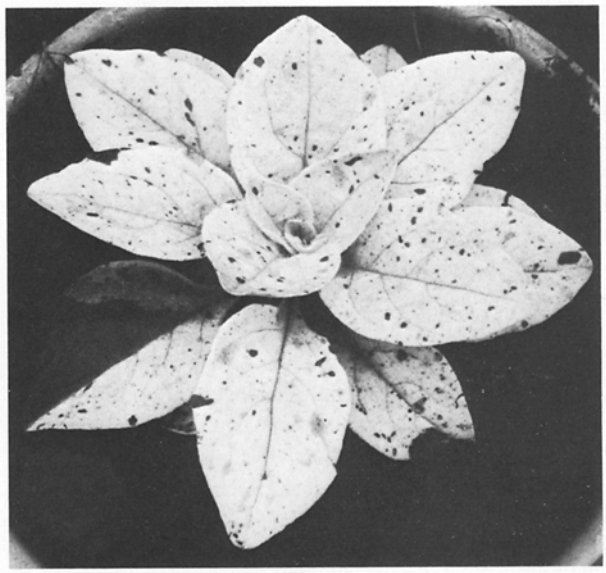

Fig. 3. Plant with yellowish green leaves on which sharply delimited spots of the normal green colour, homozygously recessive for an unstable allele of the gene *Yg*

Table 9. Segregation into yellowish green and normal green in families obtained from self-pollinations of two plants with normal green spots on yellowish green leaves

Family	Parent	Offspring	
	Yellowish green with green spots	Yellowish green with green spots	Green
B 5095	A 5140 A-71	191	1
B 5096	A 5140 B-79	194	1
Total		385	2

leaves may also occur within the sporogenous tissue, two plants with yellowish green leaves from family A 5140 were selfed. The results of these selfings are shown in Table 9. The two normally green plants must have originated from reversions of recessive alleles of the gene *Yg* within sporogenous tissue.

The observation that plants with an unstable allele of the flower pigment gene *An1* produced a number of mutants in their progenies with new recessive characteristics, one of which was in turn unstable, may be taken as indicative of the instability of *An1* being the result of the presence of a transposable element within the locus. A transposition of this element to other loci in the genome would result in recessive mutations or new instabilities.

4 Discussion

Crossings and selfings of a large number of mutants with unstable *An1* alleles permit the conclusion to be drawn that the *An1* locus is compounded of a structural gene with an adjoining regulatory region. This region must consist of two components, viz., the 'mutator' responsible for the activation at the right time and place of the structural gene, and the 'expressor' which controls the rate of activity of that gene (Bianchi et al. 1978).

Selfings of red-flowered revertants of plants with the unstable allele $an1^{s/p-+}$ yielded cogent indications of a relation between the occurrence of unstable alleles and the presence of transposable elements.

It has been established that in procaryotes, insertion of a transposable element into a gene leads to inhibition of the gene activity, and that a subsequent excision of that element will result in the repair of the gene activity. The frequency of incidence of such reversions vary from 10^{-9} to 10^{-6} (Calos and Miller 1980). The unstable *An1* alleles of *Petunia* exhibit much higher reversion frequencies, however. For instance, mutants were encountered in which over 10% of the colourless epidermal cells must have undergone a reversion. Obviously the frequencies of reversion found in this case are of a totally different order of magnitude, which renders it likely that the processes which give rise to the occurrence of reversions in *Petunia* are for the greater part not of the same kind as those operative in procaryotes. This may be attributable to the compound nature of the *An1* locus consisting of a structural gene with an adjoining regulatory region. Since a structural gene must produce a gene product after transcription it may be expected that an insertion of a transposable element disturbs the transcription to such an extent that an inactive gene product is formed, or none at all. If so, the repair of the normal gene activity would only be possible by a precise excision of this element. The recorded frequency of this process appeared to be so low in procaryotes that the chances of observation of reversions brought about in this way in higher plants are negligibly remote.

For that reason we postulate that the unstable *An1* alleles arise by an insertion within the regulatory region. Since it has been shown that mutations taking place in that region of the *An1* locus exhibit a cis-effect exclusively, i.e., only affect the expression of the structural gene at the same locus, it may be assumed that no transcriptions of the components take place out of which the regulatory region is compounded. Their function might consist of an influence upon the chromosomal structure in situ in such a way that it enables the transcription of the structural gene in a certain phase of floral ontogenesis.

That DNA replication is a prerequisite for the proper functioning of the regulatory region is evident from the observation that repair of the activity of unstable alleles exclusively takes place in dividing cells. Because the insertion of a transposable element does not necessarily result in a disturbance of the process of replication, such an insertion need by itself not result in an inactivation of the structural gene.

It is an established fact that transposable elements may induce deletions, inversions and other rearrangements in the DNA sequence. It has been found that both in procaryotes and in eucaryotes at either side of the insertion, deletions of varying lengths may originate which extend from one of the ends of the inserted element to non-random points, so that distinct classes of deletion can be distinguished (Nevers and Saedler 1977). Deletions within the regulatory region of the *An1* locus induced by an inserted, transposable element could, then, be the cause of the lack of activation of the structural gene. It follows that reversions must be ascribed to a repair of deletions.

To explain the often very high frequencies of incidence of deletion repairs it is assumed that the regulatory region is partly built up from intermediate repetitive DNA. If the deletions fall within this repetitive DNA, reparations to the original length might occur at a high rate by tandem replication of the remaining segments.

A diagrammatic representation of the structure of the *An1* locus of a number of different *An1* alleles, drawn up according to the hypothesis as developed

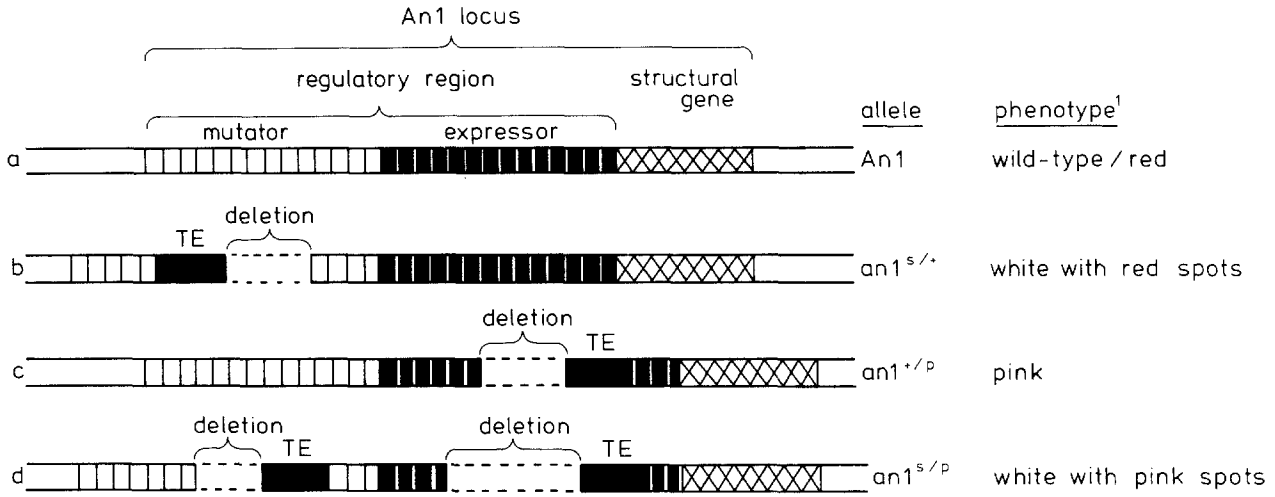

Fig. 4 a–d. Diagrammatic representation of the structure of various *An1* alleles. **a** structure of the *An1* locus of the wild-type; **b** deletion in the 'mutator', induced by an inserted transposable element (*TE*), resulting in white flowers with red spots; **c** deletion in the 'expressor', resulting in pink flowers; **d** deletions in both 'mutator' and 'expressor' induced by independently inserted transposable elements, resulting in white flowers with pink spots

[1] Phenotypical expression in combination with the allele *an1*

above, is shown in Fig. 4. Deletions in the 'mutator' give rise to the lack of anthocyanin synthesis and, hence to the development of white corollas. Reparations of deletions result in the appearance of coloured spots on the white corollas. Deletions within the 'expressor' cause a decrease of the gene activity and consequently of the hue intensity. Because nothing is known for certain of the size ratios of the elements constituting the *An1* locus and of the insertions and deletions postulated in the working hypothesis, the relative sizes in the diagram are altogether arbitrary.

Since the mutant PZ 5158 L-1 bears white flowers with red and pink spots and exhibits a range of pink to red hues, it must have undergone mutations in both the 'mutator' and the 'expressor'. The obvious corollary is that in this case the mutations are caused by a deletion extending over both the 'mutator' and the 'expressor' (Fig. 5 b). A repair process in a cell of a developing flower bud resulting in a complete restoration of the original condition, causes the appearance of a red spot on the corolla and of red revertants in the progeny (Fig. 5 c). Such a reverted allele ($an1^{+/+}$) distinguishes itself from the original wild-type allele *An1* only in the persistence of the inserted element in the locus. Partial repair of the deletion involving the 'mutator' but not the 'expressor' (or only a part of it) will phenotypically be manifest as a pink spot on the corolla and, when it took place in sporogenous cells, in self-coloured pink descendants (Fig. 5 d). When repair would proceed in the reverse direction starting in the 'expressor' and proceeding in the 'mutator', a situation might arise in which only the 'expressor' is repaired and the 'mutator' is still (partly) deleted, which would result in the arisal of descendants bearing red-spotted white flowers. Pink-flowered individuals would then not be observed. As appeared from the results of selfings of 'white-red-pink'-flowering plants, apart from 'white-red-pink'-flowered individuals and red revertants appearing in the offspring some pink descendants originated, but never plants bearing white flowers with red spots only. This infers that the repairing process leading to the removal of the deletion proceeds directionally from the 'mutator' to the 'expressor'.

The structure of the $an1^{s/p-+}$ allele and of the alleles $an1^{+/+}$ for red and $an1^{+/p}$ for pink originating from a complete or a partial repair, respectively, are diagrammatically shown in Fig. 5. The arrows indicate the direction of progression of the repairing process.

Recent studies at the DNA level substantiate the supposition that transposable elements are instrumental in the incidence of unstable alleles in higher plants, the investigation of the unstable *Sh* alleles of maize in particular contributing relevant information. It is generally accepted that the insertion of the controlling element *Ds* into the *Sh* locus results in inactivation of the gene and the subsequent excision followed by transposition of *Ds* in the recovery of its activity (McClintock 1965; Nowick and Peterson 1981). The transpositions occur through the action of one or more activator genes.

Döring et al. (1981) isolated maize DNA from the wild-type and from the unstable *Sh* mutants and found indications

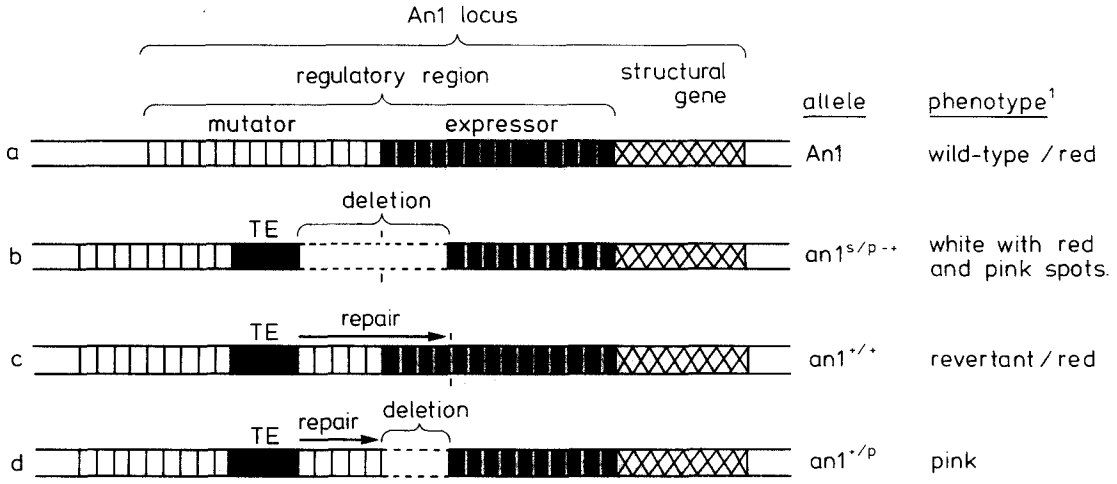

Fig. 5 a – d. Diagrammatic representation of the $an1^{s/p-+}$ allele and the alleles derived from it by a partial or complete reversion. **a** structure of the *An1* locus of the wild-type; **b** deletion extending over both the 'mutator' and the 'expressor' induced by an inserted, transposable element (*TE*); **c** reversion of $an1^{s/p-+}$ by the complete recovery of both the 'mutator' and the 'expressor'; **d** partial reversion of $an1^{s/p-+}$ by the complete repair of the 'mutator' and no, or only a partial, repair of the 'expressor'

[1] Phenotypical expression in combination with the allele *an1*

of additional DNA at the *Sh* locus of the mutants. An estimation of the minimum size of the insertion yielded a value of about 6 kb. These workers also suggested a possible incidence of deletions extending from the insertion site of *Ds* to various distances in the different mutants. They concluded that the deletion must affect a DNA segment necessary for the expression of the gene but not encoding part of the gene product. These conclusions fit in with the explanation of the regulation of gene *An1* in *Petunia* as outlined above.

Burr and Burr (1981) demonstrated by a comparison of the maize *Sh* locus of the wild-type with that of unstable mutants that the difference consists of the presence of sizeable, up to 20 kb long, insertions within the unstable alleles. Subsequent studies revealed that a revertant of a *Ds*-induced mutation had maintained a 21–22 kb long insert at the same location (Burr and Burr 1982). The presence of this insert is at variance with the premise that the recovery of gene activity must be the result of excision of the transposable element *Ds*. The authors point out that the insert in the revertant differs from its predecessor in that extensive rearrangements had taken place along two-thirds of its length, and they suggest that in this special case the readjustments would have restored the functional expression of the *Sh* gene again. However, the presence of the inserted DNA in the reverted locus agrees most satisfactorily with the explanation emanating from the working hypothesis developed in the present paper to account for the instability of the *An1* locus in *Petunia*.

The results of studies carried out by Wienand et al. (1982) are also relevant in this connection. They investigated the gene coding for chalcone synthase in *Petroselinum hortense*, *Zea mays* and *Antirrhinum majus*. In the latter two taxa unstable alleles of the responsible gene were found which arose from the integration of transposable elements. In the three unstable maize mutants studied, the size of the DNA fragment, which exhibits a homology with cloned parsley chalcone synthase cDNA, showed an increase of length by about 800 bp as compared to the corresponding wild-type fragment. Four revertants originating independently from a single mutant all appeared to have retained the 800 bp insert characteristic of the mutant. Since these workers start from the assumption that the recovery of the activity of the structural chalcone synthase gene must be ascribed to the excision of the inserted element, they drew the conclusion that the DNA fragment in question does not contain the transposable element responsible for the unstable mutation. However, their findings may also indicate a reversion of the mutated gene without a previous excision of the insert. A corresponding study of *Antirrhinum majus* in which an unstable mutant was compared with revertants descended from it yielded data which can be interpreted as an indication of the presence of an insert in the mutant which had got lost in its revertants.

The information acquired from investigations at the molecular level are still so fragmentary that no satisfactory and unambiguous answers can be given to the questions concerning processes operative during the occurrence of gene instability in higher plants. The supposition that transposable elements are involved seems to have been confirmed. Estimations of the size of the inserts studied vary from about 800 bp (Wienand et al. 1982) to 21,000–22,000 bp (Burr and Burr 1982). It is a question whether (and if so, to what extent) they are comparable to the *Is* elements and transposons found in procaryotic organisms.

Our inquiries into the genetics of the instability of floral pigment genes in *Petunia* give rise to the working hypothesis that an insertion causing an instability takes place within the regulatory region of the locus involved. The question arises whether the inhibition of the gene activity is the direct outcome of an insertion of a transposable element or if it is to be ascribed to changes in the DNA sequence induced by that insert.

In the first case a reversion must result from its excision and consequently from the loss of the transposable element. In the alternative case reversions may be the result of repair of the changes induced by the insertion, reversion being made possible with the retention of the inserted, transposable element. The results of our genetical analyses of unstable alleles of genes controlling flower pigment synthesis in *Petunia* and those of studies at the molecular level of unstable genes in maize support the latter of the two alternative suppositions.

Acknowledgements. The authors wish to express their gratitude to Mrs. H. G. Gouw-Foederer, L. E. van Oostrum and P. L. M. Verweij for their skillful technical assistance and to J. A. J. van der Meijden for his photographs and drawings. Thanks are due to Prof. Dr. A. D. J. Meeuse for the translation of the manuscript.

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