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Genetic analysis of instability in *Petunia hybrida*

4. The effect of environmental factors on the reversion rate of unstable alleles

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Summary. The effect of environmental factors on the reversion rates of several unstable alleles in *Petunia hybrida* was investigated. It is demonstrated that the reversion frequency of three unstable alleles, viz. an allele of gene *An1* and of gene *An11*, both involved in anthocyanin synthesis, and of gene *Yg3* for leaf colour, is drastically reduced when the temperature is raised from 18 °C to 25 °C. For two of the alleles it was established that this temperature effect is reversible. Changing the light period or light intensity did not have an effect on the reversion rate of the unstable allele of gene *An11* at 18 °C or at 25 °C. The results found are in contrast with those obtained in earlier experiments, in which a rise in temperature resulted in an increase in the reversion rate of another unstable allele of gene *An1*.

Key words: Unstable alleles – Reversions – Effect of temperature – *Petunia hybrida*

1 Introduction

Genetic instability resulting in variegation patterns is a frequently occurring phenomenon.

It has been thoroughly investigated in several higher plant species, e.g. *Zea mays* (McClintock 1965; Peterson 1970), *Antirrhinum majus* (Harrison and Carpenter 1979) and *Impatiens balsamina* (Sastry 1982). The occurrence of instability in these species is generally explained by the presence of a genetic element inserted at the locus in question. Such elements are assumed to suppress the gene activity. Transposition away from the locus is thought to be responsible for the restoration of activity.

In *Petunia hybrida*, variegation in flower colour as a result of instability at the *An1* locus has been extensively investigated. Gene *An1* is involved in anthocyanin synthesis (Wiering 1974). The locus is assumed to be composed of a structural gene responsible for an enzyme active during anthocyanin synthesis and an adjoining regulatory region which determines the place, point in time and rate of activation of the structural gene (Bianchi et al. 1978). Unstable *An1* alleles arose as the result of mutations in the regulatory region of the locus. Such mutations are assumed to have been induced by a transposable element already inserted at the locus. Reversions are ascribed to the frequently occurring repair of the mutations (Doodeman et al. 1984a). Indications that a relationship exists between the occurrence of instability and the presence of transposable elements in the genome were found in the discovery of new, unstable mutations at other loci in descendants of unstable *An1* mutants. Those new unstable mutations are presumed to be the result of transpositions of the element inserted at the *An1* locus to other loci (Doodeman et al. 1984b).

In earlier experiments the influence of environmental factors upon the reversion frequency of the allele $an1^{s/+}$ was investigated. This unstable allele gives rise to white flowers with a large number of red spots. It could be established that there was a significant increase in spot density at 25 °C as compared with 18 °C (Bianchi et al. 1978). Changes in the light regime and nutritional conditions also proved to have a marked effect on the reversion frequency (Bianchi et al., unpublished).

The experiments described in this paper were designed to examine the effect of environmental factors on the reversion frequencies of another unstable *An1* allele, i.e. $an1^{s/p-+}$, and of unstable alleles of two other genes that presumably arose as the result of transpositions of the element inserted at the *An1* locus.

2 Materials and methods

2.1 Plant material

Table 1 shows the genotypes and phenotypes of the different mutants that were used in the investigation. The plants homozygous or heterozygous for the unstable allele $an1^{s/p-+}$ were clones, propagated by cuttings. The mutants with the unstable allele $an11^{s/+}$ were all selected from one family

Table 1. Genotypes and phenotypes of the plant material

Genotype	Phenotype
$an1^{s/p-+}an1$	White flowers with red and pink spots and occasional unspotted, white sectors ('white-red-pink')
$an1^{s/p-+}an1^{s/p-+}$	White flowers with red and pink spots, within the pink spots smaller and darker pink and red dots ('white-red-spotted pink')
an1an1	Unspotted, white flowers
$an11^{s/+}an11$	White flowers with red spots
an11an11	Unspotted, white flowers
$yg3^{r}yg3^{r}$	Plants with yellowish green leaves bearing sharply delimited spots of normal green colour

obtained from the cross $an11^{s/+}an11^{s/+} \times an11an11$. The yellowish green plants were obtained by selfing a plant with genotype $yg3^{r}yg3^{r}$. All plants were initially reared in the greenhouse where the temperature fluctuated between 25 °C and 15 °C, with an average of 20 °C.

2.2 Conditions in the growth chambers

The experiments were carried out in growth chambers with a floor space of 3.0 m × 2.8 m and a height of 1.9 m, in which different environmental factors could be varied. Lighting was provided by 30 lamps per chamber (Philips HPI/T 400 W 9/92/2).

In the first series of experiments, the effect of temperature on the reversion frequency of $an1^{s/p-+}$ was studied at 18 °C and 25 °C. An 18 h period of light with an intensity of 14,000 lux was given, followed by 6 h of darkness.

In the second series, the effect of temperature and light on the reversion rate of $an11^{s/+}$ was investigated. The temperature (18 °C and 25 °C), light intensity (6,000 lux and 14,000 lux), and light regime (16 h days and 20 h days) were varied in several experiments.

The third unstable allele included in the experiments was $yg3^{r}$. The effect of the temperature on the reversion rate of this allele was studied at 18 °C and 25 °C. A daylight period of 18 h was provided with a light intensity of 14,000 lux.

2.3 Reversion frequencies

Each individual colour spot in a flower is the result of a single reversion, so that the number of spots on the corolla can be taken as a measure of the rate of reversion in somatic tissue. Since it was not possible to count the total number of spots per flower, only spots $\geqq 0.16$ mm were counted in a specific area of 1 cm² on each flower. In one experiment, the number of spots $\geqq 2$ mm per flower was also counted. The reversion rate of $yg3^{r}$ was measured by counting the number of green spots $\geqq 0.16$ mm/cm² in corresponding parts of comparable, young leaves.

In order to compare reversion frequencies in sporogenous tissue, pollen of two groups of plants with genotype $an1^{s/p-+}$ $an1^{s/p-+}$, reared at 18 °C and 25 °C, was used in crosses with stable white-flowering plants (*an1an1*), cultivated in the greenhouse. From the segregational ratios observed, the percentages of mutated gametes was determined. Information about the influence of the temperature on reversion rates in sporogenous tissues was obtained by comparing the number of revertant descendants.

The anthers of 40 flower buds at different stages of development were examined under the microscope (aceto-orcein squash technique) in order to determine at which stage meiosis took place. The initial stages of meiosis were never observed in anthers of buds smaller than 6 mm. Before the plants to be used as male parents in the crossing experiments were placed into the growth chambers, all floral buds exceeding a length of 2 mm were removed to ensure that microsporogenesis only took place at the given temperature.

3 Results

3.1 The effect of temperature on the reversion rate of the unstable allele $an1^{s/p-+}$

3.1.1 Reversions in somatic tissue. In order to establish the difference in rates of growth at 18 °C and 25 °C, the lengths of the corollas of 20 floral buds were measured during their development. The results are given in Fig. 1. As might be expected, the flowers at 18 °C grew more slowly and started openings six days later than those at 25 °C.

To examine the effect of temperature on the reversion rate of $an1^{s/p-+}$ in somatic tissue, white-flowering plants with red and pink spots (genotype $an1^{s/p-+}an1$) were used; 12 plants were placed at 18 °C and 12 plants at 25 °C. A 'white-red-pink' flower of a plant from each group, three weeks after transfer to the growth chambers, is shown in Fig. 2 (18 °C) and Fig. 3 (25 °C).

Each day, the flowers that had completely opened were picked from each group. The number of red and pink spots $\geqq 0.16$ mm/cm² in each flower was counted. The results are given in Fig. 4. Transfer of the plants from the greenhouse to the growth chamber at 18 °C did not have any marked effect upon the spot

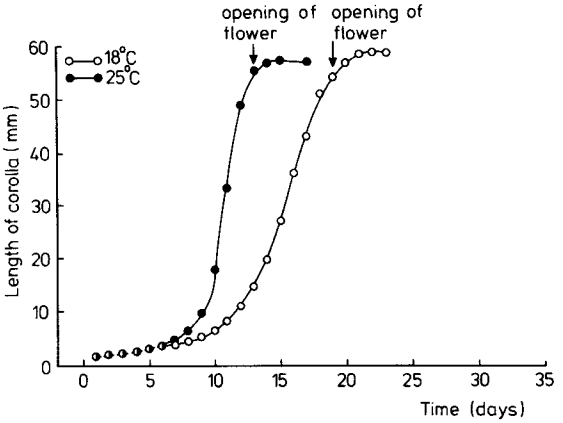

Fig. 1. Rates of growth of floral buds at 18 °C and 25 °C (20 buds/day). Each day the distance between the receptacle and the extreme end of the longest petal was measured, except for the first six days when the length of the longest sepal was taken

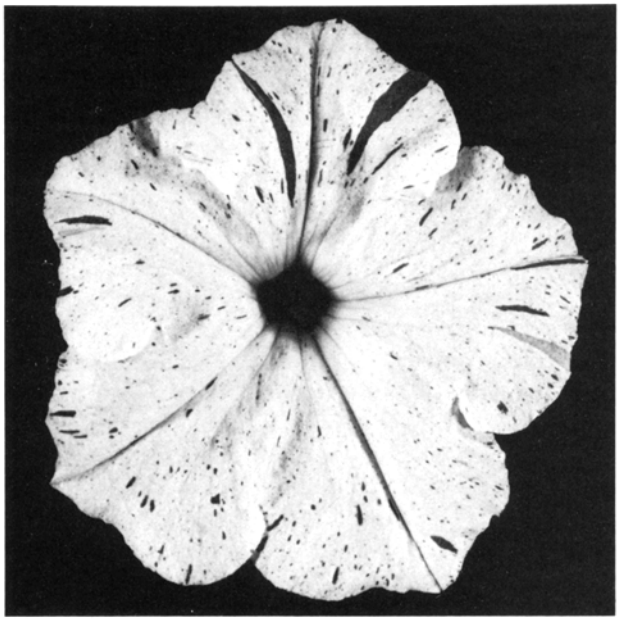

Fig. 2. A 'white-red-pink' flower of a plant with genotype $an1^{s/p-+}an1$, three weeks after transfer from the greenhouse to a growth chamber with a temperature of 18 °C

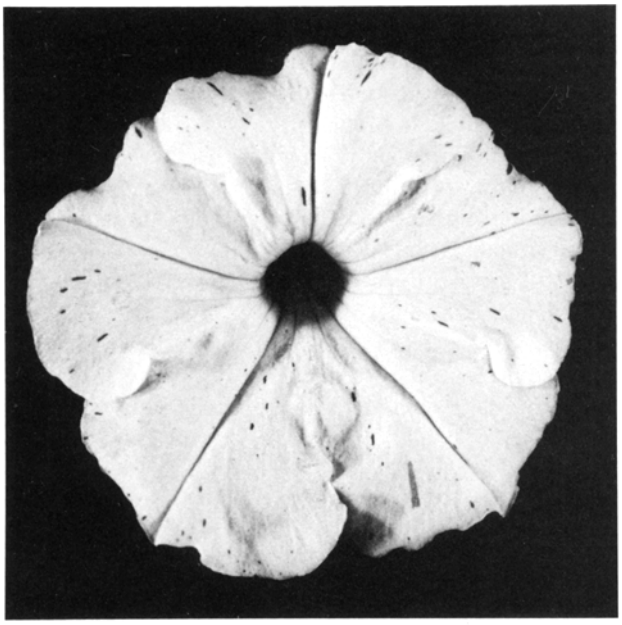

Fig. 3. A 'white-red-pink' flower of a plant with genotype $an1^{s/p-+}an1$, 3 weeks after transfer from the greenhouse to a growth chamber with a temperature of 25 °C

frequency. However, transfer to 25 °C resulted in a gradual decrease in the average number of spots ≧ 0.16 mm, starting after six days. Apparently, at the time the plants were placed at 25 °C, flowers which were within six days of opening, were too far advanced in their development to be susceptible to a change in temperature. This must be ascribed to the fact that during the last phase of floral development, growth takes place exclusively by cell expansion and therefore the variegation pattern is not subject to changes, since reversions of unstable *An1* alleles only occur in dividing cells (Bianchi et al. 1978). It follows that a shift in temperature can only exert an influence upon the spot density in the corolla during the period of flower development that is characterized by mitotic activity. From the diagram presented in Fig. 4, it can be deduced that the flowers examined on day 14 and subsequent days must have experienced their entire sensitive period at the new temperature of 25 °C. In the period between day 6 and day 14, an increasing part of the susceptible period elapsed at the given temperature. From this graph, it can also be concluded that at 25 °C, the total period of floral development lasts about 14 days. This is in agreement with the results given in Fig. 1.

In order to determine whether the temperature effect just described was reversible, a second series of experiments was carried out. A group of 12 plants from the same clone (genotype $an1^{s/p-+}an1$) was transferred from the greenhouse to a growth chamber set at 25 °C. After six days, the plants were moved to a chamber kept at 18 °C. Each day the fully grown flowers were picked and this time, in addition to the number of spots ≧ 0.16 mm/cm², the total number of spots ≧ 2 mm per flower was also counted. The results presented in Fig. 5 indicate that the average number of spots was approximately the same in both cases.

The initial decrease in spot frequency must be the result of the 6 day period at 25 °C and the subsequent increase in spot frequency the result of the transfer to 18 °C. The shape of both curves is similar. However, the effect of the temperature shift on the average number of spots ≧ 2 mm, was somewhat delayed as compared with the effect on the number of spots

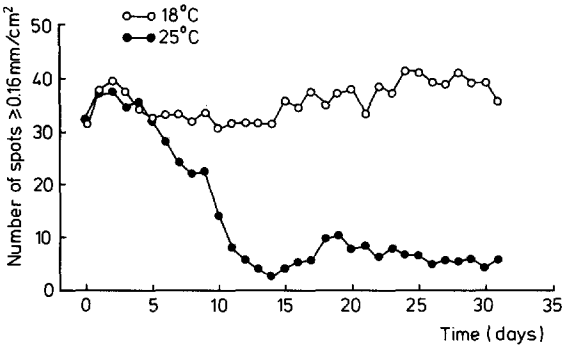

Fig. 4. The effect of temperature on the spot density in corollas of 'white-red-pink'-flowering plants (genotype: $an1^{s/p-+}an1$). On day 0 the plants were transferred from the greenhouse to the growth chambers

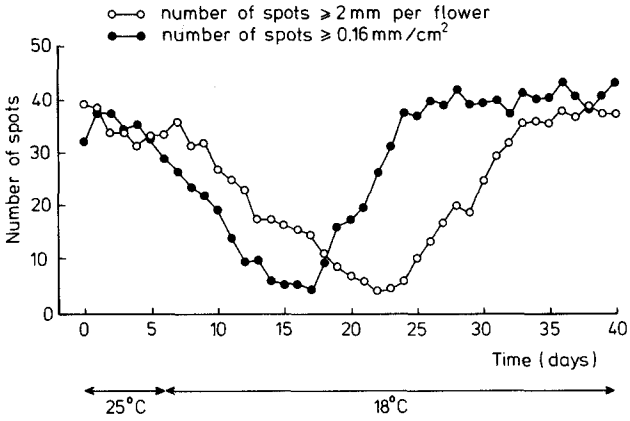

Fig. 5. The effect of temperature on the spot density in corollas of 'white-red-pink'-flowering plants (genotype: $an1^{s/p-+}an1$). On day 0 the plants were transferred from the greenhouse to a growth chamber of 25 °C. On day 6 the plants were transferred to 18 °C

$\geqq 0.16$ mm/cm². This phenomenon is easily understood when the point of time at which reversions occur, is taken into consideration. Small spots were the result of reversions that occurred in a late stage of floral development, whereas larger spots were due to earlier reversions. Consequently, the first effect of a rise in temperature on the reversion rate of $an1^{s/p-+}$ is a decrease in the number of the smallest spots. Accordingly, the larger the size of the spots counted, the later the temperature effect upon spot density. The same holds true for an increase in spot frequency as the result of the transfer from 25 °C to 18 °C.

3.1.2 The reversion rate of $an1^{s/p-+}$ in sporogenous tissue. In order to investigate the influence of the temperature on the reversion rate of $an1^{s/p-+}$ in sporogenous tissue, plants homozygous for the unstable allele were used. Such individuals could be distinguished from the heterozygotes because they bore white flowers with a greater density of red and pink spots. Moreover, within the pink spots, smaller darker pink or red dots were visible as the result of mutations of the second unstable allele. Pollen from two groups of these plants (genotype $an1^{s/p-+}an1^{s/p-+}$), reared at 18 °C and 25 °C, was applied to the stigmas of stable-white flowering plants (*an1an1*), cultivated in the greenhouse. The results from these crosses are listed in Table 2. The self-coloured, red- and pink-flowered descendants were the result of reversions of $an1^{s/p-+}$ towards red and pink in the sporogenous tissue of the variegated-flowering parent plant; the unspotted white-flowering plants must be the result of mutations towards stable white. A test of the homogeneity shows that the segregations found in the two families differ significantly (see Table 2). This justifies the conclusion that the rate of reversion of $an1^{s/p-+}$ in sporogenous tissue is larger at 18 °C than at 25 °C. These results also indicate that temperature has a similar influence on the mutation rate of $an1^{s/p-+}$ towards stable white (*an1*).

3.2 The effect of temperature on the reversion rate of the unstable allele $an11^{s/+}$

One of the new unstable mutations that were found in descendants of plants with the allele $an1^{s/p-+}$ affected another anthocyanin gene, viz. *An11*. This new, unstable allele ($an11^{s/+}$) gave rise to plants bearing white flowers with a large number of red spots. It is assumed that the element inserted at the *An1* locus was transposed to the regulatory region of the *An11* locus. Subsequently, the inserted transposable element must have induced a mutation that inhibited the activation of the structural gene. Reversions are believed to be the result of frequently occurring repair of the mutation (Doodeman et al. 1984 b).

In order to determine whether temperature exerts an influence on the reversion rate of this new mutation, two groups of white-flowering plants with red spots

Table 2. Progenies obtained from crosses of stable white-flowering plants (*an1an1*) with two groups of 'white-red-spotted pink'-flowering plants ($an1^{s/p-+}an1^{s/p-+}$)[a], reared at 18 °C and 25 °C

Parents[a]	Descendants								
	White-red-pink[a]		Unspotted white		Self-coloured pink		Self-coloured red		Total
	No.	%	No.	%	No.	%	No.	%	No.
$an1an1 \times an1^{s/p-+}an1^{s/p-+}$ (18 °C)	1,245	*86.7*	47	*3.3*	40	*2.8*	104	*7.2*	1,436
$an1an1 \times an1^{s/p-+}an1^{s/p-+}$ (25 °C)	545	*97.1*	3	*0.5*	5	*0.9*	8	*1.4*	561
Total	1,790	*89.6*	50	*2.5*	45	*2.3*	112	*5.6*	1,997

[a] See Table 1 for a description of phenotypes and genotypes
Test on homogeneity: $\chi^2_{2\times4} = 47.751$; df = 3; $P \ll 0.001$

(*an11$^{s/+}$an11*) were placed in the growth chambers, one at 18 °C and one at 25 °C. In both chambers, a daylight period of 16 h was provided with a light intensity of 14,000 lux. Reversion frequencies were again measured by counting the spots ≧ 0.16 mm/cm². The results, given in Fig. 6, are similar to those found in the experiments with *an1$^{s/p-+}$* (Fig. 4). However, in this instance, the rise in temperature ultimately led to the complete disappearance of spots ≧ 0.16 mm/cm². Some spots could still be discerned, but they were always smaller than 0.16 mm.

A group of six plants was transferred from the growth chamber at 25 °C to that at 18 °C, for a period of 6 days. This treatment restored the number of spots to the original level, but was followed by another decrease when re-transferred to the room at 25 °C (Fig. 7) These results show that the temperature effect on the reversion frequency of *an11$^{s/+}$* is reversible, just as it was for *an1$^{s/p-+}$*.

In order to obtain some information about the nature of the mechanism responsible for the frequent repair of the mutation in *An11,* an experiment was carried out in which a plant (genotype *an11$^{s/+}$an11*) was placed in a small cabinet in which the temperature was kept at 18 °C. One branch was first defoliated, led out of the cabinet and then allowed to produce flowers at 25 °C. The plant was reared at 18 °C prior to the experiment and, as a result, bore flowers with a large number of red spots. After transfer to the 18 °C cabinet, the plant continued to produce flowers with a high spot density. However, the flowers on the branch that was kept at 25 °C, showed a marked decrease in the density of spots.

Another plant of the same genotype, reared at 25 °C, had flowers with hardly any spots at all. As might be expected, transfer to the 18 °C cabinet resulted in an increase in the average number of spots, while a branch growing at 25 °C kept producing flowers without spots ≧ 0.16 mm. These results show that the influence of a particular temperature on the reversion frequency of the unstable allele remains restricted to that part of the plant kept at the temperature in question. This indicates that the repair mechanism is not influenced by factors formed elsewhere in the plant.

3.3 The effect of light intensity and light regime on the reversion frequency of the unstable allele an11$^{s/+}$

Both the light intensity and the light regime were varied in several experiments using white-flowering plants with red spots (*an11$^{s/+}$an11*). In the first series of experiments, the plants were placed in growth chambers that were kept constant at 18 °C. The results are presented in Fig. 8. Apart from the initial, small increase that must be ascribed to the transfer of the plants from the greenhouse to the growth chambers, the average number of spots ≧ 0.16 mm/cm² per flower did not deviate from that observed at 18 °C in the other experiments (compare Fig. 6). Neither a change in the

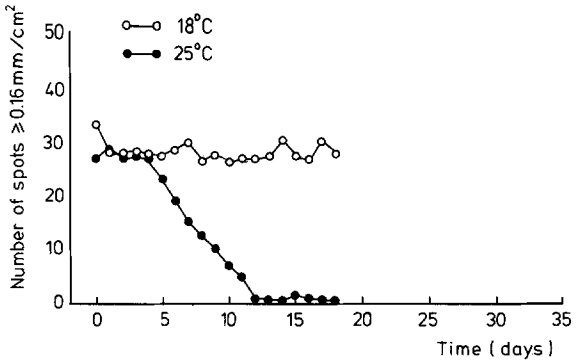

Fig. 6. The effect of temperature on the spot density in corollas of white-flowering plants with red spots (genotype: *an11$^{s/+}$an11*)

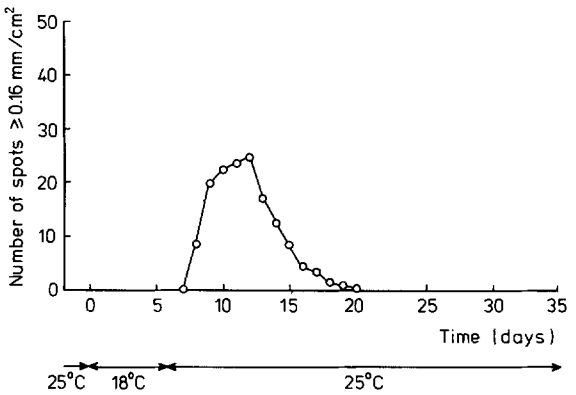

Fig. 7. The effect of temperature on the spot density in corollas of white-flowering plants with red spots (genotype: *an11$^{s/+}$an11*). On day 0 the plants were transferred from 25 °C to 18 °C. On day 6 they were re-transferred to 25 °C

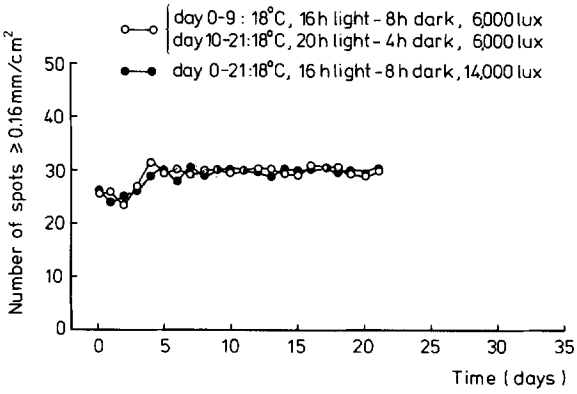

Fig. 8. The effect of the light period and the light intensity at 18 °C on the spot density in corollas of white-flowering plants with red spots (genotype: *an11$^{s/+}$an11*)

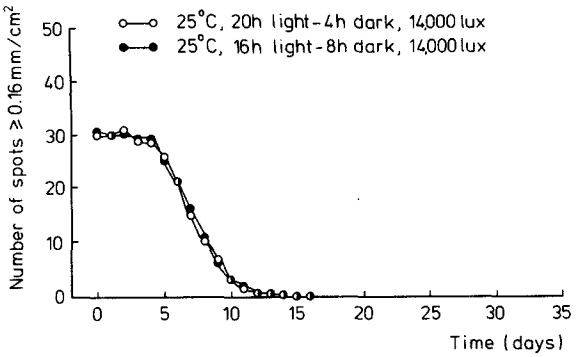

Fig. 9. The effect of the light period at 25 °C on the spot density in corollas of white-flowering plants with red spots (genotype: $an11^{s/+}an11$)

light regime nor a change in the light intensity could be shown to have any effect on the spot density at the given temperature.

In another experiment, the influence of a variation in the light regime at 25 °C was investigated. For this purpose, plants with genotype $an11^{s/+}an11$, reared at 18 °C, were transferred to 25 °C. The results (Fig. 9) show that the same decrease in spot density due to a rise in temperature was manifest irrespective of whether the light period was 16 or 20 h (compare Fig. 6). Therefore this variation in the light regime did not influence the reversion rate of $an11^{s/+}$ at 25 °C.

3.4 The effect of temperature on the reversion rate of the unstable allele yg3^r

The allele $yg3^r$ leads to plants with yellowish green leaves bearing sharply delimited spots of normal green colour. The mutation that gave rise to this unstable allele is assumed to have occurred in the same way as was described for the allele $an11^{s/+}$, i.e. by transposition of a genetic element inserted at the *An1* locus to the *Yg3* locus (Doodeman et al. 1984 a, b). Of 48 plants homozygous for the mutation and reared in the greenhouse, the number of green spots $\geqq 0.16$ mm/cm² on corresponding parts of comparable leaves was counted. This yielded an average number of 34.0 spots per leaf. Subsequently, two groups of 24 plants were transferred to different growth chambers kept at either 18 °C or 25 °C. After a period of 4 weeks, the spot density was again measured on leaves that had developed at the given temperature. At 18 °C the average number of spots $\geqq 0.16$ mm/cm² was 27.9 per leaf. However, at 25 °C the number of green spots was found to have decreased drastically, the average being 5.2 per leaf. These results clearly show that the effect of temperature on the reversion rate of $yg3^r$ at 18 °C and 25 °C is similar to the effect demonstrated for the unstable alleles $an1^{s/p-+}$ and $an11^{s/+}$.

4 Discussion

The results of the experiments described in this paper clearly show that a rise in temperature can depress the reversion frequencies of the unstable alleles $an1^{s/p-+}$, $an11^{s/+}$ and $yg3^r$. For the alleles $an1^{s/p-+}$ and $an11^{s/+}$ it was established that this temperautre effect is reversible, indicating that the influence is direct and does not change the characteristics of the alleles in question.

That the environment can influence variegation patterns has been demonstrated in several species. As far back as 1926, Eyster found that the spot densities of several strains of maize with variegated pericarps varied if they were grown under different external conditions. Rhoades (1942) studied the *a-Dt* system in maize and provided evidence that dot density was inversely related to the temperature. A similar temperature effect upon the mutation rate of an unstable allele of a gene for flower colour was demonstrated in *Portulaca grandiflora* (Fabergé and Beale 1942). In *Nicotiana* the allele v_s gives rise to white flowers with red spots due to changes from v_s to v_S (red). The density of spotting decreases with increasing temperature. The reverse change from v_S (red) to v_s (colourless) also appears and, interestingly, is increased when the temperature is raised (Sand 1957). Harrison and Fincham (1964) thoroughly investigated the type of variegation in *Antirrhinum majus* that is caused by frequent reversions of pal^{rec} alleles to the stable dominant *Pal*, giving coloured spots and sectors in otherwise acyanic flowers. They found that the spot frequency was drastically reduced when the temperature was raised from 15 °C to 25 °C. In all these examples, a rise in temperature suppresses the rate of reversion of unstable alleles, as was demonstrated to be the case for the unstable alleles $an1^{s/p-+}$, $an11^{s/+}$ and $yg3^r$ in *Petunia*.

A reverse temperature effect has already been demonstrated for the unstable allele $an1^{s/+}$ in *Petunia* and was also found in maize by Peterson (1958) for the unstable allele pg^m that gives rise to green stripes on pale green leaves. In this instance, a marked increase in the mutation rate occurred with an increase in temperature. In *Drosophila*, temperature differences could not be shown to have an effect on the instability of the miniature-3-gamma gene (Demerec 1932).

Reversions of unstable alleles in organisms like maize (McClintock 1965; Peterson 1970), *Antirrhinum* (Harrison and Carpenter 1979) and *Drosophila* (Green 1980) are generally ascribed to the excision of a transposable element inserted at the locus in question, resulting in the restoration of the gene activity. Since enzymes must be involved in such reversions, one would expect the reversion rate to be susceptible to temperature effects. Nonetheless, such effects need not be the same for the different unstable alleles, for different enzymes may be involved. Even within one species, the temperature effect can vary, as in the case of the *a-Dt* system and the mutable allele pg^m in maize.

In *Petunia*, however, for reasons extensively discussed elsewhere (Doodeman et al. 1984 a, b), reversions that lead to variegation are not ascribed to excisions of a transposable element, but to frequent repair of mutations in the regulatory region of the locus in question. Such mutations are assumed to be induced by a transposable element inserted in the regulatory region of the locus. Most likely, the temperature affects the activity of the enzymes involved in repair, and thus affects the reversion rates.

In contrast to the results described in this paper are those from earlier experiments using *Petunia* mutants having the unstable allele $an1^{s/+}$. This allele leads to a quite different phenotype than $an1^{s/p-+}$: whereas the latter leads to white flowers with red and pink spots and larger coloured sectors, $an1^{s/+}$ only gives rise to small red spots and large coloured sectors occur far less frequently. The spot density in flowers of plants with $an1^{s/+}$ reared at 25 °C appeared to be 2–3 times as large as in flowers of those reared at 18 °C (Bianchi et al. 1978 and unpublished). Thus the effect of a rise in temperature on the reversion rate of $an1^{s/+}$ is opposite to the effect on the reversion rate on $an1^{s/p-+}$.

The unstable allele $an1^{s/p-+}$ was found among the descendants of a red-flowering plant carrying an allele that arose as the result of a reversion of $an1^{s/+}$. Since reversions are ascribed to the repair of a mutation that was induced by an inserted transposable element, such a reverted allele still contains the insert. Although the insertion of another transposable element at a different position in the *An1* locus cannot be ruled out, it is assumed that $an1^{s/p-+}$ resulted from a new mutation induced by the element that, after reversion of $an1^{s/+}$, was still inserted at the same position of the locus. However, the two mutations that resulted in $an1^{s/+}$ and $an1^{s/p-+}$ must be of a different nature. This is manifest not only in the difference in phenotypic expression but also in the opposite effects that a change in temperature has upon their reversion frequencies. This difference in reaction to a temperature shift may indicate that different enzymes are involved in the repair processes leading to reversions, for the temperature optimum for the reversion of $an1^{s/+}$ was higher than that for $an1^{s/p-+}$.

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