PARAMUTAGENIC ACTION OF MUTANTS FROM MAIZE PLANTS HETEROZYGOUS $R^r R^{st}$

R.B. ASHMAN¹

Purdue University Agricultural Experiment Station, Lafayette, Indiana

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THE R alleles in maize, when present with other complementary genes, condition the production of anthocyanin pigment in both the aleurone layer of the seed and in the roots, leaf sheaths, and anther walls. The common alleles are designated R or r to indicate the presence or absence, respectively, of anthocyanin in the aleurone. A superscript indicates whether or not anthocyanin is produced in the plant parts: an r , denoting red, indicates the presence, and a g , denoting green, the absence of anthocyanin. The major alleles are thereby designated R^r , R^g , r^r , and r^g . For alleles that condition mosaics of colored and colorless aleurone, superscripts indicate particular patterns: R^{st} , stippled; R^{mb} , marbled; R^{nj} , Navajo. These superscripts have no reference to anthocyanin in the plant. The plant and seed pigmenting functions of certain R alleles have been shown to be elements of a compound locus separable by crossing over (STADLER and NUFFER 1953).

BRINK (1956) described an unusual interaction between certain R alleles. When R is made heterozygous with R^{st} its capacity to produce anthocyanin is reduced in the succeeding generations. The phenomenon has been termed paramutation (BRINK 1958, 1960).

McWHIRTER and BRINK (1962) have shown that following mutation of R^{st} to R^{sc} (self-colored aleurone) the paramutagenic action may be unchanged, quantitatively reduced, or lost, and that changes in paramutagenic action were coincident with the mutation events, rather than subsequent secondary alterations. The chromosomal basis of the coincident changes in aleurone phenotype and paramutagenic action probably does not involve crossing over, since R^{sc} mutations in $R^{st}r$ plants are not associated with recombination in regions proximal or distal to R (ASHMAN 1960; McWHIRTER and BRINK 1962).

BROWN and BRINK (1960) and BROWN (1962) have shown that a paramutable R acquires some paramutagenic action when heterozygous with R^{st} , whereas r^r does not. Crossing over is not a likely mechanism for such acquisition, even though tests did not include genetic markers to positively exclude it, because all 16 R paramutants tested had paramutagenic activity.

The tests reported here concern the paramutagenic action of seed color mutants isolated from the cross $+ R^r +/g R^{st} M^{st} \times g r^g +$. The isolation of the mutants was described elsewhere (ASHMAN 1965), but, briefly, most mutants were associated with recombination between outside genetic markers and, with one excep-

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¹ Department of Botany and Plant Pathology.

tion, could be divided into three groups based on anthocyanin in the aleurone and plant: (1) near-colorless aleurone, green plant; (2) near-colorless aleurone, red plant; and (3) colorless aleurone, red plant. These and other data were interpreted as indicating that colorless and near-colorless aleurone mutants are products of crossing over between components of the *R* locus. Characterization of the mutants for paramutagenic action provides information on the effect of intralocus crossing over on paramutation, and, ultimately, on the chromosomal basis of the paramutation phenomenon.

MATERIALS AND METHODS

To test an *R* allele for paramutagenic action it is made heterozygous with a standard *R*, known to be paramutable, and the heterozygous plants are used as male parents in testcrosses to *r r* plants. The pigmentation level of the *R r r* endosperms is compared with that of *R r r* endosperms derived from testcrosses of *R R* homozygotes or *R r* heterozygotes.

Stocks for testing the paramutagenic action of the colorless and near-colorless aleurone mutants (*r^m*) were prepared by pollinating plants homozygous for the mutant alleles with a stock culture of a standard *R^r*. One *R^r r^m* progeny of each mutant was planted, and eight to ten plants in each were self-pollinated and used as males in testcrosses to a standard *r^o r^o* tester. Similar testcrosses were also made from *R^r R^r*, *R^r r^o*, and *R^r Rst* plants.

Five testcross ears, representing five male plants, were scored from each progeny. Kernels were taken from the central part of the ears, and the *R^r r^o* kernels were compared individually with a set of six standard kernels delimiting eight pigment classes. The pigment classes were assigned arbitrary numerical values of 1 for colorless, or nearly so, through 7 for nearly colored, to 8 for fully colored. About 50 *R^r r^o* kernels from each ear were scored and the mean of the kernel frequency distribution was taken as the pigment score for the ear. All the kernel samples were assigned code numbers and randomized before scoring.

The phenotype conditioned by the near-colorless aleurone mutants is dosage dependent, and, with occasional exceptions, the aleurone is colorless when only one dose is present. The two kernel classes dealt with on the testcross ears were essentially colorless (*r^m r^o r^o*) and colored (*R^r r^o r^o*), the amount of pigment present in the latter class being determined by whether or not the particular mutant being tested was paramutagenic, and if so whether strongly or weakly. The two kernel-classes were easily separable except in testcrosses involving strongly paramutagenic alleles, in which cases some of the *R^r r^o r^o* kernels had very little aleurone pigment. Fortunately, the only mutants sufficiently paramutagenic to make visual separation of the kernel classes difficult were associated with green plant color. This made it possible to separate the kernel classes by germinating the scored kernels; seedlings from *R^r r^o* kernels are red, and seedlings from *r^m r^o* kernels are green if the mutant allele conditions green plant color. Kernels scored from *R^r Rst* testcrosses were also germinated since seedlings from *Rst r^o* kernels are also green.

RESULTS

The seed color mutants isolated from *R^r Rst* plants were given identifying numbers, which are preceded by 2 to indicate the particular series of matings from which they were isolated. The mutants fell into three groups based on anthocyanin in the aleurone and plant. The number of mutants in each group and their numerical designations are: 11 near-colorless aleurone, green plant, numbered 2-1 through 2-12; 12 near-colorless aleurone, red plant, numbered 2-13 through 2-24; and 13 colorless aleurone, red plant, numbered 2-25 through 2-38. The paramutation scores (mean color scores of *R^r r^o r^o* testcross kernels) for the mutants and for *Rst*, *R^r*, and *r^o* are shown in Table 1.

TABLE 1

Paramutation scores (mean color scores of R^r r^g testcross kernels), and also recombination data for three groups of seed color mutants isolated from $+R^r +/g R^{st} M^{st}$ plants†

Near-colorless aleurone, green plant				Near-colorless aleurone, red plant				Colorless aleurone, red plant			
Mutant No.	tation score	Paramu- Multiple range test	Outside markers	Mutant No.	tation score	Paramu- Multiple range test	Outside markers	Mutant No.	tation score	Paramu- Multiple range test	Outside markers
2-9†	2.14		<i>g Mst</i>	<i>Rst</i>	3.51		..	<i>Rst</i>	3.51		..
2-4*	2.87		$+ M^{st}$	2-14	4.33		$+ M^{st}$	2-32	5.68		$+ M^{st}$
2-3*	2.92		$+ M^{st}$	2-17	4.34		$+ M^{st}$	2-27	5.74		$+ M^{st}$
2-2†	2.93		<i>g</i> $+$	2-21	4.68		$+ M^{st}$	2-36	5.85		$+ M^{st}$
2-8*	2.96		$+ M^{st}$	2-23	4.71		$+ M^{st}$	2-38†	5.89		$+$ $+$
2-10*	3.00		$+ M^{st}$	2-22	4.85		$+ M^{st}$	<i>R^r</i>	5.90		..
2-5*	3.20		$+ M^{st}$	2-16	4.86		$+ M^{st}$	2-28	5.91		<i>g Mst</i>
2-1	3.32		$+ M^{st}$	2-24	5.17		$+ M^{st}$	2-26	5.94		$+ M^{st}$
2-11	3.34		<i>g Mst</i>	2-19	5.19		$+ M^{st}$	2-31	5.94		$+ M^{st}$
<i>Rst</i>	3.51		..	2-18	5.29		$+ M^{st}$	2-30	6.01		<i>g</i> $+$
2-7	3.51		$+ M^{st}$	2-13	5.31		$+ M^{st}$	2-33	6.06		$+ M^{st}$
2-12*	3.53		$+ M^{st}$	2-15	5.51		$+ M^{st}$	2-35	6.11		$+ M^{st}$
<i>R^r</i>	5.90		..	2-20	5.53		$+ M^{st}$	2-25	6.14		$+ M^{st}$
<i>r^g</i>	6.44		..	<i>R^r</i>	5.90		..	2-29	6.16		$+ M^{st}$
				<i>r^g</i>	6.44		..	2-34	6.41		$+ M^{st}$
								<i>r^g</i>	6.44		..

* Unstable plant color.

† Unstable seed color.

‡ Mutants within each group are compared using Duncan's multiple range test; means opposite any one line are not significantly different at a probability level of 0.01. The paramutagenic allele *Rst* and nonparamutagenic alleles *R^r* and *r^g* are included as standards for comparisons.

All mutants except 2-10 and 2-26 were tested for mutations to *R* (fully colored aleurone), and three gave such mutations. Six near-colorless aleurone, green plant mutants were unstable for plant color, mutating from green to red. Unstable mutants of these two types are identified in Table 1.

The paramutation scores from each of the three mutant groups were tested, using analysis of variance, for uniformity of paramutagenic action within each group irrespective of level of action. The calculated *F* values from the three analyses, the tabular *F* values at the .01 probability level, and the group means are in the upper part of Table 2. The *F* values from the near-colorless aleurone, green plant, and near-colorless aleurone, red plant groups exceed the tabular *F* values, indicating significant differences between the mutants within these two groups. The *F* value from the colorless aleurone, red plant group was less than the tabular value, indicating uniform action of the mutants within this group.

The paramutation score means of the three mutant groups were compared using analysis of variance. The mean square for variation among mutants within groups was used as the error terms in these comparisons, and the calculated and tabular *F* values are shown in the lower part of Table 2. All calculated *F* values were significant, indicating that the three mutant groups differ in their level of effect on *R^r*.

Duncan's multiple range test (STEELE and TORRIE 1960) was used to compare the paramutation scores of the mutants with those of the paramutagenic allele

TABLE 2

F values from statistical analyses testing the significance of variability in level of paramutation within each mutant group, and analyses comparing the means of the three groups

Comparisons	Mean	Degrees of freedom (mutants/error)	F values	
			Calculated	Tabular ($P=.01$)
Within near-colorless aleurone, green plant	3.07	10/44	3.61**	2.75
Within near-colorless aleurone, red plant	5.01	11/48	7.13**	2.64
Within colorless aleurone, red plant	5.99	12/52	1.35	2.55
Near-colorless aleurone, green plant <i>vs.</i> near-colorless aleurone, red plant	...	1/21	140.34**	8.02
Near-colorless aleurone, green plant <i>vs.</i> colorless aleurone, red plant	...	1/22	572.58**	7.94
Near-colorless aleurone, red plant <i>vs.</i> colorless aleurone, red plant	...	1/23	62.88**	7.88

R^{st} and the nonparamutagenic alleles R^r and r^g . With this test, every mean can be compared with every other mean at defined probability levels. The results are shown in Table 1, along with the outside genetic markers carried by each mutant. Eleven near-colorless aleurone, green plant mutants were tested and ten were not significantly different from R^{st} ; the one exception, mutant 2-9, was significantly more paramutagenic. All 11 mutants were significantly different from R^r and r^g . Twelve near-colorless aleurone, red plant mutants were tested and all were significantly less paramutagenic than R^{st} , but nine were also significantly different from R^r . Thirteen colorless aleurone, red plant mutants were tested and all were significantly different from R^{st} but not from R^r .

DISCUSSION

Seed color mutants isolated from $R^r R^{st}$ plants could be divided into three groups based on aleurone phenotype and plant color. Tests of paramutagenic action disclosed that the three mutant groups correspond to three levels of action. The paramutation scores within any group did not overlap those in the other groups, and the statistical analyses indicated highly significant differences between the group means. The near-colorless aleurone, green plant and near-colorless aleurone, red plant mutants were not homogeneous in level of paramutagenic action; differences between mutants within each group were statistically significant. The colorless aleurone, red plant mutants were more uniform and differences between the mutants were not statistically significant.

The multiple range test indicated that the near-colorless aleurone, green plant mutants possess the full paramutagenic action of R^{st} , and mutant 2-9 was exceptional in appearing to exceed R^{st} in paramutagenic action. Mutant 2-9 was exceptional in several other respects. Of the mutants in this group, it was one of only two not associated with recombination between outside markers, and one of only two unstable for seed color. The unstable mutants, 2-9 and 2-2, had differ-

ent levels of instability: in homozygous cultures 2-9 yielded 119 mutations to *R* from 22,609 gametes and 2-2 yielded 10 from 19,182, for respective rates of 52.6 and 5.2×10^{-4} . The seed color instability of 2-9 exceeds that of *Rst* (20.9×10^{-4} , pooled data from homozygous cultures). A near-colorless aleurone mutant with unstable seed color was isolated from a plant homozygous *Rst Rst* in an earlier test; the rate of mutation of this mutant to *R* has not been accurately measured but is about 23×10^{-4} . If these data are valid, mutant 2-9 is a noncrossover mutant from *Rst* with an altered aleurone color phenotype, and increased paramutagenic activity and seed color instability.

The differences among the near-colorless aleurone, green plant mutants in seed and plant color stability do not seem to be related to paramutation level. Likewise, the different combinations of outside markers within this mutant group are not clearly associated with characteristic paramutation levels. The different genetic alterations that gave rise to these mutants apparently were independent of the genetic material associated with the paramutation phenomenon, with the possible exception of mutant 2-9.

A direct test was made of the effect of plant color mutations on paramutation level independent of coincident seed color mutations. A mutation to red plant was isolated from each of two of the near-colorless aleurone, green plant mutants known to have unstable plant color. The paramutagenic action of each red plant mutant was compared with that of the unmutated parental allele. No change in paramutation level was observed as a consequence of the plant color mutations. These two mutants with red plant color represent a mutant class not present in the array recovered directly from *R^r Rst* plants; i.e., near-colorless aleurone, red plant, and as fully paramutagenic as *Rst*.

The multiple range test indicated that the near-colorless aleurone, red plant mutants were less paramutagenic than *Rst*, but significantly different from a nonparamutagenic *r^o*. Three of the 12 mutants were not significantly different from a nonparamutagenic *R^r*. Despite this latter fact the order of the paramutation scores, with the nonparamutagenic alleles *R^r* and *r^o* falling together at the high end of the scale, suggests that all these mutants possess some paramutagenic action but none the full action of *Rst*. The mutants in this group were all associated with recombination between outside markers, carried an identical combination of markers, and were stable for seed color. Despite the apparent uniformities of origin, phenotype, and seed color stability there were significant differences among the mutants in paramutation level. The crossover events giving rise to the mutants in this group apparently always result in a coincident reduction, but not a complete loss, of paramutagenic action, and the reduction is not always to the same level.

The colorless aleurone, red plant mutants were all significantly different from the paramutagenic allele *Rst*, and none were different from the nonparamutagenic allele *R^r*. Eleven mutants were associated with recombination between outside markers, and ten of these carried the same combination of markers. Non-recombinant mutant 2-38 was unstable for seed color; 17 mutations to *R* were recovered from 42,837 gametes for a rate of 4.0×10^{-4} .

The data support the following conclusions regarding the origin of the several classes of mutants and the chromosomal basis of paramutagenic action: (1) Near-colorless aleurone, green plant mutants that possess the potential for secondary mutations to *R* originate from *Rst* and do not involve intralocus crossing over. Two mutants from the above tests, 2-9 and 2-2, and one recovered from an *Rst Rst* plant in another test are included in this group. Mutant 2-2 was recombinant for the outside markers, but this could be an instance of a crossover occurring coincidently with a mutation. The apparent increase in paramutagenic action of 2-9 and the differences among the three mutants in rate of mutation to *R* makes it necessary to view these mutants as only a small sample of the genetic alterations that can occur coincident with mutation of *Rst* to near-colorless.

(2) Near-colorless aleurone, green plant mutants that do not possess the potential for secondary mutations to *R* originate in *R^r Rst* plants by intralocus crossing over. They may or may not possess the potential for secondary mutations to red plant. Three of the nine mutants in this group were stable for green plant color, and six were unstable; only mutant 2-11 was nonrecombinant for the outside markers.

(3) Near-colorless aleurone, red plant mutants originate in *R^r Rst* plants by intralocus crossing over. Crossovers that yield mutants of this type reduce paramutagenic activity but not to the same level. Such crossovers very likely represent alterations near, or possibly within, the genetic material associated with paramutagenic action. The resultant variability in paramutation level may be a reflection of the quantitative partitioning of such genetic material by crossover events. The physical basis of paramutation would then consist of a chromosomal region partitionable by conventional crossing over, the several parts being resolved experimentally as mutant alleles with various quantitative reductions in paramutagenic action.

(4) Colorless aleurone, red plant mutants originate in *R^r Rst* plants by intralocus crossing over. Mutant 2-38 may be an exception since it possesses the potential for secondary mutations to *R*, and this, together with the fact that it carried both outside markers from the *R^r* parental chromosome, indicates it may be a noncrossover mutation from *R^r*. Intralocus crossovers that yield colorless rather than near-colorless aleurone mutants preclude the possibility of the mutant being paramutagenic. A close association between the genetic basis of paramutation and the near-colorless phenotype is suggested.

SUMMARY

Seed color mutants isolated from *R^r Rst* plants were divided into three groups, based on anthocyanin in the aleurone and plant, and tested for paramutagenic action. The paramutagenic allele *Rst* and nonparamutagenic alleles *R^r* and *r^s* were included as standards for comparisons. Mutants within the three groups were tested, using analysis of variance, for uniformity of paramutagenic action within each group, and differences were significant within two of the groups. The means of the three mutant groups were compared and all differences were significant.

Eleven near-colorless aleurone, green plant mutants were tested; ten were as paramutagenic as R^{st} and one was more paramutagenic. Two mutants in this group were unstable for seed color and six were unstable for plant color, but the differences in stability did not seem to be related to paramutation level. The different genetic alterations that gave rise to the mutants in this group apparently were independent of the genetic material associated with paramutation, with one possible exception.

Twelve near-colorless aleurone, red plant mutants were tested and all were less paramutagenic than R^{st} , but nine were significantly different from nonparamutagenic R^r . The mutants in this group all probably possess some paramutagenic action but none show the full action of R^{st} . The genetic alterations giving rise to the mutants in this group apparently always result in a coincident reduction, but not loss, of paramutagenic action.

Thirteen colorless aleurone, red plant mutants were tested and none was significantly different from nonparamutagenic R^r . One mutant in this group was unstable for seed color. Genetic alterations that yield colorless rather than near-colorless aleurone mutants seemingly preclude the possibility of the mutant being paramutagenic. A close association between the genetic basis of paramutation and the near-colorless phenotype is suggested.

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