EFFECTS OF X-IRRADIATION ON ALEURONE PIGMENTING POTENTIAL OF STANDARD R^r AND A PARAMUTANT FORM OF R^r IN MAIZE¹

KATHERINE LU SHIH AND R. ALEXANDER BRINK

Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706

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THE aleurone pigmentation ability of certain R alleles in maize is changed heritably when these factors are passed through heterozygotes with certain other alleles. For example, a factor termed standard R^r regularly gives reduced aleurone pigmentation in the offspring of R^rR^{st} (R^{st} = stippled aleurone) heterozygotes (McWHIRTER and BRINK 1962), and enhanced pigmentation in the progeny of R^rr (r = colorless aleurone) heterozygotes (STYLES and BRINK 1966). Paramutation is manifest as a quantitative change in the phenotypic expression of an R allele, and the alteration may be to either a higher or a lower pigmentation level. Alleles inciting such a heritable change in R action are referred to as paramutagenic, and factors sensitive to the change are termed paramutable.

AXTELL and BRINK (1967) observed that R alleles whose pigmenting potential previously had been reduced by paramutation showed increased levels of action with high frequency following seed treatments with diethyl sulfate and ethyl methane-sulfonate. The results presented in this article show that X rays have a similar, apparently directed effect on R pigmenting potential.

MATERIALS AND METHODS

The R alleles employed in the present study had previously been incorporated into the W22 inbred strain by a conventional backcrossing procedure. The standard R^r allele (red anthers) and the R_g allele (green anthers), a mutant derived from it, condition a characteristic darkly mottled aleurone in single dose (Rrr) and full color in either two or three doses. The repressed (paramutated) forms of R will be symbolized R' , R'' or R''' , the number of primes corresponding to the number of successive generations of heterozygosity with a paramutagenic allele. The strongly repressed allele, $R_c^{g'}$, was derived originally from a self-pollinated R_gR^{st} individual. The subscript "c" denotes a particular stock of this origin. The stippled allele (R^{st}) is strongly paramutagenic, and also causes aleurone spotting. The r^g allele (green anthers), extensively used in the study, gives colorless aleurone.

The seeds and pollen to be irradiated were placed 25 cm below the X-ray source. For the seed treatment, the X-ray machine was operated at 250 kv and 15 ma with a .25 cm Cu and 1 mm Al filter, producing a dose rate of 600 r per min. For the pollen treatment, tassels in paper tassel bags were cut from the plants and taken to the laboratory about 1:00 PM on the day treatment was to be applied. The tassels were shaken well in the bags and the pollen thus freed from each tassel was then collected in a Petri dish. The pollen was spread evenly in the dishes. The dose rate, 420 r per min, was obtained by operating the X-ray source at 210 kv and 12 ma with a

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.25 cm Cu and 1 mm Al filter. The irradiated pollen was carried to the field in glass vials immediately following treatment and was applied to the silks before 3:00 PM.

The single dose aleurone phenotype was routinely obtained by pollinating W22 $r^g r^g$ individuals with pollen from W22 plants carrying the R allele under test. $R r^g r^g$ kernels, usually 50 in number, were taken from the resulting testcross ears and were then scored for color by matching each seed against a set of six kernels ranging in level of pigmentation from nearly colorless (class 1) to nearly self-colored (class 7).

Student's t -test (STEEL and TORRIE 1960) was used to evaluate the data statistically.

EXPERIMENTAL RESULTS

Ineffectiveness of X-irradiation on immediate pigmenting potential of standard R^r : W22 $R^r R^r$ seeds, dry or presoaked in water for 24 hrs, were X-irradiated with 10,000r. Testcrosses of $R^r R^r$ individuals reared from X-irradiated and unirradiated seeds were made on $r^g r^g$ plants. Pollen produced by $R^r R^r$ plants also was irradiated, with 1,200r. The irradiated and the unirradiated control R^r pollen was applied to the silks of W22 $R^g R^g$ plants. The $R^g R^r$ heterozygotes thus derived were then testcrossed on an $r^g r^g$ line.

The testcross data, summarized in Table 1, show that these treatments did not produce significant effects on the pigmenting potential of R^r . The scores were distributed largely in classes 6 and 7, with a few entries in class 5, and very few in class 4.

Increase in pigmenting potential of the $R^{'''}$ allele after irradiating $R^{'''}$ $R^{'''}$ seeds: $R^{'''}$ $R^{'''}$ seeds were extracted after selfing $R^{'''}$ R^{st} heterozygotes. Pollen from plants grown from X-irradiated and unirradiated dormant $R^{'''}$ $R^{'''}$ seeds was put on an $r^g r^g$ line.

Kernels from the control testcross ears fell mostly in classes 1 and 2, with a few in class 3. A small number of kernels fell in class 4 or in a higher class. Many of the kernels obtained from the testcross ears of treated material, on the other hand, were distributed in the darker classes, namely, 3, 4, and 5. In some instances, class 7 kernels were obtained; and in one case 20 out of 50 kernels scored were nearly self-colored.

TABLE 1

Mean aleurone color scores of $R^r r^g r^g$ kernels derived by pollinating $r^g r^g$ plants with pollen from $R^r R^r$ and $R^g R^r$ plants carrying X-irradiated and unirradiated R^r alleles

Male parent	Treatment	Number of matings	Mean score	t value
K3177	Control	9	6.85	...
K3178	Dry seed; 10,000r	9	6.86	0.33*
K4030	Control	8	6.85	...
K4031	Dry seed; 10,000r	9	6.78	0.85*
K4032	Control	8	6.80	...
K4033	Soaked seed; 10,000r	16	6.73	1.04*
K3306	Control	21	6.54	...
K3308	Pollen; 1,200r	61	6.64	1.21*

* Not significant ($P > .05$).

FIGURE 1.—Mean aleurone color scores of testcross matings derived by pollinating $r^g r^g$ plants with pollen from plants grown from X-irradiated and unirradiated $R''' R'''$ seeds. Each pair of observations represents the mean scores of the respective testcross matings from X-irradiated and unirradiated control groups for one family.

The mean aleurone color scores for the control and the treated material in each of the 22 families used in this study are illustrated in Figure 1. Paired comparisons of the means of the controls and the means of the treated material were made, and the results for the overall experiment and for the individual treatments are summarized in Table 2. The data show that the X-irradiation of seed often resulted in an increase in the pigmenting capacity of the R''' allele and that the effect was dose-dependent.

In the 10,000r seed treatment, for both control and treated testcross ears in each family, the percentage of ears that scored higher than the mean control score of that family was calculated. The average difference between the percentages in the control and treated material was taken as the estimate of frequency of induced changes. A value of 21% was obtained for the 11 families.

Increase in pigmenting potential of the R''' allele after irradiating R''' pollen: Two different doses of X rays, 1,200r and 1,400r, were applied to pollen produced by $R''' R'''$ plants. One to four plants from each of 14 families were selected for treatment, and for each plant several pollinations were made on an $r^g r^g$ line. The mean aleurone color scores for the control and the treated material are included in Figure 2. The data (Table 2) show an increase in R''' aleurone pigmenting ability following irradiation that is significant at the 5% level. On the average, 12% of testcross ears resulting from the 1,400r treatment showed higher levels in pigmentation than that of the controls.

Effects of X-irradiation on pigmenting potential of R''' in heterozygotes with r : Four different X-ray doses were applied to the colorless (or nearly colorless)

TABLE 2

Mean aleurone color scores of $R'''r^g$ kernels derived by pollinating r^gr^g plants with R''' pollen from irradiated and unirradiated sources

Treatment	Number of families	Number of testcross matings	Mean score	Difference (treated—control)	t value
Control	11	61	1.39	+0.30	4.03†
10,000r	11	116	1.69		
Control	6	38	1.19	+0.18	3.06*
8,000r	6	83	1.37		
Control	5	24	1.15	+0.11	2.44‡
6,000r	5	57	1.26		
Overall control	22	123	1.28	+0.23	5.09†
Overall treated	22	256	1.51		
Pollen treatments					
Control	8	41	1.31	+0.26	2.02‡§
1,400r	8	39	1.57		
Control	6	38	1.33	+0.15	1.59‡
1,200r	6	50	1.48		
Overall control	14	79	1.32	+0.21	2.57*
Overall treated	14	89	1.53		

* Significant ($P < .05$).

† Significant ($P < .05$).

‡ Highly significant ($P < .01$).

§ Not significant ($P > .05$).

§ Not significant at the 5% level but significant at the 10% level ($.10 > P > .05$).

heterozygous $R'''r^g$ seeds (class 1 on the 1–7 pigmentation scale), selected from ears following $r^gr^g \text{♀} \times R'''R''' \text{♂}$ testcrosses. These heterozygotes were then testcrossed on an r^gr^g line.

The mean aleurone color scores of the control and of the treated material for each family are illustrated in Figure 3. The average values for the 10 families are 1.39 for the control (94 testcross matings) and 1.56 for the treated material (97 testcross matings). The statistical analysis indicates a significant increase ($t = 2.30, P < .05$) in aleurone pigmentation following irradiation.

Figure 3 shows that a 10,000r dose was as effective as higher doses. This dose also was more effective than other doses in bringing about derepression (increased aleurone pigmentation) in homozygous $R'''R'''$ seeds. Since the populations were unequal in size in the treated homozygous $R'''R'''$ and heterozygous $R'''r^g$ material, however, it cannot be determined whether R''' is more or less sensitive to X irradiation in heterozygous than in homozygous form.

Heritability of the X-irradiation effects: The 15 darkest kernels (designated G_1 in Table 3) out of 50 $R'''r^g$ kernels from testcrosses of both treated and untreated $R'''R'''$ individuals were tested for heritability of the respective single-dose $R'''r^g$ phenotypes. Plants grown from these $R'''r^g$ seeds were testcrossed on an

FIGURE 2.—Mean aleurone color scores of testcross matings derived by pollinating $r^g r^g$ plants with X-irradiated and unirradiated R''' pollen from $R''' R'''$ plants. Each pair of observations represents the mean score of the respective testcross matings from X-irradiated and unirradiated control groups for one family.

FIGURE 3.—Mean aleurone color scores of testcross matings derived by pollinating $r^g r^g$ plants with pollen from plants grown from X-irradiated and unirradiated $R''' r^g$ seeds. Each pair of observations represents the mean scores of the respective testcross matings from X-irradiated and unirradiated control groups for one family.

TABLE 3

Mean aleurone color scores of $R''' r^g$ progeny of dark $R''' r^g$ kernels derived by pollinating $r^g r^g$ plants with pollen produced by plants grown from X-irradiated and unirradiated $R''' R'''$ seeds

Male family no.	Treatment	Mean score of 15 G_1 seeds tested (parent)	Number of testcross matings	Mean score of G_2 (progeny)	<i>t</i> value
K3023	Control	2.53	8	4.21	5.69†
K3024	10,000r	5.67	7	5.75	
K3028	Control	3.07	7	3.86	6.01†
K3029	10,000r	7.00	8	6.29	
K3036	Control	3.00	5	4.64	6.67†
K3037	8,000r	5.84	6	6.31	
K3039	Control	1.67	6	3.47	2.89*
K3040	8,000r	4.47	7	4.37	
K3041	Control	1.60	3	2.08	5.32†
K3042	8,000r	3.20	7	4.29	
K3044	Control	2.00	7	3.45	1.84‡§
K3045	8,000r	4.00	14	4.19	
K3017	Control	2.60	6	5.10	1.20‡
K3018	10,000r	2.53	6	4.58	
K3031	Control	3.73	6	4.13	0.98‡
K3032	10,000r	3.73	9	4.39	

* Significant ($P < .05$).

† Highly significant ($P < .01$).

‡ Not significant ($P > .05$).

§ Not significant at the 5% level but significant at the 10% level ($.10 > P > .05$).

$r^g r^g$ line. The results presented in Table 3 show that the progeny (designated G_2 in Table 3) of the treated material again were higher in pigmentation level than the corresponding controls. Another experiment was made to test the progeny of two sets of kernels (K3017 and K3018; K3031 and K3032) in which both control and treated testcross parent kernels were at the same pigmentation level. The respective progeny did not differ significantly in this test. It is clear from these tests that the dark paramutant phenotype arising in response to X irradiation is heritable.

The 50 scored kernels from the testcross ears of the progeny of the treated material were observed to fall into a unimodal distribution on the 1-7 pigmentation scale. This is the result expected if only one kind of $R''' r^g$ pollen was formed in the tassels derived from the dark $R''' r^g$ seeds (G_1). This evidence precludes the possibility that a major modifier was involved in production of the dark testcross kernels in the treated material.

R locus dependence of the paragenetic effects: Ten dark R''' paramutants

TABLE 4

Mean aleurone color scores of $R^{r''''} r^g r^g$ and $R_6^{g'} r^g r^g$ kernels derived from testcrosses of $R^{r''''} R_6^{g'}$ heterozygotes carrying either an irradiated or an unirradiated $R^{r''''}$ allele

Male parent no.	$R^{r''''} r^g r^g$			$R_6^{g'} r^g r^g$		
	Mean score	Average	<i>t</i> value	Mean score	Average	<i>t</i> value
Control						
K5101- 1	3.20	3.63	..	1.17	1.22	...
2	3.96	...	...	1.30	...	...
3	3.85	...	...	1.04	...	...
4	3.26	...	...	1.00	...	...
K5102- 5	4.48	...	...	1.08	...	...
6	3.21	...	...	1.00	...	...
7	3.41	...	...	1.46	...	...
8	3.15	...	...	1.17	...	...
9	3.94	...	...	1.42	...	...
10	3.81	...	...	1.57	...	...
	...	...	8.03*	...	...	1.19†
Treated						
K5103- 1	4.93	5.49	...	1.09	1.13	...
2	5.19	...	...	1.08	...	...
3	5.94	...	...	1.28	...	...
K5104- 4	5.64	...	...	1.22	...	...
5	5.56	...	...	1.00	...	...
6	5.55	...	...	1.10	...	...
7	5.14	...	...	1.00	...	...
8	6.68	...	...	1.29	...	...
9	5.64	...	...	1.23	...	...
10	4.58	...	...	1.04	...	...

* Highly significant ($P < .01$).

† Not significant ($P > .05$).

induced by X irradiation of $R^{r''''} R^{r''''}$ seeds were tested for *R* locus dependence in the progeny of heterozygotes with $R_6^{g'}$. Pollen from the $R^{r''''} R_6^{g'}$ plants was placed on $r^g r^g$ silks. The two resulting classes of kernels were separated following scoring for grade of aleurone pigmentation by germinating the kernels and noting whether seedling color was red ($R^{r''''} r^g$) or green ($R_6^{g'} r^g$). The results of the test-cross matings are summarized in Table 4. It will be observed that aleurone scores for the $R_6^{g'} r^g r^g$ kernels from the 10 $R^{r''''} R_6^{g'}$ heterozygotes carrying X-ray-induced dark $R^{r''''}$ alleles do not differ from those derived from the 10 $R^{r''''} R_6^{g'}$ heterozygotes carrying unirradiated $R^{r''''}$ alleles. Furthermore, mean aleurone color scores of $R^{r''''} r^g r^g$ seeds from the treated $R^{r''''}$ alleles were distinct from the mean aleurone color scores of $R^{r''''} r^g r^g$ derived from the untreated $R^{r''''}$ alleles. The difference between the mean scores is highly significant statistically. These are the results expected if the alterations in aleurone phenotype induced by the X irradiation resulted from changes in the $R^{r''''}$ allele itself.

TABLE 5

Mean aleurone color scores of $R^{r''}$ r^g kernels derived from testcrosses of $R^{r''}$ R^{st} heterozygotes involving X-irradiated and unirradiated $R^{r''}$ alleles

Male parent no.	G ₁	G ₂		G ₃		t value (G ₂ vs. G ₃)
	Mean score of parent kernels	Number of testcross matings	Mean score	Number of testcross matings	Mean score	
K4283 (Control)	1.60	3	2.08	20	1.43	...
K4284 (8,000r)	4.81	4	5.07	40	2.97	3.29*
K4289 (Control)	3.07	6	3.86	20	1.95	...
K4291 (10,000r)	7.00	10	6.29	37	2.87	7.24*
K4295 (Control)	3.00	5	4.68	20	1.55	...
K4296 (8,000r)	5.87	9	6.31	36	2.23	10.46*

* Highly significant ($P < .01$).

Paramutability of the induced dark paramutants: The 15 darkest $R^{r''}$ r^g kernels (G₁ column in Table 5) out of 50 kernels from testcrosses of both treated and untreated $R^{r''}$ $R^{r''}$ individuals were testcrossed on an r^g r^g line to verify heritability of their relatively high level of aleurone pigmentation. The progeny (G₂) were then crossed with $R^{st}R^{st}$ in order to test for paramutability of the dark forms of the $R^{r''}$ allele. The testcross data (G₃) in Table 5 clearly show that the X-ray-induced dark paramutants were still paramutable, thus supporting the conclusion that the X-ray effect was exerted on the R component involved in paramutation.

Effects of X irradiation on pigmenting potential of other paramutant alleles: Other paramutant alleles, exhibiting different levels of repression than $R^{r''}$, were tested by the same methods. In the case of treatment of $R^{r''}$ $R^{r''}$ seeds, even though a higher X-ray dose (14,000r) was used, the observed frequency of induced changes (about 16 percent) was lower than that observed in irradiation of $R^{r''}$ $R^{r''}$ seeds. A lower sensitivity, due to greater repression, of the $R^{r''}$ allele than of the $R^{r''}$ allele could be the cause of the reduced response. Possibly, in the case of highly repressed factors such as $R^{r''}$ and $R^{r''}$, derepression must reach a certain threshold before change in aleurone phenotype is observable. For the X irradiation of $R^{r''}$ pollen, produced by $R^{r''}R^{st}$ heterozygous plants, a dose of 1,200r was applied. A significant increase in pigmenting potential of the irradiated $R^{r''}$ allele was not observed. A similar non-significant result was obtained (Table 2) when $R^{r''}$ pollen was treated with 1,200r. It is likely that the 1,200r dose was not high enough to affect detectably the pigmenting potential of the repressed R allele in these latter cases.

DISCUSSION

One objective of this study was to test a particular hypothesis outlined earlier concerning the chromosomal basis of paramutation at the R locus in maize. BRINK (1964) and SASTRY, COOPER, and BRINK (1965) suggested that a paramutable R allele comprised two kinds of components. One component was a structural gene,

or gene complex, specifying a particular enzyme involved in anthocyanin formation. The other constituent was assumed to be a heterochromatic segment adjacent to the structural gene which affected expression of the latter during the development of seed and plant. It was postulated that the heterochromatic segment consisted of varying numbers of a common unit, termed a metamer for convenience, the effect of which was to repress *R* action. Degree of repression was assumed to vary according to the number of metameres making up the repressor segment. An *R* gene accompanied by many metameres was weakly expressed during development of the individual, whereas *R* expression was stronger if fewer metameres were present. According to this hypothesis paramutation is a change in the number of metameres in the repressor segment. It was suggested that such change results from misreplication during somatic mitoses. If some part, or the whole, of the repressor segment were copied more than once in a mitotic cycle a daughter chromatid would result containing an increased number of metameres. Correspondingly, failure to copy the heterochromatic repressor segment completely would result in a decrease in the total number of metameres. Potential level of *R* action would be changed accordingly.

A method of testing this hypothesis was suggested by an observation first made by STADLER (1941) and since confirmed by other investigators, namely, that in maize the point mutations induced by high energy radiations are not gene changes but are lethal in homozygous form, and thus appear to be small deficiencies. If ionizing radiations always cause deficiencies in maize, then one would predict on the above hypothesis that effective treatment with X rays of seeds and pollen carrying a repressed (weakly pigmenting) *R* allele would cause loss of metameres, and thus shift potential level of action of the gene in the plus direction. The results of irradiating standard *R*⁺ and the highly repressed *R*^{'''} allele may be examined in the light of this prediction.

The *R*^{'''} alleles used in this study were strongly repressed in aleurone pigmentation potential following propagation in *R*st heterozygotes for three successive generations. It was observed that the pigmenting action of *R*^{'''} is often significantly increased by X irradiation of seeds or pollen. Restoration of *R*^{'''} to the pigmenting level of standard *R*⁺, however, rarely occurred. According to the above hypothesis, a highly repressed allele like *R*^{'''} would consist of an *R* gene accompanied by a large number of metameres. The increase in pigmenting potential of the irradiated *R*^{'''} factor could have resulted from loss of metameres due to the treatment. The retained sensitivity of the induced dark paramutants to paramutagenic action suggests that the loss is partial only. The variation in *R*^{'''} pigmenting ability following treatment would be expected if numerous metameres were present initially and different numbers were lost in different instances.

AXTELL and BRINK (1967) observed that an *R*_c' allele whose pigmenting potential previously had been markedly reduced by paramutation showed increased levels of action with high frequency following seed treatments with either diethyl sulfate or ethyl methanesulfonate. The frequency of change was much higher following chemical treatment of seeds (about 90%) than after X-ray treatment of seeds (about 21%), as reported here. Treatments of mature pollen collected

from $R'_cR'_c$ plants with saturated diethyl sulfate vapor, however, were ineffective in producing changes in aleurone pigmentation potential. In contrast, X irradiation of pollen, in the present experiments, brought about such changes with a frequency of about 12%. A possible explanation of these differences is that the treated strains were somewhat unlike in level of aleurone color. A second possibility, which is the more likely, is that the two classes of mutagens functioned differently. The changes induced by chemical seed treatment could be explained, as suggested by AXTELL and BRINK (1967), as the result of failure to copy some of the metamereres during mitosis following partial chemical blockage of the repressor. This explanation would not apply to the effect induced by X-ray treatment of pollen in which the nuclei are nondividing. The X-ray-induced changes are understandable in terms of loss of metamereres directly or indirectly by deletion. The basis of the high frequency of postulated deletions within a presumably small chromosome segment, however, is obscure.

The standard R^r allele did not respond to X irradiation with directly detectable changes in aleurone expression. This negative result could be due to a low number of metamereres associated with this factor. Small X-ray-induced changes toward darker levels of pigmentation would not be detectable by the kernel scoring procedure used.

The present results are compatible with, but do not prove, the metamerere hypothesis of R repression. The possibility remains that, whereas the observed shifts in aleurone pigmentation potential of R'''' regularly were to higher levels, some changes to lower levels also occurred but were not detected. The irradiation of a seriated sample of paramutant R forms, ranging from weakly pigmented to color levels approaching that of standard R^r , would provide the basis for a more adequate test of the possibility that the effects of high energy irradiation are consistently in the direction of derepression of R .

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SUMMARY

Irradiation of R^rR^r seeds (10,000r) and R^r pollen (1,200r) did not produce a detectable effect on the pigmentation potential of the standard R^r allele. The pigmentation potential of a strongly repressed R'''' allele, on the other hand, was partially restored, and to different levels, after X irradiation of $R''''R''''$ seeds (6,000r, 8,000r, and 10,000r) and of R'''' pollen (1,200r and 1,400r). Complete restoration of R'''' to the pigmentation level of standard R^r , however, rarely occurred. The frequencies of detectable increases in aleurone pigmentation were 21% in the immediate testcrosses of the treated seeds (10,000r) and 12% in the immediate testcrosses of the treated pollen (1,400r). The pigmentation potential of the R'''' allele was also increased following X-irradiation of heterozygous $R''''r^o$ seeds (10,000r to 19,000r). Tests showed that the X-ray-induced increases in pigmentation were heritable and also were R -locus dependent. Furthermore,

paramutant alleles whose pigmenting ability had been significantly increased by X-irradiation were still potentially paramutable.—The experimental results are in accord with expectation on a hypothesis that level of pigmenting potential of a paramutable *R* allele is a function of the number of repeating units composing a repressor segment adjacent to the *R* gene.

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