HERITABLE CHANGES IN *R*-LOCUS EXPRESSION IN MAIZE IN RESPONSE TO ENVIRONMENT[1]

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IT is a basic assumption in genetics that hereditary units cannot be altered from generation to generation by means of environment. This assumption has resisted serious experimental questioning for the past 50 years because one or more critical components has always been lacking in experimental designs. With the availability of the paramutational system, it has become possible, in maize inbreds, to inquire whether heritable environmental effects can be assayed in the pigment expression of a single gene. Operationally, the gene is known through its expression, the phenotype; heritable phenotypic changes, regardless of how these changes are caused, will be an argument for a change in the gene.

BRINK (1956) reported in studies of the *R* locus in maize that the *R* allele, responsible for kernel color, could be changed in its ability to produce pigment by possing the *R* gene through a heterozygote with its allele R^{st} (stippled). When *R* is removed from the RR^{st} combination, less pigment is noted in the following generations. The effect is called paramutation by BRINK. The significance of the paramutation phenomenon to the work presented here is that (1) a change has been directed at a specific gene. (2) the change occurs in a high frequency—100%, and (3) the change in *R* expression is heritable (found in *all* offspring with *R*).

The paramutation events are additive from generation to generation (MIKULA 1961; BRINK 1964)—in effect the *R* gene has a "memory" of the number of generations it had been kept heterozygous with paramutagenic alleles. The important point of this work for the present paper is the demonstration of progressively accumulated genetic change directed at the single gene.

Because of this recorded behavior of the paramutable *R* allele, a most sensitive system is available for a critical test of heritable effects from the environment on a single gene expression. Experimentally, the following conditions are available: (1) an inbred background, (2) a single allele, (3) an allele sensitive enough to register small changes in expression, (4) an allele capable of summing small effects from generation to generation, (5) a pigment phenotype easily scored precisely. A question which remains is, can the environment contribute to the heritable changes in *R* expression.

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TABLE 1

History of materials used

Tables in which data are reported 1	Time of treatment 2	Genotype treated 3	Seed source 4	Genotype of testcrossed plants 5	Treatment of testcrossed plants 6	Year of testcross 7	Inbred tester 8
2,A	Spring 1965	R^1R^{st}	common ear for all treatments	as in col. 3	as given in Table 2	Fall 1965	W23
2,B	Spring 1966	R^1R^{st}	sib ear of above	as in col. 3	as given in Table 2	Fall 1966	W22
3,A	Spring 1965	R^1R^{st}	pooled seed of 3 sib-mated plants	R^1R^1 segregates of col. 3	none; planted directly in field	Fall 1966	W23
3,B	Spring 1965	R^1R^{st}	pooled seed of 3 sib-mated plants	R^1R^1 segregates of col. 3	none; planted directly in field	Fall 1966	W22
4	Spring 1965	same as line 1 above	pooled seed of 6 testcross ears	R^1r (hybrid W23 × W22)	none, raised under winter greenhouse conditions	Winter 1966	W23
5	Winter 1965	R^2R^{st}	2 sib-mated plants for each treatment	R^2R^2 segregates of col. 3	none, planted directly in field	Fall 1966	W23

MATERIALS AND METHODS

An *R* allele in inbred W22 background was made heterozygous with a paramutagenic allele, R^{st}, known to cause a reduction of *R* pigmentation to the middle ranges of pigment expression as determined by scoring methods described below. This reduction was necessary so that small changes could be observed and so that variations in *R* expression caused by treatments could be scored both toward the upper and lower ranges of color expression.

Seeds were planted in 10 cm pots and placed in two 1.2 m × 2.4 m Percival growth chambers set for a constant temperature of 21°C. The light source in each chamber was supplied by 14 200w, cool white fluorescent tubes, supplemented by 12 60w incandescent bulbs. Seedlings were placed one meter from the light source. Light conditions in one chamber were maintained at 12 hours of light and 12 hours of darkness for each daily period (referred to as LD hereafter). The other chamber was maintained in constant light conditions; this last treatment is designated LL hereafter. At weekly intervals, the same fertilizer applications were made in both LL and LD chambers.

Environmental treatments consisted of holding seedlings in LD and LL conditions for four weeks, then transplanting all treated plants to field conditions for the remainder of the life cycle until harvest in October. Mixed treatments were also employed; after two weeks in LD conditions a group of 15 plants was shifted to LL conditions for the remaining two weeks of treatment. Similarly, after two weeks under LL conditions a group of 15 plants was shifted to LD conditions for the remainding two weeks of treatment. These mixed treatments are symbolized as LD-LL and LL-LD, the symbol order indicating the treatment order for the two-week treatment-periods. At the same time seeds were planted in LD and LL conditions above, a third group of seeds was planted directly in the field.

In the field, in the first weeks of August, treated plants were testcrossed to either of two inbred testers, W22 or W23. The testcrosses were expected to show any variation in pigment level of the paramutated *R* alleles from plants which had undergone the LL and LD treatments early in the life cycle. Therefore, any pigment differences caused by the early environments must be carried through the pollen and expressed in an inbred background of a female which had been grown directly in the field. The history of the materials used, together with the appropriate tables of data to which they are related, is shown in Table 1.

Plants which received LD conditions shed pollen approximately one week earlier than those which received LL or field conditions. However, this difference in anthesis time was noticed only during the year plants received treatment; seeds derived from plants treated in 1965, flowered at the same time as the standard inbreds in 1966. Treated plants also differed in node numbers; LD plants produced 9 nodes above ground, LL plants produced 11 nodes and field grown plants produced 14 to 15. In 1966 all three groups, LD, LL and field, produced the typical 14 to 15 nodes.

The level of pigmentation (reflecting the numbers of cells in the aleurone with pigment) in each kernel was determined by matching, visually, individual kernels from testcross ears against a set of standard kernels ranging from 0 to 22, colorless through various degrees of pigmentation to completely colored. The scoring was done by persons who were not made aware of treatment backgrounds of the material being scored, so that strict objectivity could be maintained during the scoring process. Fifty kernels were shelled from each ear and matched against the standard kernels mentioned above; kernel scores from each ear were averaged and are reported as ear means.

RESULTS

Table 2 shows R^1 (one generation with R^{st}) expression with more pigment following LD treatments; less pigment was recorded for seeds of plants which received LL treatments. Plants grown under field conditions showed a pooled mean which lies between the LD and LL values and individual ear means overlapped the ear-mean scores of both the LD and LL groups.

TABLE 2

Testcross scores for R^1 *expressions from* R^1R^{st} *heterozygotes given different environmental conditions during the first four weeks of seedling development*

	Growth chamber treatments						
Inbred tester	LD	LL	Field grown	LL-LD	LD-LL	t-test comparisons	P
Part A. 1965							
W23	12.64	8.72	8.58	13.08	7.62	LD *vs.* LL	<.001
	11.90	6.66	10.38	12.96	5.54	LL-LD *vs.* LD-LL	<.001
	16.58	10.48	12.36	13.78	8.64	LD *vs.* Field	<.05
	16.14	4.48	7.72	12.80	7.86	LL *vs.* Field	<.05
	12.60	7.66	11.84	13.52	7.64	LL-LD *vs.* Field	<.05
	14.44	10.68	14.06	13.36	10.30	LD-LL *vs.* Field	<.01
	14.82	10.96	11.96	13.04	8.06		
	14.76	8.20	13.74	12.64	8.44		
Pooled $\bar{X}$	14.24	8.48	11.33	13.15	8.01		
Part B. 1966							
W22	17.14	8.38	13.98	15.36	11.58	LD *vs.* LL	<.001
	18.52	8.34	14.56	17.04	11.70	LL-LD *vs.* LD-LL	<.001
	15.78	9.92	16.38	16.20	13.70	LD *vs.* Field	<.05
	13.60	9.98	13.56	15.92	9.36	LL *vs.* Field	<.01
	17.20	11.78	9.58	15.60	11.22	LL-LD *vs.* Field	<.05
	17.26	11.78	14.52	16.52	13.82	LD-LL *vs.* Field	<.20
Pooled $\bar{X}$	16.58	10.03	13.76	16.11	11.90		

When treatments were mixed, that is, when plants were started in LL conditions for a period of two weeks, then transferred to LD conditions (LL-LD) for the remaining two weeks of treatment, the pooled mean does not differ significantly from the pooled mean of plants which received LD treatments for the entire four week treatment-period. Plants which received LD conditions for the first two weeks and LL conditions the third and fourth weeks of the treatment-period show score values essentially the same as those recorded for plants which received the LL treatment for the entire four weeks. The data show, therefore, that the effectiveness of the early environment treatment lies within the third and fourth week period during early seedling development. It can also be noted that the scores for LD and LL conditions, whether for the full four week period or for the mixed treatments, do not overlap and the differences are, therefore, highly significant.

Table 2 (Parts A and B) also permits a comparison of effects on R^1 expression from LD and LL environments for two different years. The relationships of the testcross values obtained in 1966 are the same as those observed in 1965 even though different inbred testers were used each year. The testcrosses on W22 are uniformly and consistently darker than those on W23.

The important genetic question about these data is whether the differences noted can be carried over to the next generation without further treatment. Table 3, Part A, shows testcross scores of R^1R^1 segregates from sib-mated R^1R^{st}

TABLE 3

Scores of R^1R^1 *segregates from sib-mated* R^1R^{st} *plants which had been given growth chamber treatments during the first month of development the previous year*

Inbred tester	Seedling treatments of parental R^1R^{st} plants: LL-LD	LD-LL	Field grown	t-test comparisons	P
Part A					
W23	17.34	15.52	15.30		
	16.44	11.52	13.80	LL-LD *vs.* LD-LL	<.01
	17.60	9.32	15.02	LL-LD *vs.* Field	<.001
	16.82	14.04	15.76	LD-LL *vs.* Field	<.07
	17.28	10.44	14.32		
	17.50	16.34	14.14		
Pooled $\bar{X}$	17.16	12.86	14.72		
Part B					
W22	18.24	13.72	19.02		
	19.58	13.06	15.94		
	18.34	17.48	19.48	LL-LD *vs.* LD-LL	<.001
	19.58	17.12	17.38	LL-LD *vs.* Field	<.05
	19.86	10.96	16.85	LD-LL *vs.* Field	<.02
	20.48	16.50	19.14		
	18.80	14.90	18.74		
	18.70	11.72	14.48		
	20.30	11.30	20.30		
		15.92	16.20		
		18.44			
		18.40			
Pooled $\bar{X}$	19.32	14.97	17.75		

plants treated with LD and LL conditions in the spring of 1965. While the scores are higher than the previous year (a reversion effect to be expected after the first generation of paramutation; KERMICLE 1963), the same relative differences are maintained for the three treatments as were noted in the previous year's data. Similar results are noted in Table 3,B where testcross scores were observed on inbred W22 background and were, therefore, slightly darker than those on W23 just above. The differences observed in the R^1 expressions of Table 2,A, are still present though collected a generation after environmental treatment.

The carry-over of pigment differences from 1965 treatments is reflected in another test from slightly different backgrounds and environments. Plants of R^1R^{st} (inbred W22 background), which had been treated in 1965, were test-crossed to inbred W23, *rr* (Table 2,A). The W22/W23, R^1r, testcross kernels were grown out nuder winter greenhouse conditions after an initial period of one month of LD conditions to cause early flower bud formation. Spring testcross results are shown in Table 4. Again it can be noted that the difference between LD and LL the previous year was carried over through the pollen under the winter-greenhouse conditions, as well as through the hybrid background. The significant differences noted the previous year have been maintained.

TABLE 4

Testcross scores of R^1 *expressions from* R^1r *plants derived from the testcross ears of Table 2, part A*

Growth chamber treatments of parental R^1R^{st} plants	
LD	LL
17.56	15.44
15.92	12.62
15.78	15.80
16.94	13.62
19.10	13.94
18.64	15.28
17.30	15.50
17.08	15.44
Pooled $\bar{X}$ 17.29	14.71 t-test: $P<.001$

The results of the previous tables are confirmed in another test using R^2R^2 segregates. Parental R^2R^{st} plants were grown under LD and LL conditions during late winter of 1965, then were transferred to the greenhouse to be sib-mated and complete their life cycle during the spring and early summer. The R^2R^2 segregates from the treated plants were testcrossed in the field in 1966. It can be noted in Table 5 that those plants which trace back to LD treatments are significantly darker than those which are derived from LL conditions the previous year.

DISCUSSION

Little information is available in the literature on the production, by environmental means, of male transmissible changes in specific gene expressions. HIGHKIN (1958) reported male-transmissible changes in peas in response to a constant

TABLE 5

Testcross scores of R^2R^2 *segregates from* R^2R^{st} *plants which had been given growth chamber treatments the previous year*

Growth chamber treatments of parental R^2R^{st} plants	
LD	LL
5.52	2.62
8.00	1.88
11.20	5.28
5.26	3.10
5.86	3.08
6.40	3.58
5.88	3.34
7.96	2.84
8.94	3.10
9.22	1.40
Pooled $\bar{X}$ 7.42	3.02 t-test: $P<.001$

temperature environment; DURRANT (1962) reported heritable changes in flax in response to fertilizer conditions; neither worker could register the effects of their environments on a specific gene expression. EYSTER (1926), RHOADES (1941), FABERGÉ and BEALE (1942) and VAN SCHAIK (1955) have shown alterations in mosaic patterns in response to environmental conditions; such patterns, however, were observed in somatic tissue and were not shown to be heritable.

It may be objected that the environment is affecting the paramutation process but does not affect the *R* gene itself. This is a difficult objection to put aside since little is known of the mechanism of paramutation or about the *R* gene and how it operates. But it can be argued that what is called paramutation is a phenomenon not limited to the R^1R^{st} combination but is, rather, a condition resulting from all allelic combinations with *R*; that the variations in aleurone pigmentation called paramutation are more extreme cases of the common observations that *R* produces a mosaic (mottle) when transmitted through the male gamete, and that the *R*-expression of these male gametes is determined by the mosaic of somatic tissue in the region of the tassel giving rise to the gametes (MIKULA 1966). One can now relate the mosaic phenomenon of *R* from *RR* and *Rr* backgrounds to the more extreme mosaic phenomenon noted for *R* from the combination with R^{st} (BROWN and BRINK 1960); the mosaic phenomenon from the *RR* homozygote (in testcrosses) suggests that *R* already had a low level of paramutability and paramutagenicity and that R^{st} in the R^1R^{st} heterozygote simply enhanced this inherent mosaic property of *R*. Furthermore, one can interpret the work on R^1 expression from the R^1r heterozygote (KERMICLE 1963; COOPER 1964; STYLES and BRINK 1966) or from the R^1R^1 homozygote as a reduction (reversion) in mosaic tendency for R^1. With this unified view of the *R*-locus phenomena, the objection that the environments employed above are affecting only the paramutation process does not seem relevant since some degree of mosaicism (paramutation) will attend *R* from every allelic combination. Even if the objection is allowed, the effect of environment on the paramutation process is still of considerable genetic interest, since the system demonstrates a remarkable control over cellular differentiation.

The lines of evidence presented in support of an environmentally induced, heritable change in *R*-locus expression can be summarized briefly in the following:

1. Significant pigment differences are noted in aleurone pigment expressions following environmental treatment of seedlings during the third and fourth weeks of seedling development.
2. Following treatment, pigment differences were observed in the testcrosses of R^1R^{st} (Table 2). These differences required that the *R* alleles be carried through the pollen and compared on testcross ears of tester plants which were grown in the field.
3. The changes in pigment expression were preserved in the generation derived from the treated parental plants (Tables 3, 5).
4. One test showed the carry-over of altered pigment expression of R^1 in a hybrid background (W23 × W22). This test required that R^1 be carried through the pollen twice before the comparisons of Table 4 were made.
5. Since the changes observed in *R* expression are transmitted through the male

gamete, the nucleus is implicated. Little cytoplasm is believed to be involved in fertilization (CASPARI 1948; MICHAELIS 1954).

6. The score differences imputed to the environment were observed on either of two inbred tester females which were not given environmental treatments.
7. Differences in *R*-gene expression were noted following three different environmental treatments, LD, Field and LL.
8. The effects of the LD and LL environments were confirmed on seeds matured and tested under various growing conditions: (a) plants, treated in growth chambers in spring, matured and sib-mated in the field in 1965, furnished seeds which were sown and resulting plants tested in the field in 1966 (Table 3); (b) plants were treated in the spring of 1965, matured and testcrossed under field conditions of 1965 and testcross-seeds were sown and resulting plants were tested for pigment differences under winter greenhouse conditions of 1966 (Table 4); (c) plants were treated in growth chambers in winter, matured and sib-mated in the greenhouse in the spring and early summer of 1965; seeds of these plants, in turn, were sown and the resulting plants were tested in the field of 1966 (Table 5).

From our present knowledge of the *R* system, it is possible to conclude that the LD and LL conditions, administered to the plant in the early seedling stage, have contributed heritable changes in *R* expression.

What mechanism might be invoked to explain the above changes? At least three possibilities are available, (a) assignment of mutation to the *R* gene itself, (b) assignment of control of *R* action to other "genetic" elements closely associated with *R*, and (c) assumption that only the timing of *R* action has been altered by some as yet unknown mechanism for controlling gene expression (growth substances, diurnal rhythms, polyanions, for example). The reported data do not permit a choice from among the above possibilities. Interpretations of existing paramutation literature by BRINK (1964) and MCCLINTOCK (1965) favor a repression model for explaining the phenomena reported for the *R* locus. Such a repression model may offer some consistency to the reports of AXTELL (1966) with alkylating agents on R^1, the radiation work of LINDEN (1964) with the paramutation system and the environment changes reported above, particularly in the light of the model for repression and derepression proposed by FRENSTER (1965).

Since the *R* locus is known only through its phenotype in the aleurone, and since heritable change in response to environment has been reported to be transmissible both through the male and female gametes, the results above could be used as an argument in favor of a change in the *R* gene. Such an argument, however, only reflects the limited operations available to maize genetics for the study of hereditary units. It is obvious that further work is needed to reveal the basis of the heritable changes reported; until such work is available, judgment as to the nature of the change and its relationship to *R* must be suspended.

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SUMMARY

Changes in paramutated *R* expression were recorded following exposure of seedling R^1R^{st} and R^2R^{st} maize plants to LD environments (12 hr light and 12 hr darkness) or LL (constant light) environments for a period of one month. Temperatures were held at constant 21°C during this period. Upon transplantation to the field for the completion of their life cycle, testcrosses of treated plants show that the environmental effects occurred during the third and fourth weeks of seedling development; more cells were conditioned to produce pigment following LD conditions, fewer pigmented cells were found in the aleurone following LL conditions. The change in *R* expression is pollen transmissible in the generation which received the LD and LL treatments; the change persists in the testcrosses of the progeny of the R^1R^{st} and R^2R^{st} plants, the R^1R^1 and R^2R^2 segregates, as well as in the plants raised from R^1r testcross kernels. It is concluded that the LD and LL environments have been responsible for a heritable change in paramutated *R* expression.

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