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Regulation of Gene Action in *Petunia hybrida*: Unstable Alleles of a Gene for Flower Colour

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Summary. In a progeny of a selfed individual of the dark red-flowered cultivar 'Roter Vogel' some white-flowered plants appeared as the result of a mutation of the genetic factor *An1* involved in anthocyanin synthesis. The white flowers of these plants had red spots owing to back-mutations in the dermal cells of the young corolla.

Owing to a striking unstability of the new allele of *An1*, a number of mutants originated which differ mutually in the frequency of reversion, which expressed itself in the very substantial differences in the spot density of the limb of the corolla. Between a mean number of less than one spot per cm^2 of the limb and a mean number of over 10.000 spots/cm^2, a series of transitions was found.

The reversions did not remain restricted to the young epidermis but also occurred in sporogenous tissues. This resulted in the appearance of selfcoloured red descendants of plants with red-spotted white flowers. There is a positive correlation between the spot density of the parent plants and the percentage of plants with completely red corollas.

The red spots on the corolla usually have the same colour as the wild type ('Roter Vogel'), but occasionally mutants occur with paler spots, the colour varying from a very pale pink to a red nearly as deep as in the wild type. The selfcoloured descendants of such mutants also show this colour variation from pale pink to red.

On the grounds of these observations a theory was formulated which postulates that the *An1* locus consists of a structural gene responsible for an enzyme active during anthocyanin synthesis and a regulatory element built up from intermediate repetitive DNA. This regulatory element in turn is built up of two components, one of which, the 'mutator', decides the activation of the structural gene while the other, the 'expressor', modifies the rate of activation. The mutations must be considered representative of larger or smaller deletions within one or both of these components. Reversions are the result of the restoration of the deletions by means of an amplification of the repetitive DNA in dividing cells of the developing flower buds.

Key words: Unstable alleles – Flower variegation – Gene regulation – *Petunia hybrida*

1 Introduction

A number of genes are known which are responsible for the occurrence of coloured flowers in *Petunia hybrida*. In the developing flower these genes are only active for a short time and their activity remains restricted to the epidermis of the corolla. Since the same genes for flower colour are not only present in the cells of the developing corolla of the flower buds but also in all other living cells of the plant, there must be some regulating mechanism responsible for the activation of genes at the proper place and at the right time.

Mutations of one of the basic genes for anthocyanin synthesis (Wiering 1974) may result in the formation of flowers without pigmentation, i.e. of white-flowered mutants. If these mutations involve structural genes coding for enzymes taking part in anthocyanin synthesis, back-mutations resulting in the re-establishment of the pigment production can only occur when, by the restoration of the original nucleotide sequence, or otherwise, the effect of the first mutation has been wholly or partially annulled. Since reversions of mutated structural genes will be rare, one may expect that coloured spots attributable to the back-mutation of a structural gene can only occur in very low frequencies. However, in *Petunia hybrida* mutants occur whose white flowers show so many coloured spots that one must accept a frequency of back-mutation as high as 10 percent and over. Such a high frequency ren-

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ders a reversion of a structural gene in these cases highly improbable. One must rather accept the possibility of a disturbance of some regulation mechanism, in other words of mutations in genetic elements responsible for the activation of the structural genes for flower pigmentation.

It has been tried, by means of a genetical analysis of these mutants, to gain some insight into the nature of the disturbance and in this way into the structure and activities of the regulatory elements.

2 The Mutants with Spotted Corollas Included in the Investigation

2.1 The Origin of the Plant Material

From a red-flowered cultivar ('Roter Vogel') a progeny was obtained after three generations of inbreeding, and subsequent selfing in which, apart from 16 red-flowered specimens, 3 white-flowered ones were also present. Since in the previous generations red-flowered plants were found exclusively, these white-flowered descendants must have originated from a mutation of one of the genes involved in anthocyanin synthesis in the flower. Two of these mutants produced seed after selfing and from their progenies the lines W17 and W28 were obtained by further inbreeding.

From crossing experiments with white-flowered individuals of known genetic composition it could be established that it was a mutation of the gene *An1*. It is known that this gene is, like the gene *An2*, situated in chromosome VI (Smith et al. 1974). Both *An1* and *An2* are strongly linked with the gene *Rt* responsible for the alteration of the red flower colour into magenta (Wiering 1974). The *An1* allele originating from the mutation appears to be unstable as in the epidermis of the corolla, reversions to a red colour occur in a high frequency. Since the reverted allele exhibits a great stability, the cell families derived from a cell with a repaired anthocyanin synthesis will almost invariably also be coloured and this results in the presence of red spots on the white corolla.

These reversions are by no means restricted to the epidermal cells of the young corolla and may also occur in the sporogenous tissue of the developing flower bud. In this way reverted gametes originate so that the progenies of W17 and W28 also contain individuals with completely red corollas.

2.2 The Size of the Coloured Spots

An early occurrence of a reversion of *An1* in a dermal cell of the developing corolla will result in a large red area. The later the reversion occurs, the smaller the coloured spot, the smallest consisting of a single cell only. Since the anthocyanin remains contained in the cell in which it is formed, the red spots are always sharply delimited.

The reversions are not restricted to the epidermis cells of the corolla in which they can express themselves, but also occur in the dermal layer of a vegetative shoot apex. In this way stems may result with sectors containing the active allele of *An1*. When flower primordia are initiated within such a sector, this ultimately results in completely red-coloured flowers and when it happens that they are formed on the border line between a reverted and a non-reverted zone, the resulting flowers will show red sectors of various sizes (Fig. 1a, b). An axillary bud originating within a reverted sector of a stem results in the development of a lateral branch with wholly red-coloured flowers (Fig. 2).

From experiments with plants grown under standardized conditions (Bianchi et al. unpublished) it could be

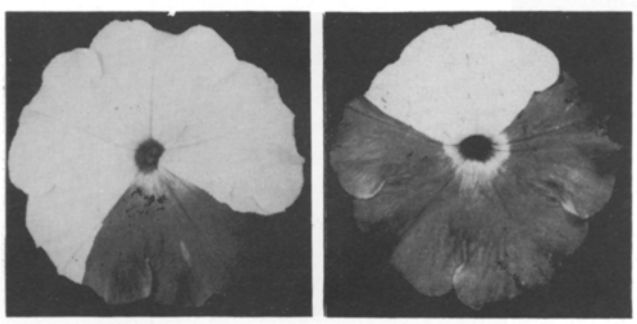


Fig. 1. Flowers of *Petunia* whose corollas contain red sectors resulting from reversions of the instable *An1* allele in the dermal layer of the shoot apex

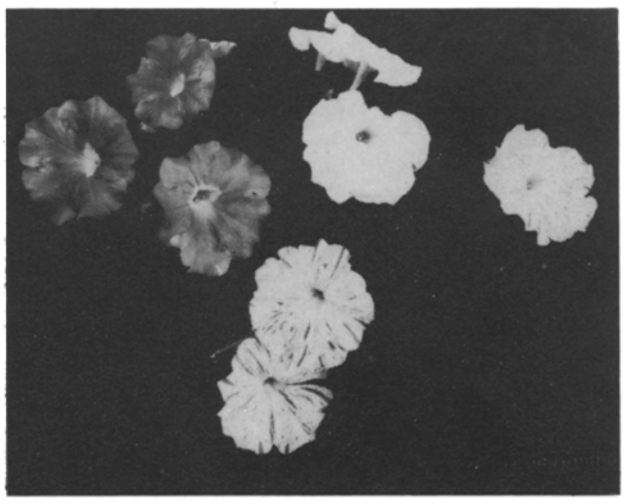


Fig. 2. A white-flowered plant bearing a red-flowered branch owing to the formation of a growing point with an epidermis in which all cells contain a reverted allele of *An1*. The flowers on other branches show different spot densities

deduced that during the last phase of floral development when the mitotic activity has ceased and growth takes place exclusively by vacuolisation and stretching of the cells, the number of coloured spots does not increase any more. This justifies the conclusion that reversions occur exclusively in dividing cells. Taking into account that there are always numerous one-celled spots numbering about twice as many as the two-celled ones, it follows that if a reversion takes place in a dividing cell this reversion remains restricted to only one of its daughter cells.

2.3 The Number of Coloured Spots

Since every coloured area, irrespective of its size, must be considered to be the result of only a single mutational event, the number of spots is determined by the reversion frequency. As a measure of this frequency the spot density expressed in the number of spots per square centimetre of the upper epidermis was taken. Such an area contains about 170,000 cells. The counts were always performed in the same place on the corolla and as peripherically on a corolla lobe as possible. A sample area of this size proved to be sufficiently representative to obtain reproducable counts.

The counts were made microscopically with transmitted light using a magnification of 10 × 10. For the delimitation of the area to be counted, a cell finder film slide was used on which each cm^2 was divided in 25 vertical and 25 horizontal strips of 0,4 mm wide. Counts of the number of spots from 50 plants of W17 and W28 showed that there may be appreciable differences in spot density of the corollas of different individuals, the number of spots varying from 20 to 280 per cm^2.

2.3.1 Effect of External Factors on the Reversion Frequency

Part of the variation observed must be attributed to the influence of external conditions. Of 23 plants from the line W17, the number of spots per cm^2 of the corolla was determined at the beginning of May, the end of May and the end of June, 1974. The results are shown in Figure 3. It is quite evident that this number increased from early May till late June. Since at this time of the year there are important changes in the environmental factors, such as temperature and day length, the obvious conclusion is that the increase in reversion frequency must be ascribed to these changes. Indeed it was shown to be possible to find a relationship between the temperature and the number of spots by growing plants under standardized conditions at 18°C and at 25°C: the number increases with the temperature. Also changes in light intensity proved to have a marked effect on the reversion frequency (Bianchi et al. unpublished).

2.3.2 Effect of Genetic Factors on the Reversion Frequency

With four W28 plants of which the number of spots had been determined as 88, 104, 232 and 261 per cm^2, respectively, crosses were made between the two plants with the highest two numbers of spots, the two with the lowest two numbers and the two with the highest and the lowest number. The results are shown in Figure 4. In order to

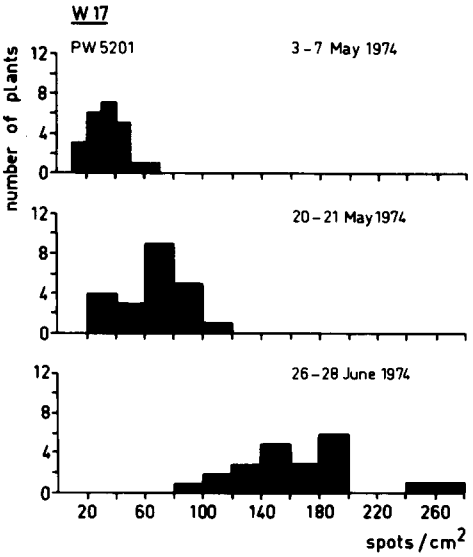


Fig. 3. Effect of external conditions on spot density in plants of line W17

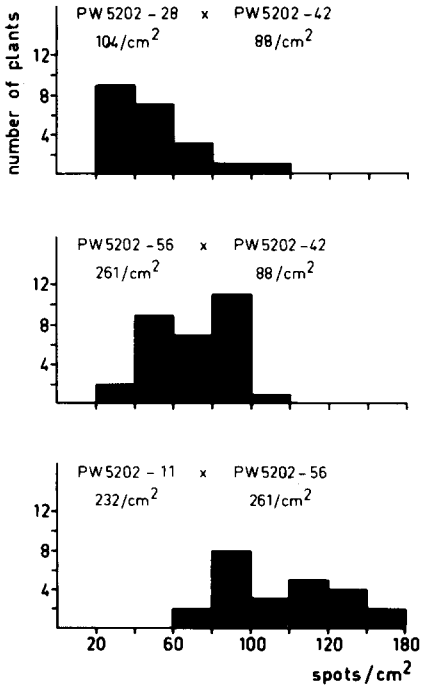


Fig. 4. Spot densities in progenies obtained from crosses between W28 plants with different reversion frequencies of *An1*

render them comparable the counts were made on all descendants on the same day. Since all flowers used had developed under more or less identical conditions the effect of environmental factors on the variation must have been, therefore, negligible. Since there is a manifest correlation between the reversion frequency of the parent plants and that of their descendants, certain genetic factors must also control the differences in spot density observed in the progenies. It is rather striking that there was a lower mean spot density in the offspring than there was in the parent plants, but this is most probably attributable to the differences in the external conditions under which the two generations were grown.

2.4 Distribution of the Spots over the Corolla

Counts made on 300 flowers of the number of spots in 25 strips of 0,4 × 10 mm in two perpendicular directions showed that their density increases in the centrifugal direction (Fig. 5a, b); the density in the outermost strip being about twice as high as in the innermost one. Counts made in the tangential direction, starting from the midpetaline vein, did not show such a gradient (Fig. 5b).

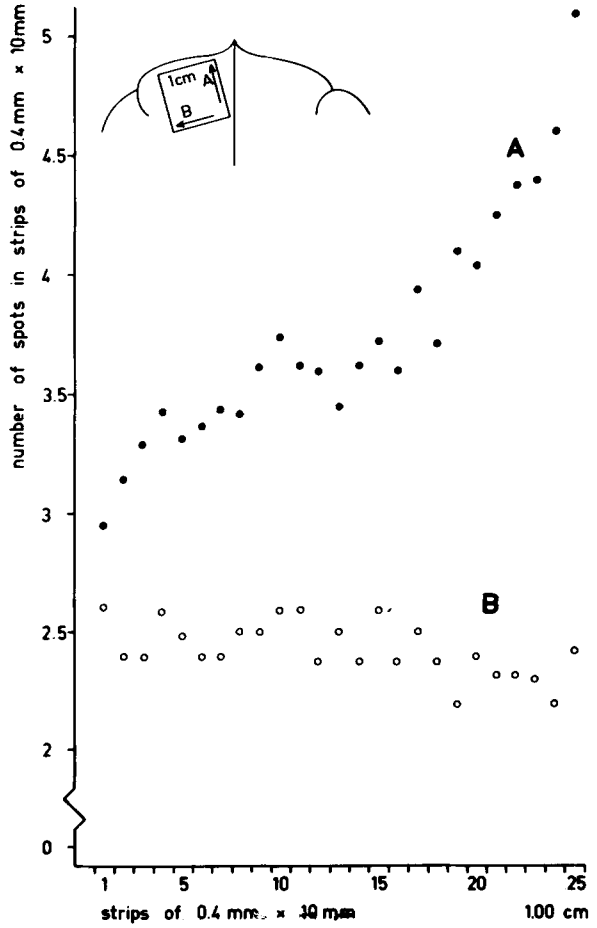


Fig. 5. Increase of spot density from the centre towards the periphery. The counts of the spots were made in strips of 0.4 × 10 mm in a sequence indicated by the arrows A and B

A study of floral development made it clear that the more peripheral part of the corolline epidermis is formed by a greater number of mitoses than the more centrally situated part (Cornelissen et al. unpublished). The gradient in the reversion frequency recorded might indicate that the larger the number of mitoses preceding the formation of a cell of the corolla, the greater the chance of a reversion occurring.

2.5 The Pleiotropous Effect of the Mutation of An1

The mutation responsible for the incidence of spotted corollas has a pleiotropous effect. The *An1* mutants exhibit, apart from the almost complete absence of anthocyanin pigments in the flower, changes in the seed coat. It is of a pale colour and is so badly developed that desiccated seeds become wrinkled and shrivelled and loose their germinating power sooner than the normal dark-coloured seeds. The abnormal seed coat structure also causes the seeds to stick together. That this must be ascribed to pleiotropy of *An1* is manifest from the fact, that after a back-mutation in the dermal layer of a shoot apex, not only flowers with a completely red colour are formed but also seeds with a normal dark-coloured testa.

3 Experimental Results

3.1 Differences in Reversion Frequencies of Instable Alleles of An1 in Somatic and in Sporogenous Tissues

As mentioned already reversions are not restricted to the outer cell layer of the shoot apex and the developing corolla, but may also take place in the subdermal layer from which the sporogenous tissue and later the gametes are derived. This results in the formation of gametes with an allele of *An1* which had regained its activity by a reversion. These gametes cause the appearance of descendants with corollas which are red throughout.

Table 1 shows the results of selfings of 11 W17 and 12 W28 plants. In all progenies red-flowered descendants appeared distinct from the plants with red-spotted, white corollas. It appears from this table that there is a certain relation between the number of spots on the corolla limb and the percentage of revertants since on the average slightly more spotted W28 plants yielded progenies with likewise, more red-flowered individuals.

Also, in the sporogenous tissue mutations are likely to occur at different times during the development of the flower. An early reversion will result in a group of re-

verted cells and this group will be more numerous because the reversion took place earlier. The incidence of smaller or larger clusters of reverted gametes may also be anticipated.

Since the number of red-flowered descendants is not determined by the number of clusters but by the number of gametes containing the reverted *An1* allele, for the comparison of the reversion frequencies in the somatic and in the sporogenous tissues, not the number of red spots but the number of red epidermis cells must be compared with the number of revertants in the progeny. Counts of the number of red spots and the number of red cells per cm^2 of the corolla limb of 15 different flowers of the lines W17 and W28 showed that there is a remarkably constant relationship between the number of spots and the number of red cells. At densities of up to 500 spots per cm^2, the number of red cells is about 2.2 times that of the red spots. Since the total number of cells of that area has been determined as about 170,000, it was possible to calculate the mean percentage of coloured cells in the corolla from the average spot density of the corollas of W17 and W28 plants.

Table 2 shows that the percentage of red descendants after selfing is 85 to 104 times that of the percentage of red epidermal cells of the parent plant. This appreciable difference might conceivably be the result of certation, resulting in a much larger proportion of pollen tubes derived from pollen grains with the reverted allele of *An1* reaching the ovules. However, reciprocal crosses between plants with different spot densities did not result in differences in the percentages of revertants in the progenies, or hardly so, so that certation cannot explain the great dif-

Table 1. Percentages of red revertants obtained from selfings of plants of the lines W17 and W28

A. Selfings of W17 plants (spot density 52-135/cm^2)

Family	Parent	Descendants			
		Total. no.	White	Red	% of red
PX 5088	PW 5201- 4	365	328	37	10.1
„ 5089	„ „ - 5	809	726	83	10.3
„ 5090	„ „ - 6	702	622	80	11.4
„ 5091	„ „ -22	634	588	76	12.0
„ 5092	„ „ -17	541	482	59	10.9
„ 5093	„ „ -21	1247	1109	138	11.1
„ 5094	„ „ - 3	788	692	96	12.2
„ 5095	„ „ - 1	1043	917	126	12.1
„ 5096	„ „ - 7	875	775	100	11.4
„ 5097	„ „ - 9	288	257	31	10.8
„ 5098	„ „ -16	1039	914	125	12.0
Sum total		8331	7380	951	11.4

B. Selfings of W28 plants (spot density 84-261/cm^2)

Family	Parent	Descendants			
		Total no.	White	Red	% of red
PX 5099	PW 5202-13	183	162	21	11.5
„ 5100	„ „ -19	141	120	21	14.9
„ 5101	„ „ -27	121	101	20	16.5
„ 5102	„ „ - 6	300	256	44	14.7
„ 5103	„ „ -11	348	285	63	18.1
„ 5104	„ „ -14	378	306	72	19.0
„ 5105	„ „ -56	509	425	84	16.6
„ 5106	„ „ -59	221	192	29	13.1
„ 5107	„ „ - 5	257	227	30	11.7
„ 5108	„ „ -21	220	178	42	19.1
„ 5109	„ „ -36	326	270	56	17.2
„ 5110	„ „ -57	302	252	50	16.1
Sum total		3306	2774	532	16.1

ference observed. It is not very likely either that the higher percentage of reverted descendants is the result of a greater viability. In the numerous progenies in which they grew side by side, we never saw any indication of differences in the rate of development between the reverted and non-reverted specimens. The obvious conclusion is that the reversion frequency is much higher in the sporogenous tissue than it is in the epidermis of the corolla.

Considering that reversions can only come about during cell divisions, one may accept the probability that the changes of a reversion of an unstable allele of *An1* are greater by far during a meiosis than during a mitosis.

3.2 Mutations of Unstable Alleles of An1

3.2.1 Mutations to a Stable White Corolla

Among the several tens of thousands descendants of selfings and crossings of W17 and W28 parents, a few plants were found which were utterly devoid of any red pigment in the corolline epidermis. From one of these individuals (PX 5088D-19), after selfing and subsequent mutual crosses between the descendants, 800 white-flowered plants were obtained divided over 4 successive generations. All flowers produced were completely white, apparently because reversion had not taken place. Mutations in the sporogenous tissue were not apparent since there was not a single red-flowered revertant in the successive progenies. This warrants the conclusion that these white-flowered plants arose after a mutation had taken place changing an unstable *An1* allele, with an appreciable tendency towards reversion, into a stable allele unable to regain its original activity during anthocyanin synthesis. These white-flowered mutants in question are homozygous for this stable and recessive allele of *An1*.

3.2.2 Mutations to the 'Wild Type'

The hue and intensity of the red colour appearing as spots on white flowers and of the red flowers of reverted descendants are, in the great majority of the cases, exactly like those in the flowers of the original cultivar (here called 'wild type'). This is indicative of a complete regeneration of the biosynthetic activity of *An1*. Although this reversion exhibits a certain degree of stability, there are some indications of some small differences with the original 'wild type' allele as found in unspotted and unvariegated cultivars of *Petunia*. Revertants which are heterozygous for the reverted allele exhibit, namely, regularly red flowers with some minute, but occasionally larger, white spots. This implies that at least in somatic cells the reverted allele can again lose its activity although only in a very low frequency. Such white spots on the red corolla have not been encountered in plants heterozygous for the original 'wild type' allele.

In order to get some idea of the stability of the reversion in the sporogenous tissue, heterozygous 'wild type' revertants of W17 and W28 were crossed. Of the F1 progeny, 28 red-flowered plants were selfed. Nine of these produced exclusively red-flowered descendants. It may be assumed that these plants were homozygous for the reverted allele of *An1*. Four of these homozygotes were back-crossed on a large scale with a stable white mutant. If in the sporogenous tissue of these red-flowered homozygotes a gamete originated which again contained an inactive allele of *An1*, this would express itself in the progeny by the incidence of a white-flowered individual. The seeds of the nine white-flowered plants of Table 3 came from as many different capsules. It may be accepted that they originated by as many different mutations. No significant differences in the mutation frequencies between the reciprocal crossings were noticed. The mutation frequency of 1 in 4395 indicates that the stability of the reversion, though large, is yet clearly smaller than that of the original 'wild type' allele *An1* occurring in the normal, unspotted flowers of *Petunia*.

3.2.3 Mutations Towards Higher and Towards Lower Reversion Frequencies

Flowers of the lines W17 and W28 regularly show a sector in which the spot density, as the result of the change in the reversion frequency of an unstable allele of *An1*, is clearly different from the remainder of the corolla. The sharp delimitation of these sectors clearly indicate that the alteration of the reversion frequency is to be ascribed to a mutation in a dermal initial of the developing flower bud. When such a mutation takes place in a shoot apex instead of the flower primordia, this may result in flowers whose corollas show a different spot density throughout (Fig. 2).

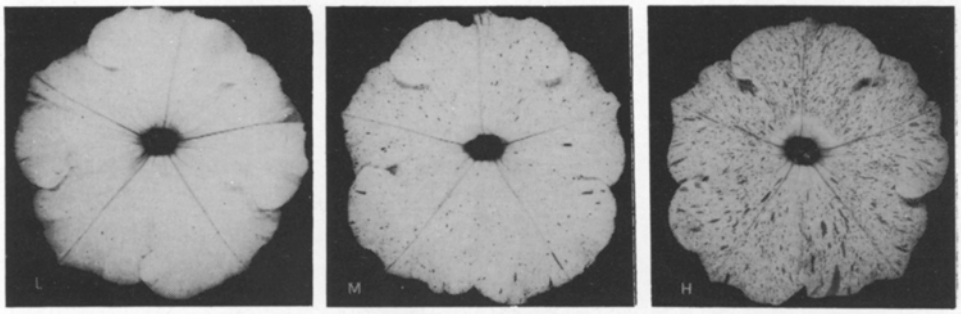
All flowers with unstable alleles of *An1* appeared to be

Table 2. Comparison of the reversion frequencies in somatic and in sporogenous tissue in plants of the lines W17 and W28

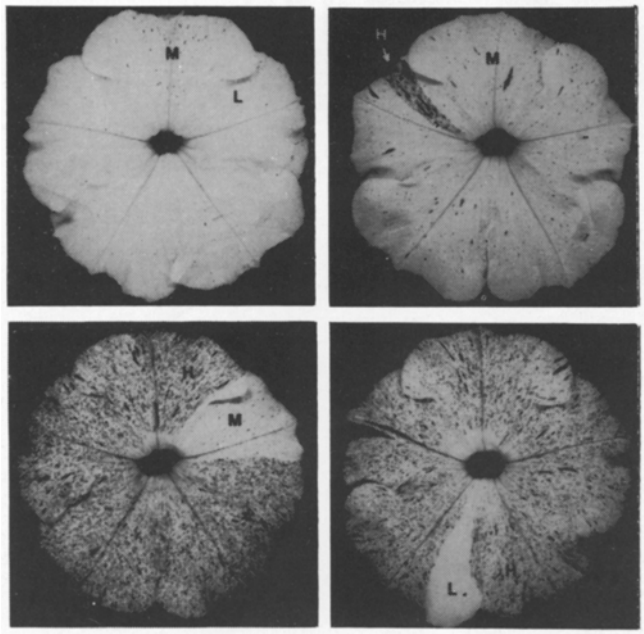
Line	No. of plants	Mean no. of spots/cm²	Mean no. of red cells/cm²	a % red cells	b % revertants	b/a
W17	11	86.3	190	0.11	11.4	104
W28	12	146.7	323	0.19	16.1	85

Table 3. Crosses between a homozygous red revertant with a stable, white mutant

Parents		Number of		
		Capsules	Plants	White mutants
stable white ♀	× homoz. red ♂			
PX 5088D-19	PX 5085A- 7	154	5965	1
PX 5088D-19	PX 5085B- 2	240	8407	1
PX 5088D-19	PX 5085B- 9	238	8612	3
homoz. red ♀	× stable white ♂			
PX 5085A- 7	PX 5088D-19	25	2845	2
PX 5085B- 2	PX 5088D-19	39	4735	–
PX 5085B- 9	PX 5088D-19	45	5541	–
PX 5086 -10	PX 5088D-19	28	3267	2
Sum total		796	39372	9



Figs. 6, 7, 8. Flowers with a low, a medium and a high spot density respectively



Figs. 9, 10, 11, 12. Flowers showing sharp delimitated sectors with different spot densities caused by mutations in dermal initials of developing flower buds

classifiable in three different classes characterized by a low, a medium and a high spot density, respectively (Figs. 6, 7, 8). Although within each class there is a certain degree of variation, the distinction can be made on the basis of a clear discontinuity in the respective spot densities.

The mutations go either way: flowers with low spot densities may form sectors with a higher density (Figs. 9, 10) or vice versa (Figs. 11, 12). Mutation to a high spot density were rare. Sectors of medium spot density in corollas with low densities were regularly encountered.

Since the reversion frequency is strongly influenced by the prevailing external conditions such as the temperature and the amount of light, it proved to be impossible to indicate fixed limits for the three classes. Dependent on the circumstances the limit between 'low' and 'medium' may shift from about 300 to about 500, and that between 'medium' and 'high' from about 1200 to about 1800 spots per cm^2. Flowers of the class 'high' may exhibit spot densities attaining 10,000 per cm^2.

The incidence of mutations resulting in alterations of the reversion frequencies is not restricted to somatic cells.

Such mutations may also occur in sporogenous tissues since in progenies of parents with a low spot density plants appeared with medium densities. So far no plants were observed which showed a high density owing to a mutation in sporogenous tissue. In an experiment in which 11,637 descendants were obtained from selfings of 23 plants with a low spot density, two plants appeared whose flowers consistently exhibited a medium density. If these plants did arise from mutations within the *An1* locus, the new allele must be dominant and the two mutants heterozygous for this allele. From selfings of these plants one may therefore expect to obtain, in addition to red-flowered revertants, white-flowered descendants with a low spot density next to plants with medium densities. The results of such selfings of one of these mutants (PX 5105A-5) are shown in Tabel 4. Although all families tabulated were raised from selfings of the same individual, they show differences in the red: white segregation ratios. These differences are to be expected because there is no mendelian segregation but a separation caused by reversions to the 'wild type' allele. If, during the development of the sporogenous tissue an early back mutation to the 'wild type' occurs, a big cluster of reverted gametes will result which will, after selfing, produce a higher percentage of red-flowered descendants than selfings with flowers which do not contain such early reversions. The mean percentage of 44.4 red-flowered revertants found in the progenies is appreciably higher than the percentages of 10%-20% recorded in progenies of selfings of individuals with a low spot density (Table 1). All this clearly shows that the new allele is not only subject to reversion in a higher frequency in somatic tissues but also in sporogenous ones. Of the white-flowered descendants, the percentage of individuals with a medium spot density was not 75% but only 55%. This deviation from the normal 3 'medium' : 1 'low' segregation must partly be explained by the fact that of the two alleles of *An1* represented in the genotype, the one responsible for a medium spot density in the corolla reverts more frequently to the 'wild type' than the other one.

Although within the classes distinguished there is a manifest variation, the classification of most of the individuals into 'medium' and 'low' did not cause great difficulties. From all plants of the family A 5066 the density was determined. The results are shown in a diagram (Fig. 13). A marked discontinuity in the class frequencies justifies the distinction of two classes, but within the classes all possible transitions between the extreme values occur. The variation is partly attributable to environmental factors during floral development. That genetic factors also contribute to the variation within the classes has already been mentioned (Fig. 6). Apart from modifiers present elsewhere in the genome and influencing the

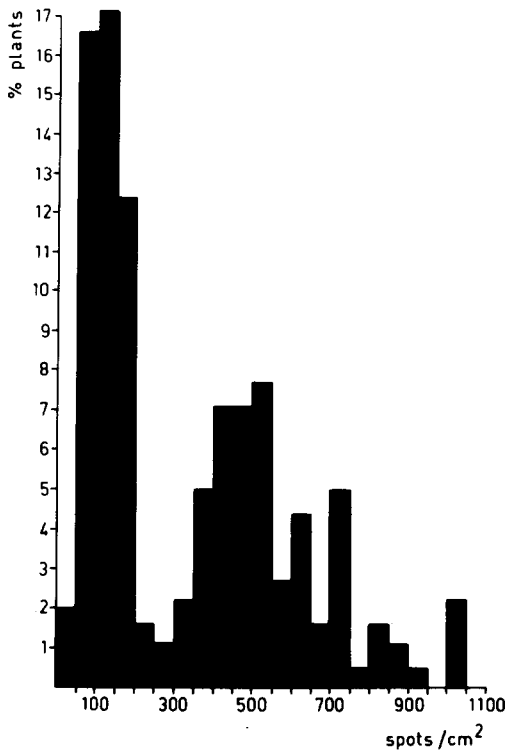


Fig. 13. Spot densities of 190 white-flowered plants of fam. A 5066, obtained from a selfing of a mutant with 'medium' spot density (PX 5105A-5)

Table 4. Progenies obtained from selfings of a mutant with a medium spot density (PX 5105A-5)

Family	Total	Red		White 'medium'		White 'low'	
		No.	%	No.	%	No.	%
PZ 5387	131	66	50.4	39	29.8	26	19.8
PZ 5388	71	26	36.6	26	36.6	19	26.8
PZ 5389	83	33	39.8	32	38.5	18	21.7
PZ 5390	104	46	44.2	31	29.8	27	26.0
PZ 5391	71	36	50.7	20	28.2	15	21.1
PZ 5392	113	40	35.4	35	31.0	38	33.6
A 5066	354	164	46.3	101	28.5	89	25.2
Sum total	927	411	44.4	284	30.6	232	25.0

reversion frequency, repeated mutations within the *An1* locus also occur which result in such small alterations of the reversion frequencies that they do not cause a transgression beyond the class limits (Bianchi et al. unpublished).

3.2.4 Mutations to Differences in Colour Intensity

The line W17 and W28 are characterized by white corollas with red spots. The colour of the large majority of these spots has the same intensity as that of the 'wild type' (which corresponds with Hortic. Colour Chart no. 819/1). Only on rare occasions was a spot of a lighter hue encountered. The frequency of occurrence of such lighter spots cannot so easily be estimated because the difference in hue can only clearly be observed in the spots of a larger size which represent a very small minority among the spots.

The incidence of mutations resulting in a paler colour is not restricted to epidermal cells of the flower. They also occur in sporogenous cells; in the latter case giving rise to descendants with pale red self-coloured corollas. From selfings of plants with red-dotted white corollas, 7,420 red-flowered revertants were obtained of which 52 (0.7%) had a lighter colour than the original 'wild type' flowers. The corollas of these 52 revertants varied from a very pale pink to a deep rose-pink (in the range H.C.C. 21/3-020/1). On those pink flowers dark red spots could be observed.

Table 5 shows the results of selfings of two mutants with pink flowers and of a pink-flowered descendant obtained, by selfing, from one of these mutants. The pink-flowered plants were placed in three classes: light, medium and dark. Within each class there was some distinct variation so that the classification is arbitrary and only serves to illustrate manifest differences in colour between the parent plants and part of their descendants. This Table shows, according to expectation, that the mutants were heterozygous for the allele responsible for the incidence of a pink hue. Both the light pink mutant and the dark pink one showed, after selfing, a segregation into red-spotted white, pink and wildtype red. That fewer than 25% white-flowered plants were found is to be expected since the allele for unstable white has reverted to the wild type in some of the offspring. The plants with wild type red corollas did not only originate from mutations of white but also from mutations of the allele for self-coloured pink. This is evident from the segregation ratios in the family PZ 5412. As no white descendants were found it may be assumed that the parent plant was homozygous for the allele for self-coloured pink. The 1.8% of 'wild type' plants can, therefore, only have arisen from mutations of the pink allele. Table 5 also shows that from dark pink-flowered parents light pink-flowered may descend, and vice versa. The reversion of pink to wild type, in addition to the differences in hue between parents and offspring and between descendants of the pink-flowered plants clearly point to a considerable degree of instability of the allele responsible for the self-coloured pink flower.

From the numerous crossings with W17 and W28 plants not only mutants with self-coloured pink flowers were raised. A cross between a plant with red-spotted white corollas and a plant with stable white flowers also yielded a plant with pink-spotted flowers (Fig. 14). From a cross between a plant homozygous for the back-mutation to the wild type colour and a stable white-flowered plant a mutant was obtained with white flowers which were invariably both red- and pink-spotted (Fig. 15).

The characteristics of the above-mentioned mutants warrant the conclusion that mutations within the *An1* locus may result in both the appearance of coloured spots on white corollas and changes in colour intensity, and that these mutations may occur independently.

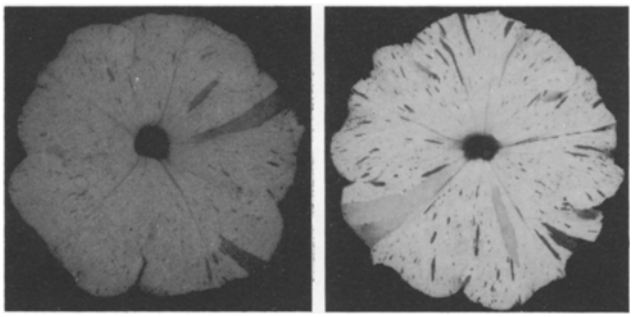


Fig. 14. Mutant with only pink spots on the white corolla

Fig. 15. Mutant with both pink and red spots on the white corolla

Table 5. Selfings of pink-flowered individuals

Family	Parent	Total	Descendants									
			White		Light pink		Medium pink		Dark pink		Red	
			No.	%	No.	%	No.	%	No.	%	No.	%
PZ 5406	dark pink	527	114	21.6	10	1.9	231	43.9	105	19.9	67	12.7
PZ 5407	light pink	763	175	22.9	496	65.0	27	3.6	7	0.9	58	7.6
PZ 5412	dark pink	379	–	–	1	0.3	195	51.5	176	46.4	7	1.8

4 Discussion

The experimental results reported above indicate that the *An1* locus must consist of a structural gene and an associated regulatory element. The structural gene codes for one of the enzymes involved in the anthocyanin synthesis. Mutations in this part of the *An1* locus result in an inhibition of the formation of anthocyanins, i.e., in white-flowered phenotypes. A local reparation of the pigment production by a reversion of such mutations, if it occurs at all, must be so rare that one may hardly expect to come across it.

The regulatory element serves to activate the structural gene in the right place and at the right time, i.e. in the dermal cells of a developing corolla. Mutations of this element may also lead to white-flowered phenotypes, but in this case the white flowers may also have numerous red spots owing to frequent reversions in somatic cells. The colour intensity of the coloured areas of the white corolla may equal that of the wild type but may also be of a lighter hue. This suggests a dual nature of the regulatory element: one of its components, the 'mutator', being responsible for the activation or inhibition of the structural gene, and the other one, the 'expressor', deciding the degree of intensity of the gene action.

The high reversion frequencies responsible for the numerous spots on white corollas suggest that changes induced by mutations of the regulatory element are far less specific than those resulting from mutations of the structural gene.

Since the number of coloured spots on the corolla may vary in the several mutants obtained, and also since a series of transitions exist from a very pale pink to the dark red of the wild type, the impression was gained that the differences between the mutations in both the 'mutator' and the 'expressor' are of a quantitative rather than a qualitative nature.

The low specificity of the mutations and the quantitative nature of the difference between the various mutants might point to a constitution of the two components of the regulatory part of the *An1* locus of intermediate repetitive DNA.

On different grounds, several authors have already pointed to the possibility of intermediate repetitive DNA playing a role in regulating gene activity (Britten & Davidson 1969; Strom & Dorfman 1976; Klukas 1977). The mutations may accordingly be considered to be the result of smaller or larger deletions in this repetitive DNA. A deletion in the 'mutator' will result in the complete inhibition of the structural element for the production of flower pigment. The anthocyanin synthesis can only begin again after the deletion has been restored by an amplification of the repetitive DNA during cell divisions in the growing flower bud. The larger the deletion, the smaller the change of reparation and the smaller the number of spots on the corolla. Differences in spot density are, accordingly, attributable to differences in the size of the deletions in the 'mutator' component.

As regards the 'expressor', the size of a deletion may decide the rate of suppression of the anthocyanin synthesis. Also in this component a reparation of the original conditions may occur by an amplification of the repetitive DNA which renders the pigmentation the same intensity as the wild type colour again.

The mutator-expressor model for the regulation of the flower pigment gene *An1* resembles in a number of respects the situation in maize described by McClintock (1965) in which a suppressor-mutator element is supposed to be responsible for the incidence of spotted grains. One of the most striking properties of this controlling element was its capacity to shift its position in the genome. For an explanation of the phenomena concerning spotting in *Petunia* flowers, the postulation of transpositions of the controlling element is unnecessary. On the other hand, our experiment does not permit the conclusion that in *Petunia* no transpositions of regulatory elements takes place.

The occurrence of spotted flowers in Petunia as the result of frequent reversions is not restricted to mutations in the *An1* locus. Cornu (1977) described plants with spotted flowers resulting from an instability of mutations of *An2*. In the collection of *Petunia* lines of the Institute of Genetics, University of Amsterdam, there are plants mutated in the floral pigment loci *An3*, *An6* and *Ph4* with spotted flowers caused by reversion.

The phenomenon is not at all restricted to genetic factors controlling stages of the anthocyanin synthesis either. Bianchi et al. (1974) reported the frequent appearance of spontaneous back-mutations of a recessive dwarf of *Petunia hybrida* to normal dimensions. That in this taxon and in other plant species especially mosaics resulting from disturbances of genes controlling the corolline pigmentation are so frequently recorded is due to the local effect of these genes so that reversions can readily lead to sharply delimited, and consequently striking, spot patterns.

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