INSTABILITY AT THE *PAL* LOCUS IN *ANTIRRHINUM MAJUS*

I. EFFECTS OF ENVIRONMENT ON FREQUENCIES OF SOMATIC AND GERMINAL MUTATION

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STRAINS of *Antirrhinum majus* with flowers showing magenta flakes and stripes on an ivory background were first described by de Vries (1901) and later by Baur (1924) and members of his school (see review by Stubbe, 1938). The gene responsible for the variegation was identified by the German workers as an allele of the *pallida* series, which they called *pallida-recurrens* (pal^{rec}). It was established by these early workers that the instability of gene expression in somatic tissues was associated with the appearance of occasional fully-coloured plants among the progeny derived by self-fertilisation. Numerous other cases of alleles which appeared to be mutable both in somatic tissues and in the cell layer giving rise to the mega- and microspores, have long been known, both in *Antirrhinum* and in other flowering plants (Stubbe, 1938). Apparently analogous situations are known in *Drosophila* (Demerec, 1941).

There has always been uncertainty as to whether genetic changes which are sufficiently frequent to give obvious somatic variegation can be regarded as in the same category as gene mutations happening at more ordinary frequencies. In spite of the enormous quantitative differences there have never been any satisfactory criteria for establishing qualitative differences between high and low frequency mutational events. However, recent developments tend to strengthen the belief that such differences do exist.

On the one hand, the discovery of the structure and (at least in bacteria) the mode of replication of genetic DNA, and the extensive work on chemical mutagenesis in bacteria and viruses (*e.g.* Freese, 1961), have led to the formulation of very precise and convincing models to account for mutations occurring a low frequencies. These models involve the rare mis-pairing of purine and pyrimidine bases during DNA replication and they account for many of the observations on the specificity of chemical mutagenesis in micro-organisms. They provide no basis, however, for a persistent and extreme instability at a particular site. As Schwartz (1960) has pointed out, one could account for persistent instability by postulating a mis-matched base pair with the DNA duplex replicating conservatively so as to transmit

the pairing error. With semi-conservative DNA replication, which is almost universally accepted as the much more probable mechanism, such an error should be corrected in the next replication after it occurred. Even accepting Schwartz's suggested explanation, the unstable pair should be able to resolve itself into only two different stable derivatives, while there are many systems, one of them described in this paper, in which an unstable gene can probably mutate to several different alleles.

On the other hand, the work of the last two decades by McClintock (1951, 1956) and Brink (1960), on unstable loci in *Zea mays*, has revealed a host of previously unsuspected properties of mutable genes. These properties include the occasional transfer of mutability from one locus to another, an extreme sensitivity of gene expression and mutation rate to the presence or absence of other elements in the genome and often to common environmental variables, and sometimes an induction by the mutable allele of mutation in previously stable alleles of the mutable gene in heterozygotes (" paramutation "). They suggest that the basis for the instability in such cases is quite different from that of ordinary gene mutation.

Both McClintock (1951) and Brink (1960) have suggested that mutations of the kind involved in variegation patterns are normally of significance only in ontogeny. Occurring in a strictly controlled way, they could be responsible for cellular differentiation, but the tissues giving rise to the germ cells must normally be immune to such changes. From this point of view it is desirable to know as much as possible of the conditions favouring mutation of the high-frequency type, since such information may well tell us something of the factors governing cell differentiation. In addition the question arises whether, in situations of high mutability, the germinal mutations are events of the same type as those responsible for the somatic variegation.

The work reported in the present paper is an extension, and in part a repetition, of experiments performed by Mather (1933) at the John Innes Horticultural Institution some 30 years ago. Mather's results were not published except in the form of a Ph.D. thesis. Working on stocks of *Antirrhinum* carrying pal^{rec} he showed: (1) that the intensity of spotting was highly variable, and that more or less heavily spotted lines could easily be obtained by selection; (2) that mutations were transmitted through the germ cells as well as appearing in the epidermis, and that the frequency of germinal mutation shown by a given line was correlated with its intensity of somatic spotting; (3) that mutation could occur not only to the form of the gene giving the full red phenotype, but also to other alleles associated with intermediate levels of stable pigment production. Our experiments have confirmed several of Mather's observations and have further shown that the occurrence of the mutations, both somatic and germinal, can be controlled by the manipulation of environmental variables of which temperature seems likely to be the most important. By appropriately

timed environmental shifts mutations can be caused to occur, or prevented from occurring, at any desired stage during the development of the flower.

1. MATERIALS AND METHODS

(i) *Controlled temperature rooms*

For controlled environment studies, plants were grown in two constant temperature rooms set respectively at 15° and 25° C. In one experiment, during which the temperature control was not working well, the mean temperatures were closer to 14° and 24°. Lighting was provided by banks of fluorescent tubes giving an intensity of approximately 1000 foot candles (somewhat more to the tops of the plants and somewhat less to the lower leaves). Since growth was very much slower at the lower temperature, given equal light, an attempt was made to equalise the rates of development in the two rooms by giving the 25° plants 10-hour days, and the 15° plants 16-hour days. Even with the greater day length, the plants in the 15° room were somewhat slower in their development, but the difference in times to flowering was no more than a few weeks in a total time of development of about three months. Thus both temperature and light were varied together in our experiments, and we cannot, with any assurance, attribute the effects obtained to one variable rather than the other. For reasons to be mentioned later we think that temperature is likely to have been the decisive factor.

(ii) *Method of estimating spot frequencies*

In order to obtain an objective record of the density of spotting on individual flowers, counts were made on the upper (forward facing) surface of *one* of the upper corolla lobes. The lobe was carefully cut from the corolla, flattened beneath a transparent cover ruled with parallel lines spaced 1 mm. apart, and counted with the aid of a dissecting microscope. When the spot frequency was low a complete count of the lobe could be made. When the frequency was too high for a complete count a 1 mm. lane running transversely mid-way up the lobe was counted, and the number of spots on the entire lobe surface was estimated by multiplying by a factor corresponding to the approximate total area divided by the area of the sample transect. The density of spotting usually appeared to be sufficiently uniform for this procedure to give a fair estimate. So far as possible the number of pigmented cells composing each spot was also recorded.

(iii) *Antirrhinum stocks*

We have used two main stocks homozygous for alleles of the pal^{rec} type. Both have been maintained for many years by self-pollination at this Institute.

H3 plants show a low frequency of spotting on the flowers. Usually some spots are present on every flower, but the number is always easily countable. No germinal mutations have ever been detected in this stock. H5 plants have a generally high frequency of spotting. The level of spotting and sectoring is rather variable from plant to plant, but is always too high for complete counts to be easily made. This stock is evidently the one worked on by Mather. Since it is not certain that the difference between H3 and H5 is even partly due to a difference at the *pal* locus itself, we will refer to both lines as pal^{rec}, with the prefix (high) and (low) to refer to H5 and H3 respectively. The H3 and H5 stocks are rather different in growth habit and flowering time, and are undoubtedly of different horticultural origins. Both (low) pal^{rec} and (high) pal^{rec} stocks are homozygous for delilah (*del*) a recessive on another chromosome which has the effect of eliminating anthocyanin in the corolla tube while not affecting the corolla lobes. The other stocks listed below are all homozygous for *Del*, the dominant allele giving fully coloured flowers.

Seeds from which we have established stocks carrying three other alleles of the

pal series were kindly provided by Dr D. R. Sampson of the Canadian Department of Agriculture.

(1) *pallida-carnea* (pal^{car}) was supplied as seed of the garden variety "Windmiller's Early Lilac". This allele, which is stable in its expression, gives a pale magenta flower, considerably lighter than that associated with the dominant "wildtype" allele *Pal*.

(2) *pallida-tubocolorata* (pal^{tub}) is a recessive allele giving flowers which are acyanic except for a ring of magenta pigmentation round the base of the corolla tube beneath the calyx. The homozygous stock carrying pal^{tub}, supplied to us by Dr Sampson, is also homozygous for *sulfurea* (*sulf*), a recessive allele at an unlinked locus which spreads the yellow pigment aureusidin, normally confined to the corolla lip, over the whole of the corolla lobes. Our other stocks are all homozygous *Sulf*.

(3) *pallida-tincta* (pal^{tin}) is a recessive giving completely acyanic flowers. The original seeds which we obtained carrying this allele were from a segregating family, and we have established a homozygous pal^{tin} stock from them. The stock is, presumably, even less likely than the others to be isogenic at other loci. pal^{tin} and pal^{tub} cannot be distinguished in homozygous *del* plants.

2. THE GENETIC BASIS OF THE DIFFERENCE BETWEEN (HIGH) AND (LOW) pal^{rec} STOCKS

An analysis of the cross (high) pal^{rec} × (low) pal^{rec} showed that the difference in spot frequency between the two stocks was largely controlled by a single locus. The F_1 generation was fairly uniform with

TABLE 1

Analysis of (high) pal^{rec} × *(low)* pal^{rec} *cross*

Cross	Class		
	High	Medium	Low
(high) × (low)	...	All	...
(low) × (high)	...	All	...
F_1 × (low)	...	20	29
(low) × F_1	...	29	21
F_1 × (high)	22	26	...
(high) × F_1	26	24	...
F_2	9	19	13

The figures in the body of the table show the number of plants in each spotting class.

an intermediate spot frequency; reciprocal crosses gave identical results. The back-cross to (low) gave a good fit to a 1:1 ratio of "low" to "medium", the two classes being very clearly distinct. The back-cross to (high) gave a fairly clear segregation of "high" versus "medium", again with a good fit to a 1:1 ratio. The F_2 showed a fair approximation to the expected 1:2:1 ratio. The results are summarised in table 1.

It thus seems that the (low) pal^{rec} and (high) pal^{rec} stocks differ in one gene of major effect. It does not follow, however, that this gene difference resides at the *pal* locus itself. One would expect to be able to answer this question by examination of the spotted segregants

in the F_2 and backcross generations from crosses of (high) and (low) pal^{rec} to other tester stocks. If the frequency of spots were controlled mainly at the *pal* locus itself, such segregants should always show, more or less, spotting frequencies similar to that of the original spotted parent. However, outcrosses of the two mutable lines to other stocks have not given any such simple result. It soon became clear that our stocks carrying other *pal* alleles also contained modifiers, either positive or negative, of the mutation frequency of pal^{rec}. F_1 plants from either (low) pal^{rec} or (high) $pal^{rec} \times pal^{tin}$ showed spot frequencies somewhat lower than in the corresponding pal^{rec} parent. A rather similar result was obtained in $pal^{rec} \times pal^{car}$ F_1 plants (in this case the full magenta spots appeared against a pale magenta background). This sort of result is understandable on the basis that, in the F_1 plants, only one mutable allele was present in each nucleus, as opposed to two in the original pal^{rec} stock. However, when either (low) or (high) pal^{rec} was crossed to pal^{tub} the F_1, in each case, showed a considerably higher grade of spotting than in the pal^{rec}/pal^{tin} or pal^{rec}/pal^{car} heterozygotes, and even somewhat higher than in the original pal^{rec} homozygote (cf. table 3). Further genetic analysis, which is still in progress, has shown that the "plus" modifier in the pal^{tub} stock is not pal^{tub} itself but is probably a complex of factors segregating independently of *pal*.

F_2 and back-cross generations derived from these crosses showed a very wide spread of spotting frequency among the spotted segregants. To take one example, many of the more highly spotted segregants in the (low) $pal^{rec} \times pal^{tin}$ F_2 were considerably "higher" than the original (low) stock, and were more densely spotted than the "lower" segregants in the (high) $pal^{rec} \times pal^{tin}$ F_2, some of which were 10-100 times less densely spotted than the (high) pal^{rec} stock. This does not answer the question whether there is any difference, residing at the *pal* locus itself, between (low) pal^{rec} and (high) pal^{rec}, but it does show that, if there is any such difference it must either become very unstable in other genetic backgrounds, or its effect must be capable of being masked by segregation of modifiers at other loci. It remains possible that we have only one pal^{rec} allele and that the major gene difference between the (high) and (low) stocks is at a modifier locus. This aspect of the problem is still under active investigation.

3. CHARACTERISTICS OF THE SOMATIC VARIEGATION

(i) *Size distribution of spots*

Each flower spot consists of a definite number of strongly pigmented cells, easily distinguishable against the ivory or yellow background. The cells adjacent to the fully pigmented cells were themselves faintly pigmented, presumably because of diffusion of anthocyanin, but there was no difficulty in deciding the number of cells in a spot. The most numerous class of spots were those having only one fully pigmented cell. Two-, four- and eight-celled spots were, in decreasing order of

frequency, also common. A tendency of the cell numbers to be close to powers or two would, of course, be expected if each spot originates as a mutation in a single cell which may then undergo, 2, 3, 4 or more complete rounds of division. Deviations from exact powers of two would result if not all parts of a clone of mutant cells necessarily go through the same number of divisions before cell multiplication is completed. In our material, spots of more than 16 cells show very little clear division into size classes. The uniformity of cell division seems to be a good deal less perfect than in the otherwise analogous case of *rose-alpha* of *Delphinium ajacis*, studied by Demerec (1931). Table 2 shows a representative count on (low) pal^{rec} plants.

If the mutation rate per cell generation were consistent throughout corolla development, the one-cell spots should be twice as numerous as the two-cell spots, which in turn should be twice as numerous as the

TABLE 2

Distribution of spot size in (low) pal^{rec} at 15°—sum of 30 flowers

Number of cells	1	2	3	4	5	6	7	8	9-12	c. 16	20-30	c. 32
Number of spots	176	111	10	94	3	11	3	62	9	27	4	9

four-cell spots, and so on. The data of table 2 seem to indicate some falling off in mutation rate during later cell divisions, though other sets of data have approximated more closely to the pattern expected for a constant mutation rate. It is clear that the distribution of spot size can vary significantly from one clone to another, but this variation has not been given detailed attention.

Spots and flakes which are small compared with the area of a corolla lobe are usually confined to just one epidermal surface. Sectors covering a substantial proportion of a lobe more often affect both epidermal layers simultaneously (most of the corolla is only two cells thick). In (high) pal^{rec} plants very much larger fully pigmented sectors are often seen, affecting a large sector, sometimes a perfect half, of a whole flower, or even a radial sector of an entire flowering shoot. Side-shoots which develop on (high) pal^{rec} plants late in the growing season frequently bear entirely self-coloured flowers. Flowers of several such shoots have been self-pollinated, but the resulting seeds have always been found to be homozygous for pal^{rec}, apart from sporadic mutants. This observation was also made by Mather (1933). It seems likely that the mutations responsible for somatic variegation are usually confined to the epidermal cell layer, and that the apparently fully mutant side shoots are periclinal chimæras.

(ii) *Kinds of mutant sector*

In the (low) pal^{rec} stock the only kind of spot or sector which has ever been seen is the fully pigmented *Pal* type. In (high) pal^{rec}, grown

under conditions favouring a high spotting frequency, several different kinds of sector have been distinguished. These same kinds of sector are still more frequent and obvious in (high) *pal*^{rec}/*pal*^{tub} F_1 plants which, as has been mentioned, show an especially high spot density. The following sector types have been distinguished:

(*a*) Fully pigmented, as if carrying standard *Pal*.

(*b*) Unspotted white, as if carrying a stable allele giving an acyanic phenotype. Such sectors are common in (high) *pal*^{rec}/*pal*^{tub} plants, where they can be most plausibly attributed to mutation to alleles of the stable recessive *pal*^{tin} or *pal*^{tub} type. More puzzlingly, white sectors have also been noticed occasionally in homozygous (high) *pal*^{rec} plants, and, on one occasion, an unspotted white (rather than pale magenta) sector was observed in (high) *pal*^{rec}/*pal*^{car}. In these cases the sectors must either be explained as due to concurrent mutation of both alleles, or as mutations to a *dominant* "acyanic" allele. In connection with the possibility of concurrent mutation of both alleles in the mutable homozygote to a stable recessive condition, it is interesting to note that Mather (personal communication) observed one case of mutation of both *pal*^{rec} alleles to a stable allele resembling *pallida-maculosa* (see below).

(*c*) Uniformly pigmented but pale, as if carrying *pal*^{car} or an allele of similar effect. Sectors of this type seem much more common in (high) *pal*^{rec}/*pal*^{tub} than in (high) *pal*^{rec}; in the latter stock they nearly always appear as mauve sectors with superimposed full magenta spots. At least three different grades of stable pale sector have been recognised, and sometimes two or three different ones can be observed in the same corolla.

(*d*) Very lightly spotted, full magenta on white. Sectors of this type are common in the (high) *pal*^{rec} stock, and often include whole shoots or substantial sectors of whole shoots. Here there is the same difficulty of explanation as in (*b*) above, since it would seem that the mutability of both *pal*^{rec} alleles in the homozygous nucleus must have been inhibited at the same time.

(*e*) Very low frequency of definite spots, but with the development of a light, rather blotchy, flush of pigment especially near the margins of the corolla lobes. Such a phenotype has not been recognised in small sectors, but it has been seen in a whole sideshoot on a (high) *pal*^{rec} stock plant, and, as we shall see, it has turned up among the whole-plant mutants in the progeny from self-pollination of (high) *pal*^{rec}/*pal*^{tub}.

4 ENVIRONMENTAL CONTROL OF SOMATIC VARIEGATION

The first indication of a strong environmental effect was seen when plants of (high) and (low) *pal*^{rec} clones were cultured from an early

stage of growth until flowering in the 15° and 25° growth rooms. The effect of the environmental difference on spot frequency is shown in plate I and table 3. In the 15° room both lines showed spot frequencies which were high for the line but hardly outside the range seen in similar plants grown in outside plots. In the 25° room, in contrast, the spot frequency in both (high) and (low) *pal*^{rec} plants was drastically reduced; on the (low) plants there were almost no spots at all, while on the (high) plants the spot frequency was reduced by a factor of about a thousand as compared with the 15° set (plate I). Since the day length given to the 15° plants was longer than that given to the 25° plants it is possible that a part of the large effect observed was due to this factor. We have shown in another experiment that, under

TABLE 3

Environmental control of somatic mutability in three genotypes

Genotype	24°-10 hour days		14°-16 hour days	
	Spots	Total pigmented cells	Spots	Total pigmented cells
(low) *pal*^{rec}/(low) *pal*^{rec} . .	0	0	13	72
(high) *pal*^{rec}/(high) *pal*^{rec} .	15	45	c. 18,000	c. 33,000
(high) *pal*^{rec}/*pal*^{tub} . .	640	c. 2000	>30,000	>100,000

The figures in the body of the table are mean counts per flower, based on a number of representative flowers. Only one surface of one upper corolla lobe was counted (see Methods).

glasshouse conditions, complete darkening of developing inflorescences (leaving the leaves exposed to natural daylight) does not have any marked effect on spot frequency, though it does cause a considerable reduction in intensity of pigmentation. It thus seems that illumination of the inflorescences themselves is not an important factor, but this, of course, does not rule out the possibility that the day length regime to which the whole plant is subjected may have an effect.

In a later experiment, using the two similar environments except that the temperatures were 14° and 24°, the (low) *pal*^{rec} and (high) *pal*^{rec} lines were compared with several other spotted genotypes including (high) *pal*^{rec}/*pal*^{tub}. The representatives of each genotype were a clone, propagated by cuttings. Counts on representative flowers are shown in table 3. It will be seen that the (high) *pal*^{rec}/*pal*^{tub} plants maintained a fairly high level of spotting even in the 24° environment, but in this genotype, as in the other two, the frequency was enormously greater in the 14° room.

(i) *Control of the timing of somatic mutation by environmental shifts*

Experiments were designed to show at what stages of development plants are susceptible to the environmental effect described in the

preceding paragraphs, and how long it takes for a shift in environment to show an effect. Seedlings of the (high) and (low) pal^{rec} lines were germinated in the greenhouse, pricked off into individual pots as soon as possible, and transferred to the constant temperature rooms under the same conditions of illumination as already described. Different sets of plants were subjected to different regimes as summarised in table 4.

In both (high) and (low) pal^{rec} lines, set (3) resembled set (2), and set (4) resembled set (1) in the flower spotting density. In other words the early environment of the plants had no effect on the mutation patterns shown by the flowers which developed later. It was apparent

TABLE 4

Environmental shift experiment

Environment A: 15° with 16-hour days
B: 25° with 10-hour days

Plant set	Conditions under which plants grown
1	A throughout
2	B throughout
3	A for first 2 weeks, then B
4	B for first 2 weeks, then A
5	A till flower bud formation, then B
6	B till flower bud formation, then A
7	A till opening of first flower, then B for various times, then A
8	B till opening of first flower, then A for various times, then B

from inspection of (high) plants in sets (3) and (4) that the appearance of spots on the epidermis of the stem internodes depended on the environment in which the internodes were formed. Thus plants in set (3) continued for some time to show spots and streaks of purple pigment on the lowest internode, which had been formed at 15°, but later internodes, formed at 25° were entirely green. Conversely, plants in set (4) continued to have entirely green lower internodes, but showed spots and streaks on the internodes which developed after the transfer to 15°. The fact that no effect of early environment showed in the flowers presumably means that the early mutational events were largely confined to the differentiating epidermal tissue and did not accumulate in the meristematic cells which later were to give rise to the flower initials.

The patterns of flaking in the flowers of both lines in sets 7 and 8 were all entirely consistent with the idea that the degree of mutability at a given stage of development is dependent on the environment prevailing at that stage. Just a few examples will be taken by way of illustration. Table 5 shows the pattern of spotting in successive flowers of a (high) pal^{rec} plant of set 8 which was transferred back to

25° after just one day at 15°. The flowers already open at the time of the 15° period developed no additional spots as a result of the cool treatment, and neither did any of the flowers which opened during the first five days after the return to 25°. It thus seems that flowers which were within five to six days of opening were too far advanced in their development to be susceptible to a shift in environment. The first flower to show any clear effect was No. 18 (table 5) which opened at day 6 after the start of the 15° period, and showed a somewhat increased

TABLE 5

Effect of a one-day transfer to 15° on a (high) pal^{rec} plant otherwise grown at 25°

Day flower recorded	Flower No.	No. of cells in spot								
		1	2	3-6	8	12	c. 16	c. 32	c. 64	>100
5	15	18	7	4	3	...	...	...	...	...
	16	6	6	...	1	...	...	...	...	...
6	17	18	23	3	1	...	...	...	...	...
	18	**41**	9	2	1	...	...	...	...	...
	19	**56**	8	...	...	...	...	1	...	...
7	20	20	8	3	2	...	...	...	...	...
	21	**47**	**28**	...	...	...	...	...	...	...
	22	**40**	**42**	3	1	...	...	...	...	...
	23	**64**	**36**	1	...	...	...	...	...	...
9	24	**29**	**80**	**20**	2	...	...	...	...	...
	25	**35**	**50**	**37**	**8**	...	...	...	...	...
	26	**31**	**67**	**35**	4	**1**	**1**	...	...	...
	27	2	**12**	**41**	1	**1**	**1**	...	...	...
14	28	...	2	22	**18**	5	**2**	**1**	...	...
	29	5	5	...	1	...	...	**1**	...	**1**
	30	11	3	4	2	1	...	...	...	**2**
21	31	19	11	10	2	...	...	...	...	...
	32	18	13	3	2	1	...	...	...	...
29	33	9	4	2	...	...	...	...	...	...

The days are numbered from the 15° period (day 1). The figures in the body of the table are spot counts on the upper surface of one upper corolla lobe. Figures in heavy type are those which seem to be in excess of the typical 25° value.

frequency of one-cell spots. This increase was more marked in flower 19 opening shortly afterwards. Flower 21, opening on day 7 or 8, was the first to show an excess of two-cell spots, while in flower 24, opening on day 8 or 9 the increase was extended to four-cell spots. Flower 28, opening on day 13 or 14, had a relatively large number of eight-cell spots, and at this point in the flower sequence the number of one- and two-cell spots had decreased once again to the " background " level. Later flowers continued to show a few large spots and flakes with no excess in the smaller size classes. The effect of the 15° period finally died away at around day 24; flowers opening after that time were indistinguishable from flowers on plants which had been at 25° throughout their development.

Table 6 shows the pattern of spotting on another plant of the same set which was given three days at 15°. The general picture was very much the same as for the one-day treatment except that, as might have been expected, the number of extra spots induced on each flower is considerably greater, and there is an indication that the range of spot sizes found on a single flower is also increased. Flower 16, for example, has a considerable number of spots in the eight-cell class while still showing some stimulation in the one-cell class.

TABLE 6

Effect of a 3-day period at 15° on a (high) pal^{rec} plant otherwise grown at 25°

Flower No.	Day flower recorded	Number of cells in spot							
		1	2	4	5-12	c. 16	c. 32	50-100	>100
4	4	2	4	3	2	...	...	...	...
5		11	8	5	2	...	...	...	...
6	6	12	5	...	...	...	...	...	...
7		110	16	...	1	...	...	...	...
8		77	5	...	...	...	...	...	...
9		274	15	...	1	...	...	...	...
10		154	8	...	...	...	...	...	...
11		189	44	...	...	...	...	...	...
12		419	237	...	...	...	...	...	...
13		247	267	...	...	...	...	...	...
14	10	180	439	...	...	...	...	...	...
15		62	290	290	...	...	...	...	...
16		27	174	316	140	10	...	...	...
17	13	2	103	261	134	5	2	...	...
18		...	4	19	20	2	1	...	...
19		1	...	7	18	6	3	...	...
20		...	...	...	6	6	2	2	...
21		...	...	...	2	5	3	...	...
22	18	1	...	1	1	3	1	1	...
28	49	1	2	1	...	...	...	...	...

The period at 15° was during days 1, 2 and 3. Other details as for Table 5.

Plate II shows two plants which were subjected to a 6-day period at 15°. One feature well shown in this photograph, and one which has been noted on several occasions, is that it is the margins of the corolla lobes which retain their sensitivity to mutation for the longest time. This may be an indication that cell divisions are still continuing near the periphery of the lobes after they have ceased elsewhere in the flower.

The reverse type of experiment, with plants transferred for periods of a few days to the 25° environment, after being grown to flowering at 15°, gave results which were the mirror image of those just described. Table 7 shows the effects of an 8-day 25° period on a plant of the

(low) *pal^rec* strain. The earliest flowers to show an effect showed a relative deficiency of spots in the one- and two-cell classes, and the deficiency was extended to the larger spot classes in later flowers. Those opening ten days after the transfer back to 15° had no spots at all, and may be supposed to have undergone the entire sensitive period of their development during the 25° period. During the next few days, one-cell spots began to appear on newly opening flowers, and, with the progressive appearance of the larger spot classes on later flowers, the typical 15° pattern was restored 15 days after the end of the 25° treatment. The time of the susceptible period of flower development is not necessarily expected to be the same in the (low) and (high)

TABLE 7

Effect of an 8-day period at 25° on a (low) pal^rec plant otherwise grown at 15°

Flower No.	Day recorded	Number of cells in spot					
		1	2	3-7	8	c. 16	c. 32
1	7	1	3	5	5	14	4
2	9	...	...	...	7	2	2
3		...	1	2	...	2	...
4		...	...	2	...	...	1
5		...	...	1	...	2	3
6		...	...	...	...	1	...
7	16	...	...	...	...	1	3
8		...	...	...	...	...	...
9		...	...	...	...	...	...
10		2	...	...	...	...	...
11	21	...	...	...	...	...	...
12	24	9	...	1	...	...	...
13		20	14	9	7	1	1
14	29	11	7	7	...	1	...

The 25° period was during days 1-8.

pal^rec stocks, nor in the same stock at 15° and 25°, but the qualitative agreement of the results of the two kinds of temperature transfer experiment is good.

Some very striking effects of a kind which could be expected on the basis of the experiments just described, were obtained in plants of sets 5 and 6, which were permanently transferred at the time of flower bud formation from one environment to the other. Plate III illustrates typical effects on a (high) *pal^rec* plant subjected to the 15°→25° shift. The 25°→15° experiment gave the " mirror image " type of result.

(ii) *Discussion of the results of environmental shifts*

It is evident that mutation can be induced or inhibited at any desired stage of flower development by appropriate manipulation of the environment. The simplest interpretation would be that a temperature change has an almost immediate effect on mutation rate—that

mutations begin to appear in greater frequency at once, or at any rate at the next cell division when transfer is made to 15°, and that this enhanced mutability is as rapidly switched off on return to 25°. To reconcile such a hypothesis with the present data one has to suppose that some cells delay much longer than others before dividing after a temperature shift, and that different cell lineages may go through different numbers of rounds of cell division in the same time. This is because the span of time during which the frequency of spots of a given size is affected is generally greater than the time of the inducing treatment. Indeed, it seems very likely that there is sufficient non-uniformity in cell division for the data to be reconciled with the hypothesis of a rather direct effect of temperature. There is certainly no critical proof of this hypothesis, however, and we cannot rule out the possibility that the effect of the environmental shift may be delayed for several cell generations in some lineages.

There is some evidence of another kind suggesting that the effect of a temperature shift on mutation rate cannot be long delayed. Some rather approximate cell counts on whole corollas, macerated at different stages of development by the method of Brown and Rickless (1949), have shown that there is very little increase in cell number, though a large increase in mean cell size, during the last six days before opening of the flower. This virtual cessation of cell division during the last days of flower development is no doubt connected with the failure to induce any changes in mutation frequency by treatment during this time. Tables 5 and 6 show that the stimulation of two-cell spots is near-maximal eight or nine days after the inducing treatment at 15°. Thus most of the mutations giving rise to such spots must have been established within two or three days of the start of the 15° treatment, and may well have occurred during the very next cell division. More extensive studies on the timing of cell division during flower development would be necessary to place these provisional conclusions on a firm basis.

One anomaly for which there is at present no explanation is that in the 25°→15°→25° experiments on the (high) pal^{rec} stock, flowers, which between them must have been at 15° during all stages of development, do not, even in sum, seem to have as many spots as are usually seen on plants of the same stock grown throughout development at 15°. The deficiency is not confined to large sectors such as might have been initiated before flower initiation. The whole habit and, presumably, the physiological state, is rather different depending on whether plants are raised entirely at 15° or predominantly at 25°, and it may be that long previous growth at the lower temperature makes some difference to the mutational responsiveness of the developing flowers. The 15°→25° shift experiments showed, however, that prior growth at 15° could not substitute, so far as mutation induction was concerned, for exposure to the 15° environment during flower development.

5. ENVIRONMENTAL CONTROL OF GERMINAL MUTATION

Seed was collected from self-fertilised plants grown at 15° and 25° in the early experiment illustrated in plate I, and the resulting seedlings were scored for full red mutants. Among 758 progeny of (high) pal^{rec} plants of the 15° set there were no mutants; less surprisingly there were no mutants among a similar number of progeny of the 25° set. This result was later shown to be quite uncharacteristic of (high) pal^{rec} and is quoted here to show that a high somatic spotting frequency is not *necessarily* associated with a high frequency of germinal mutation. It may be relevant that the (high) pal^{rec} plants used in this experiment were all members of one clone.

Following this negative result the experiment was repeated using (high) pal^{rec} plants of a different clone—one which had been propagated by cuttings from a spotted plant from a family in which several of the sister plants were full red mutants. Seeds from different capsules were sown separately and several capsules were tested from each plant. Scoring of the seedlings was made easier by the discovery that full red mutants can be recognised at the early seedling stage by their uniform, rather than spotty, pigmentation in the cotyledons. Seedling scoring is simple and reliable provided the seedlings are grown under conditions favouring anthocyanin development, that is a good light intensity and not too high a temperature. The accuracy of the scoring was checked by growing a number of plants to flowering, and it was confirmed in every case. The results are shown in table 8.

The calculation of the significance of the results, which at first glance seems obvious, is complicated by the fact that the mutants were obviously not all independent events; the marked clustering of mutants, some capsules yielding many and others none, indicates that some flower initials must have contained sectors of mutant cells in the sporogenous layer, each sector leading to many mutant germ cells. Even mutants arising in different capsules on the same plant cannot be assumed to be of independent origin, though in most cases such an assumption would probably be true. The only valid procedure is to consider the proportion of plants in each set which yielded at least one mutant. While the numbers of seedlings tested from each plant were not exactly the same, it will not introduce much error to assume that each plant would have had the same chance of yielding at least one mutant if the environmental difference were having no effect. Thus we compare the 7 out of 11 plants of the 15° set which yielded mutants with 0 out of 13 in the 25° set. The difference is significant at the 1 per cent. level, even though the number of independent mutational events represented by the mutants in the 15° set was certainly greatly underestimated.

After the discovery of the very high frequency of somatic flaking in F_1 plants from (high) $pal^{rec} \times pal^{tub}$, cuttings were taken from one such plant and grown in the two different environments and self-pollinated. The large difference in flaking frequency caused by the

TABLE 8

Full red mutants in progenies of (high) pal^{rec} plants grown at 15° and 25°

Plant no.	15°						Plant no.	25°					
	Capsules			Seedlings				Capsules			Seedlings		
	No. tested	No. with mutants	Per cent. with mutants	Total	Mutants	Per cent. mutants		No. tested	No. with mutants	Per cent. with mutants	Total	Mutants	Per cent. mutants
1	6	5	83	488	44	9·0	12	1	0	0	84	0	0
2	6	3	50	514	18	3·5	13	7	0	0	532	0	0
3	6	0	0	332	0	0	14	6	0	0	510	0	0
4	6	0	0	402	0	0	15	3	0	0	252	0	0
5	6	3	50	403	15	3·7	16	2	0	0	163	0	0
6	6	0	0	519	0	0	17	6	0	0	504	0	0
7	6	3	50	343	9	2·6	18	7	0	0	583	0	0
8	6	0	0	517	0	0	19	3	0	0	280	0	0
9	6	3	50	462	6	1·3	20	3	0	0	252	0	0
10	6	1	13	490	1	0·2	21	6	0	0	361	0	0
11	4	3	75	272	17	12·5	22	7	0	0	549	0	0
							23	6	0	0	428	0	0
							24	6	0	0	595	0	0
Total .	64	21	32·8	4742	110	2·3	Total	63	0	0	5193	0	0
Excluding plant 1	58	16	27·5	4254	66	1·5							
Fraction of plants with at least 1 mutant	6/11							0/13					

Plants 2-24 were all from the same clone; plant 1 was from a different but closely related clone.

environmental difference was shown in table 3. Seed was collected from one cutting of the 25° set and from three different cuttings of the 15° set. Seedlings were grown to maturity in order to see whether mutant types other than the usual full red would appear. Table 9 shows the results.

TABLE 9

Scoring of F_2 plants from the cross pal^{tub} × (high) pal^{rec}

Temperature at which F_1 grown	F_1 Plant No.*	Capsule No.†	Total plants	Phenotypes§				
				pal^{tub}	pal^{rec}	Full red	Other‡ mutants	Per cent. mutants
25°	1	1	183	46	92	45	0	24·6
		2	270	66	204	0	0	0
		4	194	42	142	10	0	5·2
		5	312	90	222	0	0	0
		6	200	46	154	0	0	0
		7	332	70	262	0	0	0
		8	229	72	157	0	0	0
		9	297	67	230	0	0	0
		Totals	2017	499	1463	55	0	2·7
15°	2	1	230	52	134	30	15	19·6
		2	237	63	63	110	1	46·8
		3	183	62	101	20	0	10·9
		5	245	68	117	59	1	24·5
		8	212	54	108	46	4	23·6
		9	162	37	115	10	0	6·2
		10	146	35	97	13	1	9·6
	3	1	173	47	55	70	1	41·0
		2	132	38	44	41	9	37·9
		3	194	49	59	62	24	44·3
	4	1	182	44	121	17	0	9·3
		3	223	58	75	78	11	39·9
		5	150	38	86	21	5	17·3
		7	84	31	48	0	7	8·3
		8	204	51	152	1	0	0·5
		9	164	35	76	53	0	32·3
		11	80	26	47	5	2	8·7
			198	55	92	36	15	25·8
		Totals	3199	843	1589	672	96	24·0

* All plants were raised from cuttings and were members of one clone.
† In order of development.
‡ With or without superimposed red spots and flakes.
§ Segregation of yellow (*sulf*) and *delilah* (*del*) is ignored in this table.

The data as a whole show a good fit to the expected 3:1 ratio of cyanic to acyanic types. Among the pigmented class, however, there were many fully coloured plants as well as spotted ones. A number of full-red mutants occurred in two of the capsules of the plant grown at 25°, but the incidence of such mutants is obviously very much higher in the progeny of the plants grown at 15°. Thus far the results parallel those obtained with the homozygous (high) pal^{rec} stock,

although the germinal mutation rate, like the somatic spot frequency, is considerably higher in the (high) pal^{rec}/pal^{tub} plants. However, an even more significant feature of the data is the appearance, in 12 out of 18 capsules of the 15° set but in none out of 8 in the 25° set, of mutants of kinds other than full red. At least three different grades of stable light pigmentation were observed, either with or without superimposed full red spots, depending, presumably, on whether pal^{rec} or pal^{tub} was present as the other allele. In addition a number of plants were observed which had a markedly different pattern of variegation. In these plants there were few sharply defined spots, but there was a faint and rather patchy tinge of colour distributed rather generally over the flower but often particularly obvious near the margins of the corolla lobes. These " tinged " mutants appeared to be of several distinguishable kinds, but a definite classification must await further work. They would probably be difficult to detect in the presence of a heavy superimposed flaking, and many may have been missed for this reason. There is an obvious tendency for clusters of mutants of the same kind to occur in the same capsule, but several of the capsules yielded more than one kind of mutant. The mutants observed in the progenies of the 15° plants parallel, in their range of phenotypes, the variety of somatic sectors observed in the same plants (table 10). Some of them may resemble previous reported alleles of *pal*, such as *rubra* (light red), *carnea* (mauve or " flesh coloured "), *rhode* (light mauve), *malacea* (pink tinged) and *maculosa* (indistinct and irregular areas of light pink spotting) (Kuckuck and Schick, 1930).

(i) *The genetic status of the germinal mutations*

The many mutants picked up in the experiment just described have not yet been analysed genetically. It is not known whether all the different types are due to different alleles at the *pal* locus. From limited results already available it seems that at least some of them have such a basis. Relatively extensive genetic analyses have been performed on the common type of full red mutant, both by Mather (1933) and ourselves. In every case examined it has been clear that the mutation was to the stable " wildtype " allele *Pal*, or to an allele of similar effect. In our experience the *Pal* which arises by mutation from pal^{rec} is quite stable. One plant homozygous for a *Pal* allele of mutant origin was crossed both to pal^{tin} and to (low) pal^{rec} stocks. Four hundred seedlings were grown from each cross, and all were full red. No obvious acyanic sectors were present in the flowers, such as might have been expected if the *Pal* allele were tending to revert to pal^{rec}.

One plant of the (high) pal^{rec} stock had previously been observed in which the full red flakes were barely visible against a light red background. This plant, which was similar to one of the types referred to in table 8, was self-pollinated. Among 102 progeny, 26 were typical (high) pal^{rec}, 52 had dark red flakes on red, like the parent, and 24

TABLE 10

Classification of " Other Mutants " of Table 9

Plant no.	Capsule no.	Phenotypes* A Light red		B Mauve or " flesh coloured "		C Light mauve		D Many pale blotches	E Few pale blotches	F Tinged	G Dark patch near base of upper lobe	Total
		with ‡ flakes	no flakes	with ‡ flakes	no flakes	with ‡ flakes	no flakes	no flakes †	no flakes †	no flakes †		
2	1	2	3	0	1	3	4	1	1	0	0	15
	2	0	0	0	0	0	1	0	0	0	0	1
	5	0	0	0	0	1	0	0	0	0	0	1
	8	0	0	0	0	0	0	4	0	0	0	4
	10	0	0	0	0	0	0	1	0	0	0	1
3	1	0	1	0	0	0	0	0	0	0	0	1
	2	7	0§	0	0	0	0	1	1	0	0	9
	3	2	7	3	12	0	0	0	0	0	0	24
4	3	1	0	0	0	0	0	5	4	0	1	11
	5	0	1	0	3	0	0	0	0	1	0	5
	7	0	0	1	4	1	1	0	0	0	0	7
	11	0	0	1	0	0	0	0	1	0	0	2
		4	7	0	0	0	0	4	0	0	0	15
Totals .		16	19	5	20	5	6	16	7	1	1	96

* A, B, C, (D+E) and F seem to correspond to the published descriptions (Kuckuck and Schick) of the *pal* alleles *rubra*, *carnea*, *rhode*, *maculosa* and *malacea* respectively. Some variation occurred within each class.

† These phenotypes would probably not have been detected in the presence of flaking.

‡ Classification of the background colour was more difficult and uncertain in the presence of flaking.

§ The colour here may have been too dark to distinguish from full red except where darker flakes were also present.

had full red flowers. A progeny plant with red flakes on red gave, on selfing, the same 1:2:1 ratio again in the next generation, and gave 13 typical (high) pal^{rec} and 6 rather light red progeny on crossing to pal^{tin}. The other two types of segregant bred true on selfing. Thus everything suggests that the mutation in this case was to an allele of *pal*, possibly resembling *pallida-rubra*. It seems highly likely that the more recently detected mutants with various grades of light pigmentation will prove to have a similar genetic basis.

6. GENERAL DISCUSSION

We have shown that under certain environmental conditions, and in certain genetic backgrounds, pal^{rec} becomes quite extraordinarily unstable, tending to mutate with high frequency to a number of different states. Under other conditions, and in other backgrounds, the same allele can be relatively stable. The genetic factors controlling the degree of instability have not yet been analysed, but more than one chromosome locus seems likely to be involved. It may be that multiple elements of cumulative effect, perhaps like " modulator " (Brink, 1960) or " activator " (McClintock, 1956) in maize, are present in our *Antirrhinum* material.

There are strong indications that the somatic changes which are visible in corolla spots and sectors are similar in kind to the germinal mutations which show up in the whole plants and are, so far as we have tested them, stably transmitted through further sexual generations. Firstly, as Mather (1933) has already shown, the frequencies of somatic and germinal mutations are correlated. Secondly, both respond in the same way to environmental change. Thirdly, all the quite numerous kinds of somatic sector have their counterparts in germinal mutants.

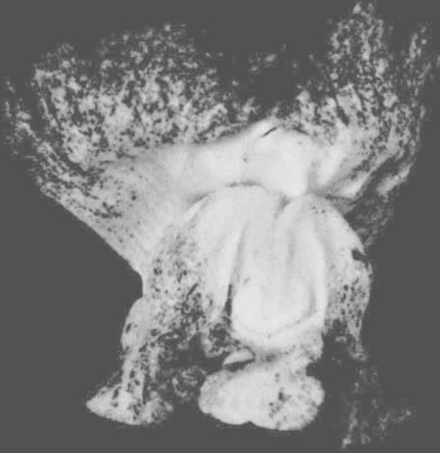
There seem to be no valid grounds for denying the title of " mutation " to the kind of genetic change involved here. However, it hardly seems likely to resemble, at the molecular level, the kind of very infrequent mutation of which some understanding has been achieved as a result of work on chemical mutagenesis in micro-organisms. The general conclusion derived from the microbial work is that mutations consist of base substitutions (or sometimes additions or deletions of sequences of bases) in the genetic DNA. There is no obvious mechanism for transmissible instability in a gene given the generally accepted semi-conservative mechanism of DNA replication. The problem of explanation becomes still more difficult when the unstable gene can mutate to more than two other relatively stable states. Mutable loci which can mutate to several other alternative states have been reported in several cases in maize (McClintock, 1956; Peterson, 1963) and the present work appears to add another example.

It is difficult to avoid the impression that the type of very frequent mutation which finds expression in obvious somatic variegation is due to an order of genetic determinants different from, and in a sense superimposed on, the DNA which carries the basic information for

specific macromolecular structure. Brink (1960) has, in a useful review of the field, proposed the terms *orthochromatin* and *parachromatin* to denote the self-replicating genetic materials concerned respectively with carrying the permanent genetic information of the organism and with the regulation and timing of the expression of that information. *Parachromatin*, in this sense, would include the controlling elements in maize, now well known through the work of McClintock, and possibly also the heterochromatic elements responsible for variegated-type position effect in *Drosophila*. *Parachromatin* is thought to differ from *orthochromatin* in its susceptibility to environmental factors and to other elements in the genome. While this is hardly the place to argue the point in detail, there seems to be no good reason to put variegated-type position effects in *Drosophila* in a fundamentally different category from mutable gene situations in flowering plants. Position effect variegations seem to have a cell-lineage basis (Baker, 1963) as if due to somatic mutation, they are known to be sensitive to both genetic and environmental factors (Gowen and Gay, 1934), and the fact that they do not seem to involve the germ cells may merely reflect the greater degree of insulation from environmental effects enjoyed by the animal germ line.

Flaked and spotted varieties are widespread in flowering plants. Most are attributed to mutable genes, but in one case (Catcheside, 1947) a position effect of the *Drosophila* type is responsible. In several cases it is known that the somatic mutation frequency responds to changes in temperature in much the same way as pal^{rec} of *Antirrhinum*. Fabergé and Beale (1942) showed that the flaking frequency in a mutable variety of *Portulaca* was much reduced by culture at higher temperatures. Sand (1957) studied a *Nicotiana* variety of hybrid origin in which speckled red-on-white sectors appeared in plants which otherwise had full red flowers. A reversible mutation appeared to be responsible. It was shown that the frequency of the speckled sectors increased, but that the density of spotting within these sectors decreased with increasing temperature. Rhoades (1941), in the classical case of the *Dotted*-a_1 system of maize, provided evidence that dot frequency was inversely related to temperature. One experiment of our own, on a strain of *Primula sinensis* homozygous for the mutable allele *e* (De Winton and Haldane, 1935), showed that the frequency of purple sectors was very much lower when the plant was cultured at 25° than it was at 15° or under greenhouse conditions. All these examples have in common a decreasing frequency of mutation to anthocyanin production as the temperature is increased. One is tempted to suggest a connection between this apparently rather general rule and the fact that lower temperatures tend, in general, to favour anthocyanin synthesis in higher plants. Such a link between a genetic mutation and a developmental process is not unexpected if the mutation in question represents in distorted form a type of change which is normally only of ontogenetic significance. If this kind of

HIGH MUTATION LINE



LOW MUTATION LINE

15° C.

25° C.

Representative flowers of the (high) pal^{rec} and (low) pal^{rec} stocks grown at 15° with 16-hour days, and at 25° with 10-hour days. The (high) pal^{rec} clone used in this experiment gave a lower frequency of large flakes than the one used in the experiment illustrated Plates II and III.

Two (high) *pal*^{rec} plants grown at 25° (10-hour days) and transferred for 6 days to 15° (16-hour days) during flower development. The plant on the left was transferred to 15° 3 days after the opening of the first (lowest) flower. Only the latest flowers show spots, mostly 1-celled and concentrated near the margins of the corolla lobes. The plant on the right was transferred *back* to 25° 10 days before the opening of the first flower, which shows a moderate number of rather large spots. Later flowers show fewer and fewer spots of progressively larger mean size.

Plate III

Two (high) pal^{rec} plants grown at 15° (16-hour days) and transferred permanently to 25° (10-hour days) during flower development. The first (lowest) flower of the plant on the left opened 17 days after the transfer, and that of the plant on the right 22 days after transfer. The first flower of the plant on the right roughly coincided in time with the last flower of the plant on the left. Note the decrease in flaking frequency in successive flowers, and the increase in mean flake size.

idea has any validity, it may be that a study of the factors which influence mutation rate in highly mutable systems may give information relevant to the understanding of the stimuli which initiate normal cell differentiation.

7. SUMMARY

1. In *Antirrhinum majus* alleles of the *pallida-recurrens* (pal^{rec}) type tend to mutate frequently to the stable dominant *Pal*, giving magenta spots and sectors in the otherwise acyanic flowers. Different stocks show widely different spot frequencies. At least part of this variation is due to modifiers at other loci; whether differences at the *pal* locus itself are also involved is still to be determined.

2. Within a stock showing a characteristic and fairly constant spot frequency, far fewer spots appear if the plants are cultured at 25° with 10-hour days than if they are cultured at 15° with 16-hour days. By manipulating the environment, mutations can be induced, or prevented from occurring, at any desired stage of flower development.

3. The same environmental difference has a large effect, in the same direction, on the frequency of mutations to *Pal* among the germ cells.

4. In F_1 plants from the cross of a pal^{rec} stock with a high flaking frequency to another stock homozygous for the stable recessive pal^{tub}, an even higher flaking frequency is observed. This enhancement of somatic mutation rate is not due to pal^{tub} itself but to other factors present in the pal^{tub} stock.

5. When grown in the 15° environment the pal^{rec}/pal^{tub} F_1 plants showed an extremely high frequency of somatic sectors of many different types. Selfed seed matured on these plants at 15° and subsequently germinated in the greenhouse and grown in outside plots, showed over 20 per cent. mutants. Most of the seedling mutants were of the *Pal* type, but several other mutant types, some of them resembling the phenotypes of other known alleles of the *pal* series, were found. Plants of the same genotype grown in the 25° environment gave far fewer somatic flakes and germinal mutations, and such mutations as there were were all of the common *Pal* type.

6. The theoretical significance of mutable genes is considered in the light of these results.

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