INSTABILITY OF Hh HETEROZYGOTES IN FLAX GENOTROPHS

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SUMMARY

The induction of large (L) and small (S) genotrophs by the environment from the plastic (Pl) genotroph is accompanied by changes in a gene determining hairy (H) and hairless (h) septa in the capsules. S and Pl are HH, L is hh. The heterozygote Hh is unstable and three heterozygous classes, I, II, III, are recognisable in the F_2 with approximately 30, 40, 50 hairs per septum. $H \rightarrow h$ and $h \rightarrow H$ changes occur and class III F_2 Hh heterozygotes with the highest hair numbers give H : h ratios in F_3 of about 6 : 1. Selection of class III F_2 Hh heterozygotes also selects for Hh plants with a high $Hh \rightarrow HH$ potential, but only about 33 per cent. of these F_2 plants have this potential in so far as it leads to an overt and immediate change of $Hh \rightarrow HH$ by the next generation. In the F_3 of $L \times S$ and $S \times L$ crosses a further proportion of Hh heterozygotes has the $Hh \rightarrow HH$ potential, but not in the $Pl \times L$ and $L \times Pl$ crosses. Thereafter no further changes are found. The fade-out in F_3 and F_4 may be due to stabilisation at the locus or of associated factors, to the different environments in which the generations are grown, or because selection of class III was discontinued after F_2 and all three heterozygous classes reappeared in F_3 . These orderly, high-frequency changes occur in the Hh heterozygotes only, and are probably essentially the same as paramutation in maize.

1. INTRODUCTION

HERITABLE changes in plant weight and amount of nuclear DNA (Durrant, 1962*a*, 1971; Evans, Durrant and Rees, 1966; Evans, 1968) induced by growing flax plants in different environments may be accompanied by changes in a gene H-h, determining hairy-hairless septa in the capsules (Durrant and Nicholas, 1970). The variety Stormont Cirrus, a plastic genotroph (Pl), *i.e.* one in which heritable changes can be induced, has hairy septa (HH); the small (S) stable genotroph induced from it also has hairy septa, but the large (L) stable genotroph induced from Pl is hairless (hh). The two alleles appear to be fairly stable in the homozygous state but heterozygotes (Hh) are not. The heterozygotes have fewer hairs per septum than the HH homozygote, *i.e.* dominance is not complete, and in the F_2 the Hh plants can be separated into three distinct classes with mean hair numbers of 33, 41 and 49 hairs per septum, compared with 60 for the HH homozygous class. They are called respectively class I, II and III heterozygotes, and presumably reflect different levels of gene activity. The hair number of one septum of one capsule is normally sufficient to classify a plant because the hair numbers of septa within a capsule, or capsules on the same plant, do not vary by more than two or three.

Plants of the three heterozygous classes give different H : h ratios in the next generation, as shown by the summarised results in table 1 (Expt. 1

TABLE 1
Mean hair numbers of class I, II and III heterozygotes in F_2 and $H:h$ ratios in F_3 , in three experiments

	Expt. 1		Expt. 2		Expt. 3	
	F_2 Mean hair number	F_3 Ratio (H/h)	F_2 Mean hair number	F_3 Ratio (H/h)	F_2 Mean hair number	F_3 Ratio (H/h)
I	29.8	2.3	31.8	3.3	30.9	3.5
II	40.4	3.0	40.5	3.5	40.1	2.7
III	51.5	5.0	50.8	6.8	50.5	6.9

and 2) of two experiments done by Durrant and Nicholas (1970). Summing over the appropriate groups of F_3 families, class I F_2 heterozygotes with fewest hairs per septum give the lowest number of H plants in the F_3 ; class III with most hairs per septum give a large excess of H plants, and class II an intermediate number. More information on the unstable nature of this gene has been obtained from the F_2 and later generations.

2. F_2 AND F_3 GENERATIONS

Another set of F_3 families (Expt. 3) was grown from seed from independently scored F_2 plants. The hair numbers of capsules taken, one from each of three plants of each of the three F_2 heterozygous classes and of the F_2 HH homozygotes, from the four crosses $L \times Pl$, $Pl \times L$, $L \times S$, $S \times L$, are given in table 2a. The mean hair numbers of the three heterozygous classes and homozygous HH plants are close to those of Expt. 1 and 2 (table 1). The seed was sown in November 1967 in a heated greenhouse with supplementary lighting and the F_3 capsules scored in March 1968. The numbers of H and h plants in each F_3 family of about 9 plants are given in table 2b; the position of each F_2 plant and its F_3 family in tables 2a and 2b correspond. The overall F_3 ratios are entered in table 1 (Expt. 3), where class III F_2 heterozygotes again give a large excess of H plants in F_3 , and classes I and II fewer H plants, but here class I gives more than class II.

TABLE 2a
Hair numbers of F_2 capsules of the three heterozygous classes I, II and III, and HH homozygotes, used in Expt. 3

	$L \times Pl$	$Pl \times L$	$L \times S$	$S \times L$	Mean
I	{ 30 32 34 }	{ 32 30 31 }	{ 27 30 32 }	{ 32 29 32 }	30.9
II	{ 42 40 38 }	{ 42 41 40 }	{ 41 44 42 }	{ 44 41 39 }	40.1
III	{ 52 52 49 }	{ 48 50 52 }	{ 50 51 51 }	{ 49 50 52 }	50.5
HH	{ 68 80 64 }	{ 74 73 60 }	{ 62 67 72 }	{ 62 72 65 }	68.5

The F_3 ratio of class III summed over the three experiments and four crosses is significantly different ($P < 0.001$) from the combined class I and II ratios which are not significantly different themselves. Consequently, as far as the present data are concerned, the evidence for the dependence of F_3 H:h ratios on the F_2 heterozygous class rests upon the high ratios given by class III.

In the three experiments there are altogether 38 F_3 families from class III F_2 heterozygotes, 16 of which contain only H plants, *i.e.* they are non-segregating (table 3). The overall numbers of H and h plants given by the remaining 22 segregating families is 153 H and 48 h, a ratio of H:h of 3.18:1. This suggests that the high ratio given by the 38 F_3 families could be because some homozygous HH F_2 plants have hair numbers within the F_2 class III heterozygotes range and their inclusion among the class III

TABLE 2b

Numbers of H and h plants in F_3 families of Expt. 3 given by seed from capsules in table 2a. Positions of parent capsule and F_3 family correspond in the two tables

	L × Pl		Pl × L		L × S		S × L		Total	
	H	h	H	h	H	h	H	h	H	h
I	8	2	8	1	7	1	7	3	86	24
	9	0	6	2	5	5	8	0		
	8	2	8	2	6	3	6	3		
II	5	3	6	2	7	3	8	0	82	30
	4	4	7	3	6	4	8	2		
	6	4	10	0	7	3	8	2		
III	8	1	8	2	7	2	9	0	97	14
	6	1	10	0	7	3	9	0		
	9	1	9	0	5	4	10	0		
HH	9	0	9	0	9	0	8	0	112	0
	9	0	10	0	10	0	10	0		
	10	0	10	0	8	0	10	0		

plants has increased the H:h ratio from 3:1 to 6:1 by the presence of their non-segregating F_3 families.

The heterogeneity of the 38 class III F_3 families is tested in table 4. The average number of plants per family is 8.9, and the overall H:h ratio is 6.1:1. Rounding off to 9 plants per family and a H:h ratio of 6:1 the table gives the expected frequencies, from the binomial expansion, of families with different numbers of h plants per family, and the χ^2 testing agreement of the frequencies obtained with them. The χ^2 is significant ($P < 0.01$) due to the large excess of non-segregating families and therefore the data do not agree with the hypothesis that F_2 class III heterozygotes are homogeneous in giving 6:1 ratios in F_3 families.

Another hypothesis is tested in table 4, assuming that F_2 class III heterozygotes segregate to give 3:1 ratios in F_3 families but there are included in the F_3 some additional non-segregating families. Some of the class III heterozygotes are also expected to give non-segregating families, and calculations show that at least one, but not more than three, of these is expected. Accepting that two non-segregating families are due to this

Total numbers of plants, and numbers of segregating and non-segregating families, descended from class III F₂ heterozygotes. F₄ plants descended from F₃ plants in Expt. 2 only

	Class III Hh							
	38 plants							
	(12 in Expt. 2)							
F ₂								
	16 non-seg. families				22 seg. families			
	(4 in Expt. 2)				(8 in Expt. 2)			
F ₃								
	H		h		H		h	
	136		0		153		48	
	(31 in Expt. 2)				(58 in Expt. 2)		(13 in Expt. 2)	
	28 as used parents				52 as used parents			
F ₄								
	27 non-seg. families		1 seg. family		29 non-seg. families		23 seg. families	
	H h		H h		H h		H h	
	245 0		9 1		263 0		159 59	

cause, 24 of the 38 F_3 families may be assumed to be produced by class III Hh F_2 heterozygotes, the remaining 14 non-segregating families being due to other causes. The expected frequencies of the 24 families with 9 plants per family and an H:h ratio of 3:1 are given in table 4, and the χ^2 testing agreement of the data is not significant.

The conclusion is that F_3 families assumed to have come from class III F_2 heterozygotes are heterogeneous, containing one group which segregates

	Number of h plants in family					
	0	1	2	3	4	5+
Expected numbers of families (6:1 ratio)	9.5	14.3	9.1	3.7	0.9	0.5
Numbers obtained	16	8	5	6	3	0
	$\chi^2_{(5)} = 15.74$		P < 1 per cent.			
Expected numbers of families (3:1 ratio)	1.8	5.4	7.2	5.6	2.8	1.2
Numbers obtained less 14 non-seg. families	2	8	5		3	0

$$\chi^2_{(4)} = 3.18 \quad P = 50 \text{ per cent.}$$

in agreement with a mendelian 3:1 ratio and another group which is homozygous HH. The second group occurs because either homozygous HH F_2 plants were contained among the class III heterozygotes, or a proportion of the class III F_2 heterozygotes change from Hh to HH. Studies on later generations described below show that the last interpretation is correct.

3. F_3 AND F_4 GENERATIONS

The F_4 generation was grown to examine the behaviour of the genetic factors determining H-h in F_3 and F_4 . For this and later generations descendants of the F_3 generation of Expt. 2 only were grown. In this experiment the 12 F_3 families gave a mean ratio of H:h of 6.8:1; it contained 4 non-segregating families and the H:h ratio of the remaining 8 segregating families was not significantly different from 3:1. It therefore conforms to the average pattern of the three F_3 experiments.

An F_4 family of up to 10 plants was grown from a capsule taken from each of 80 of the 89 F_3 H plants of Expt. 2 (table 3). Twenty-eight of these were from the 31 H plants in the four non-segregating families; the remaining 52 were from among the 58 H plants in the eight segregating families (table 3). Each F_4 family was grown in a separate pot in John Innes compost from November to March in a heated greenhouse with supplementary lighting.

Twenty-seven of the 28 F_4 families from non-segregating F_3 families showed no segregation (table 3). The single segregating family gave a ratio of H:h of 9:1 and was from one of eight H plants in the non-segregating $L \times S$ F_3 family. None of the other seven H plants gave segregating F_4 families. The probability of its occurrence being due to heterozygosity of the F_2 grandparent and mendelian segregation thereafter is very small, and it must be due to a different type of event than those under consideration. Excepting this single hh plant, the 28 F_4 families are members of true breeding homozygous HH lines.

The H parent plants of the 52 F_4 families from segregating F_3 families should be HH:Hh in a ratio of 1:2 and therefore give non-segregating to segregating families in the F_4 in a ratio of 1:2. The ratio is 29:23 (table 3). The probability of a selfed heterozygous plant giving no homozygous recessives in a family of ten plants is $(0.75)^{10}$, approximately 5.7 per cent., so that 23 segregating families must represent 94.7 per cent. of the true frequency of segregating families, giving corrected frequencies of non-segregating and segregating F_4 families of 27.7 and 24.3. Rounding off in the direction of the expected 1:2 ratio this gives a ratio of 27:25, which deviates significantly ($P < 0.01$) from a 1:2 ratio. These F_4 families were from segregating F_3 families and there is no possibility of HH homozygotes being among their F_2 parents. Therefore the disproportionate number of non-segregating F_4 families must have been due to a change of Hh (or hh) to HH. It is also reasonable to conclude that the disproportionate number of non-segregating HH families in the F_3 was due to a similar cause, viz., a change of Hh $\rightarrow$ HH, rather than to the inclusion of HH plants with low hair number among the F_2 class III heterozygotes, for which there is no supporting evidence elsewhere.

In table 5 the F_4 families have been split into the two sets of crosses, $L \times Pl$, $Pl \times L$, and $L \times S$, $S \times L$, and interpreted in terms of genotypic

frequencies in the F_3 segregating families. Although no F_4 families were grown from F_3 hh plants, these are expected to give all hh F_4 families and are included to complete the F_3 generation. Since 52 out of a total of 58 H plants were used as parents the 13 hh plants should be reduced proportionately to 12, approximating to 6.5 and 5.5 in the two sets of crosses respectively but the adjustments would make no difference to the overall conclusions.

Table 5 shows that there is no deviation from a 1:2:1 ratio in the $Pl \times S$ and $S \times Pl$ crosses, only in the $L \times S$ and $S \times L$ crosses which now separated out reveal a ratio approximating closely to 2:1:1; in both sets of crosses the H:h ratio is close to 3:1 and is a reflection of the 3:1 ratio given by the segregating families in F_3 . Table 3 shows that the overall ratio of H:h among the segregating F_4 families is 159:59, which agrees with a 3:1 ratio. Therefore, apart from the difference between the two sets of crosses in F_3 ,

TABLE 5
Frequencies of HH and Hh plants in segregating F_3 families interpreted from the numbers of segregating and non-segregating families in F_4 . The number of hh F_3 plants in the segregating F_3 families are given

	F_3 genotypes		
	HH	Hh	hh
Unadjusted	29	23	13
Adjusted	27	25	13
$Pl \times S$ } $S \times Pl$ }	7	17	7
$L \times S$ } $S \times L$ }	20	8	6

the F_3 repeats the pattern of the F_2 in giving rise to an excess of non-segregating families in the next generation but within any one generation a normal 3:1 segregation occurs among the segregating families.

4. F_4 AND F_5 GENERATIONS

One hundred and forty-eight F_5 families of up to 10 plants each were grown out of doors in pots from April to August in compost without John Innes fertiliser or lime. Fifty-five families were from hh plants (one capsule from each) from non-segregating hh F_4 families taken at random over the four crosses. Fifty-five families were from HH plants from non-segregating HH F_4 families taken at random over the $L \times S$ and $S \times L$ crosses. Thirty-eight families were from H plants from segregating F_4 families taken at random over $L \times S$ and $S \times L$ crosses.

The 110 F_5 families from non-segregating HH and hh F_4 families bred true, confirming the relative stability of this locus when homozygous. The 38 F_5 families from H plants in segregating F_4 families consisted of 10 non-segregating families and 28 segregating families, which do not deviate significantly from a 1:2 ratio. Within the F_5 segregating families there was an overall ratio of H:h of 176:59, which is an almost exact 3:1 ratio. Therefore H-h segregates normally in F_4 families; unlike the F_2 and F_3 there is no evidence of change of Hh to HH, or of Hh to hh.

5. DISCUSSION

The overall frequencies of homozygotes and class I, II and III heterozygotes in the F_2 , grown in the field from April to September, of reciprocal crosses between L and Pl, and between L and S (Durrant and Nicholas, 1970) are given in table 6. The segregation of H:h deviates significantly from a 3:1 ratio with excess h plants, and the homozygotes HH and hh deviate significantly from a 1:1 ratio with excess hh plants. Genetic instability in plant weight occurs in the F_1 of L $\times$ S and S $\times$ L crosses (Durrant, 1962*b*; Durrant and Tyson, 1965), and it is likely that major instability at the *h* locus also occurs in the first instance in F_1 Hh heterozygotes to give the non-mendelian segregations and three heterozygous classes in the F_2 shown in table 6. It is possible, but unlikely from the present observations, that the state of the Hh heterozygote is uniform over all F_1 plants, instability developing during the growth of the F_2 plants. This could be resolved by examining descendants of individual F_1 plants. If instability occurs in the F_1 , the distribution in table 6 will be determined by the pattern of instability among the F_1 plants and their contributions to the seed samples used for F_2 . The instability is modified by the genotrophic background, *i.e.* Pl $\times$ S,

TABLE 6
Class frequencies in F_2 summed over all crosses
(Durrant and Nicholas, 1971)

HH	Hh			hh
	III	II	I	
167	147	204	81	243

S $\times$ Pl compared with L $\times$ S, S $\times$ L and the environment in which the parents and F_1 plants are grown (Durrant and Nicholas, 1970), but there are no differences between reciprocal crosses.

F_2 class III heterozygotes give an excess of non-segregating F_3 families; there are no differences between the two types of crosses, nor between reciprocals, and the F_3 families which segregate do so in a 3:1 ratio. There must therefore be some plants among the F_2 class III heterozygotes which have a capacity, or potential, for the change Hh $\rightarrow$ HH, but the change in any one plant is not realised until later in development after its hair number has been determined. The F_2 class III heterozygotes which do not have this potential give a normal 3:1 ratio in the F_3 . If all F_2 class III heterozygotes had the same but moderate potential all F_3 families would give, within the limits of sampling error, uniform excess of H plants, which is not the case. Assessed over all crosses and experiments (table 3), 14 out of 38 (37 per cent.) F_2 class III heterozygotes have the Hh $\rightarrow$ HH potential (Expt. 2 by itself, 33 per cent.).

The F_4 generation shows however that among the F_3 families descended from the remaining two-thirds of F_2 plants apparently without the Hh $\rightarrow$ HH potential there are Hh plants in the L $\times$ S, S $\times$ L crosses, but not in Pl $\times$ S, S $\times$ Pl, which give non-segregating F_4 families. These must have since acquired the Hh $\rightarrow$ HH potential, or only now is it realised. Here a ratio of HH:Hh:hh among the F_3 heterozygotes of 2:1:1 indicates that half the HH plants arise from the change Hh $\rightarrow$ HH, that is approximately 10

out of 18 heterozygotes (57 per cent.) changed to HH. In the F_4 no changes are found. The Hh→HH potential first disappears in the F_3 of the $L \times Pl$, $Pl \times L$ crosses, and one generation later in the F_4 of the $L \times S$, $S \times L$ crosses.

Summarising, when the two alleles are brought together in the F_1 with their associated genotrophic backgrounds, the Hh heterozygote is unstable and changes may occur in either direction, $H \rightarrow h$ or $h \rightarrow H$. In the present environments there was an excess of hh homozygotes in the F_2 (table 6) so that in the F_1 generation the change was predominantly, or entirely, $H \rightarrow h$. The three heterozygote classes in the F_2 reflect further levels of instability and in selecting class III Hh plants, with the highest hair numbers, there is selection also for plants with a high Hh→HH potential. Only some F_2 plants have this potential in so far as it leads to an overt and immediate change of Hh→HH by the next generation. In the $L \times S$, $S \times L$ crosses a further set of Hh plants in the F_3 show this potential. Thereafter none is found.

The fade-out could be due to four reasons. First, the instability of the Hh heterozygote is not a function of the locus, *i.e.* a unit cistron, but a function of associated elements closely linked to the locus or elsewhere on the chromosomes, the apparent stabilisation of the locus in later generations being due to the stabilisation of the other elements following the initial instability brought about by heterozygosity. Second, the H-h locus may be compounded of a number of repeated segments, differing in number between the two alleles and in the different genotrophs, and interacting in the heterozygote to produce repeated sequences with different numbers again, eventually stabilising and rationalising into stable H and h alleles once more. If the repeated segments are not the gene itself but in one way or another influence its activity, they may be the associated elements of the first interpretation. Third, no classification of heterozygote hair number was carried out after F_2 and consequently there was no further selection for class III. Hence the initial impetus and potential for Hh→HH is gradually lost, and the data contain once more three heterozygous classes; one would, however, have expected the analysis to have picked up changes in both directions. Fourth, the Hh→HH potential is influenced by the different environments in which the generations are grown. The differences between the two sets of crosses, $Pl \times S$, $S \times Pl$ compared with $L \times S$, $S \times L$ in the F_2 ratios, and generation in which stabilisation, or apparent stabilisation, occurs, are compatible with these interpretations.

The instability of the heterozygote compared with the homozygote parallels, as mentioned elsewhere, the instability of mating type heterozygotes in *Tetrahymena* (Nanney and Caughey, 1955) and, particularly with regard to the H-h locus, to paramutation in maize (Brink, 1960), the differences between the two possibly being only a matter of degree. All R^r alleles paramutate when heterozygous with R^{st} whereas only a proportion of h change to H, or vice versa depending on conditions, but if the heterozygous Hh classes are taken into account probably all alleles change in activity, and varying degrees of paramutation can be obtained at the R locus (Mikula, 1961). Heritable changes in plant height and flowering time have been induced in a highly inbred variety of *Nicotiana rustica* (Hill, 1967). Crossing between the induced types gives F_1 instability (Perkins, Eglington and Jinks, 1971) similar to that given by the induced plant weight changes in flax. It seems, therefore, that environmentally

induced changes, including those at the *h* locus, which are stable in themselves, may be generally unstable in the heterozygous state.

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