MUTANTS FROM MAIZE PLANTS HETEROZYGOUS $R^r R^{st}$ AND THEIR ASSOCIATION WITH CROSSING OVER[1]

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THE R locus alleles in maize, together with certain complementary genes, condition the production of anthocyanin pigment in the aleurone layer of the seed and in such plant parts as the roots, leaf sheaths, and anther walls. In the designation of the common alleles (EMERSON, BEADLE and FRASER 1935), R and r are used to indicate the presence and absence, respectively, of anthocyanin in the aleurone. A superscript is added to this notation to indicate whether anthocyanin is present in the other plant parts. An r superscript, denoting red, indicates the presence, and a g superscript, denoting green, the absence of anthocyanin. The four major alleles are thereby designated R^r, R^g, r^r and r^g.

Unfortunately, the designation of certain other R alleles varies from the above notation. The genetic symbols for alleles that condition mosaics of colored and colorless aleurone tissue use superscripts to indicate particular patterns, and these superscripts have no reference to anthocyanin pigment in other plant parts. Stippled aleurone conditions colored spots of varying size and number and is designated R^{st}. The R^{st} allele used in these studies does not produce anthocyanin in plant parts other than the aleurone, but this is not indicated in its gene symbol.

The structure of certain R alleles has been the subject of intensive investigation by STADLER, EMMERLING, and others (STADLER 1951a, 1951b; STADLER and NUFFER 1953; STADLER and EMMERLING 1954, 1956; EMMERLING 1958). Data from these studies have generally been consistent with a gene model in which the plant (P) and seed (S) pigmenting functions of the alleles are elements of a compound locus and are separable by crossing over. The structure of R^{st} became of special interest when it was observed that the phenotype of an R is different when extracted from an $R R^{st}$ heterozygote than when extracted from an $R r$ heterozygote or an $R R$ homozygote (BRINK 1956). This unusual interallelic interaction has been termed paramutation (BRINK 1958, 1960).

In an earlier test, eight seed color mutants were isolated from plants heterozygous $R^r R^{st}$ (ASHMAN 1960). The mutants conditioned colorless or near-colorless aleurone and red plant color, and carried a genetic marker from the R^{st} chromosome distal to the R locus. If the red plant color is assumed to have come from the R^r allele the genetic change responsible for these mutants must have involved crossing over between functional components of the R locus. The data in this paper are primarily from a similar test with the addition of genetic markers

proximal and distal to the R locus. As will be seen, mutations to colorless and near-colorless aleurone in $R^r R^{st}$ plants are, with some exceptions, associated with recombination between the outside markers.

MATERIALS AND METHODS

An $R^r R^{st}$ stock was prepared incorporating the genetic markers g, 14 units proximal, and M^{st}, six units distal to R. The gene golden (g) conditions yellowish-green plant color, and M^{st} is a major modifier of the R^{st} phenotype. The parent $R^{st} R^{st}$ line carried both g and M^{st}. The $+ R^r +/g R^{st} M^{st}$ plants were pollinated with $g r^g +$ pollen, and the ears produced were examined for nonparental kernels, i.e., neither stippled nor fully colored. Kernels selected as presumed mutants were planted and tests made to verify the mutation and determine the constitution of the outside genetic markers. Verification of the mutation was accomplished by pollinating the plants grown from the selected kernels with pollen from $R^r r^g$ plants and examining the resulting kernels for segregation on the ear of either of the parental alleles, R^r or R^{st}. This mating also identified any mutant phenocopies resulting from pollen contamination; ears from outcrossed kernels segregated one of the parental alleles and some allele other than r^g.

The constitution of the proximal marker, golden, was determined by observing the plants grown from the selected kernels. Plants homozygous for g are pale yellow and have deep yellow silks in contrast to the green silks of plants carrying a normal allele. Testing for the constitution of the distal marker, M^{st}, required an additional generation for test crosses to R^{st}. Plants carrying each of the verified mutant alleles, heterozygous with an r^g known not to carry M^{st}, were pollinated with R^{st} pollen. The stippled pattern produced by R^{st} is much darker (more colored spots) when M^{st} is present. If the kernels from the matings to R^{st} of plants carrying a particular mutant allele showed a uniform stippled pattern the mutant was classified as not carrying M^{st}. If the kernels from the mating to R^{st} showed a 1:1 segregation ratio for two stippled patterns, one with a higher frequency of colored spots than the other, the mutant was classified as carrying M^{st}.

The mutant phenotypes identifiable by the above procedure were limited to changes to or toward colorless aleurone. Mutations of R^{st} to self-colored aleurone (R^{sc}), a mutation known to occur, would not be distinguishable since they would have the same aleurone phenotype as the parental R^r kernels. Likewise, if R^r mutated to stippled aleurone, or to any level of mosaicism approximating the R^{st} phenotype, such mutations would not be recoverable from the above test. Certain other classes of mutations were isolated by germinating the nonstippled kernels, composed predominantly of the $R^r r^g$ parental class, and scoring the seedlings for anthocyanin pigment in the coleoptile and first leaf sheath. Seedlings from $R^r r^g$ kernels contain anthocyanin pigment, and those lacking anthocyanin were suspect as possible mutants. The green seedlings were reared to maturity for verification of a mutant phenotype, and for classification of the proximal marker. Some mutants were also classified for the distal marker.

RESULTS

The kernels on 262 ears were scored for mutations to colorless and near-colorless aleurone. The parental classes totaled 50,515 self-colored and 49,446 stippled kernels. The scoring yielded 95 possible mutant kernels that were separated into four groups: colorless aleurone (35); near-colorless aleurone, only a few pigmented aleurone cells (7); a few small darkly pigmented spots (45); a few small pigmented spots of variable intensity (8). The kernels were planted for verification of the nonparental phenotypes, and the plants were scored for golden and for anthocyanin pigment in the anthers. Five kernels either failed to germinate or the seedlings died.

Verified mutants from kernels classified as near-colorless were invariably mutations to near-colorless aleurone. Many of the kernels classified as having colorless aleurone proved in the following generation to be mutations to near-colorless aleurone. The near-colorless aleurone phenotype consists of usually a few, but occasionally many, faintly pigmented aleurone cells on kernels carrying three doses of a near-colorless allele. The phenotype is dosage dependent and the frequency of pigmented cells is reduced in kernels carrying only two doses, and kernels with one dose are, with rare exceptions, colorless.

The kernels classified as carrying a few small darkly pigmented spots all proved to carry the R^{st} parental allele. The eight kernels showing small pigmented spots of variable intensity proved to be of two kinds: four were mutations to near-colorless aleurone, and four carried the R^r parental allele. The total number of seed color mutants recovered was 43. The mutants were planted the following year and crossed to R^{st} to test for M^{st}.

The data on recombination, aleurone phenotype, and plant color are presented in Table 1. The column totals show that 37 of the 43 mutants were recombinants for the outside genetic markers, and that 35 of these 37 mutants received the proximal marker from the R^r parental chromosome and the distal marker from the R^{st} parental chromosome. The 35 + M^{st} mutants were subdivided into three classes based on aleurone pigment and plant color: (1) near-colorless aleurone, green plant; (2) near-colorless aleurone, red plant; and (3) colorless aleurone, red plant.

The row totals show that the mutants with near-colorless aleurone were divided about equally between those with green and those with red plant color, and that the mutants with colorless aleurone, with one exception, had red plant color. The exceptional mutant with colorless aleurone and green plant color was lethal when homozygous and showed some reduction in transmission through the pollen. It is probably a small deficiency for the *R* locus, and apparently did not arise by crossing over.

Several of the mutants have exhibited either seed or plant color instability. One of the three nonrecombinant mutants with near-colorless aleurone and green

TABLE 1

Classification of 43 seed color mutants isolated from the cross + R^r + / g Rst Mst × g r^g + *for aleurone phenotype, plant color, and the distribution of proximal and distal markers*

	Constitution of proximal and distal markers				
	Nonrecombinants		Recombinants		
Plant color	+ +	g Mst	+ Mst	g +	Totals
Near-colorless aleurone					
Green	0	3	9	1	13
Red	0	0	15	0	15
Colorless aleurone					
Green	0	1	0	0	1
Red	1	1	11	1	14
Totals	1	5	35	2	43

plant color showed mutations to fully colored aleurone in preliminary observations on the stability of the mutant alleles. The frequency of such changes was comparable to that observed for the R^{st} allele (ASHMAN 1960), and, while critical tests are not yet complete, it appears that three other near-colorless mutants may also be mutating to fully colored aleurone but at a lower rate. A mutant of similar phenotype and stability was isolated in an earlier test from a plant homozygous R^{st} R^{st}. The occurrence of this mutant shows that mutations of this type are not exclusive to R^r R^{st} heterozygotes and probably represent noncrossover mutations from R^{st}.

Plant progenies representing 11 of the 13 near-colorless aleurone, green plant color mutants were examined in homozygous stock cultures. Occasional plants in six progenies had tassels mosaic for red and green anthers. The six mutants exhibiting unstable plant color were all from the $+ M^{st}$ recombinant group of mutants; two of the other three $+ M^{st}$ mutants did not show instability for plant color, and one was not in the planting.

Isolation of green seedling mutants by germination of the nonstippled parental class of kernels was done in two similar but separate tests. Kernels germinated in the first test were from a series of matings that did not carry a proximal marker; kernels germinated in the second test were from the same matings as the above seed color mutants and carried g for a proximal marker. Both groups of matings were marked distally with M^{st}. Since R^{st} mutates to self-colored aleurone (R^{sc}), and R^r mutates to R^g, it was anticipated that both of these mutations would occur in R^r R^{st} plants. It was further anticipated that mutants of these two classes could be identified as to source by their phenotypic expression. Many R^{sc} mutants have been isolated and they invariably have given fully pigmented aleurone when present in only one dose in the triploid endosperm. On the other hand, R^g mutants from R^r give incompletely pigmented aleurone (dark mottled) when present in a single dose. Also, R^{sc} mutants are not susceptible to the paramutagenic action of R^{st}, while R^g mutants are paramutable and will thereby be further reduced in aleurone pigmenting capacity. The two classes of mutants were separable on this basis.

Unexpectedly, a third type of green seedling mutant was found. Some of the self pollinated ears from green plants segregated the expected ¼ colorless (r r) kernels but the colored class contained kernels with a stippled pattern of spots on a light mottled background. Progeny tests of the colored kernels showed them to be carrying a compound R locus allele, composed of a stippled and a solid-colored aleurone component. The compound allele was designated $R^{st} \cdot R^g$. (The dot is used in the symbol to prevent possible confusion between the designation of the compound allele and the designation of merely an R^{st} R^g heterozygote). In crosses to r r plants to give $R^{st} \cdot R^g/r/r$ endosperms, the colored spots characteristic of the R^{st} allele were superimposed on the very light mottling characteristic of one dose of a paramutant R.

The frequencies of the three classes of green seedling mutants recovered are summarized in Table 2. The R^{sc} mutants were not classified for the distal marker in either test. The 30 R^{sc} mutants from matings marked with g were not associ-

TABLE 2

Mutants isolated from the cross $R^r R^{st} \times r^g r^g$ *on the basis of absence of anthocyanin pigment in seedlings grown from the nonstippled kernels*

	No. of seedlings scored‡	Number of mutants		
		R^{sc}	R^g	$R^{st} \cdot R^g$
No proximal marker*	8,378	10	0	4
Proximal marker	10,552	· 30	3	9
Pooled	18,930	40	3	13

* One group of matings was marked proximally with golden (*g*) and one was not; both groups of matings were marked distally with M^{st}.
‡ Adjusted for proportion of selected seedlings verified.

ated with crossing over proximal to *R*. The three R^g mutants were classified for both outside markers and each carried a different combination: $g +$, $+ +$, and $+ M^{st}$. The $R^{st} \cdot R^g$ mutants from the test with both proximal and distal markers were all recombinant for the outside markers, the mutant strand carrying the proximal marker from the R^{st} parental chromosome and the distal marker from the R^r parental chromosome.

One characteristic of R^{st}, the basis of which is unknown, is that it mutates to R^{sc} more frequently when homozygous than when heterozygous with *r* (ASHMAN 1960; McWHIRTER 1961b). Two near-colorless aleurone, red plant mutants were tested for their effect on R^{st} to R^{sc} mutation frequency. Plants heterozygous R^{st} r^g were included in the test for a control. The data are shown in Table 3. The frequency of mutations in heterozygotes with the near-colorless mutants was significantly higher (29.7×10^{-4}, pooled data) than that obtained in the heterozygotes with r^g (6.3×10^{-4}), the respective minimum and maximum limits of expectation being 21.4 and 14.8 at the .05 probability level (STEVENS 1942). The mutation frequency in the heterozygotes with the near-colorless mutants was comparable to that observed earlier in R^{st} homozygotes, 20.9×10^{-4} (pooled data).

DISCUSSION

The column totals in Table 1 show that 37 of 43 seed color mutants isolated from $R^r R^{st}$ plants were recombinant for outside genetic markers. Furthermore,

TABLE 3

Frequency of R^{st} to R^{sc} mutations when R^{st} is heterozygous with each of two near-colorless aleurone mutants having red plant color, $r^r(nc)$, and with r^g

Heterozygous combination	No. of stippled kernels*	No. of R^{sc} mutants	Frequency of mutation $\times 10^{-4}$
$R^{st} r^r(nc)$[1]	7,179	17	23.7
$R^{st} r^r(nc)$[3]	7,318	26	35.5
Pooled	14,497	43	29.7
$R^{st} r^g$	7,898	5	6.3

* Adjusted for proportion of mutants verified.

35 of the 37 recombinant mutants were of one crossover class, $+ M^{st}$. The 35 mutants clearly are products of crossing over in or near the R locus. The heterogeneity within the $+ M^{st}$ mutants relative to aleurone phenotype and plant color indicates at least two crossover sites to be involved: crossovers at one site yielding near-colorless, and at another site colorless, aleurone mutants. Plant color differences within the near-colorless aleurone mutants suggest that a third crossover site may also be involved. However, the significance of the plant-color differences is questionable because at least six of the nine $+ M^{st}$ mutants isolated with green plant color were unstable and mutated to red. An anthocyanin-inhibiting R locus component may be involved that is genetically or transpositionally unstable.

The eight nonrecombinant and $g +$ recombinant mutants could be instances of a mutation producing crossover occurring coincidentally with one or more cross-overs between R and the outside markers. The data are not critical because of the small number of mutants. Also, as noted above, two $g M^{st}$ mutants were exceptional in certain respects indicative of a noncrossover origin, and preliminary data suggest a similar origin for several other mutants.

Available data are not sufficient to elucidate the relationship between the gene structures of the near-colorless and colorless aleurone crossover mutants and those of the two parental alleles. The occurrence of near-colorless mutants in R^{st} homozygotes proves the source of this phenotype to be the R^{st} allele, or a component closely associated with it, but the phenotype was observed to occur with several different combinations of seed and plant color stability. The one near-colorless mutant isolated from an R^{st} homozygote and at least one isolated from an $R^r R^{st}$ heterozygote were unstable for seed color, but stable for plant color. On the other hand, the near-colorless $+ M^{st}$ crossover mutants all appear to be stable for seed color, but may be either stable or unstable for plant color. The near-colorless phenotype is independent of the variations in seed and plant color stability, and, therefore, very likely has an independent genetic basis.

Results of a mutation study by McWhirter (1961a) on the stability of R^{sc} mutants also suggest independent R components for the near-colorless and R^{sc} phenotypes. Over one million R^{sc} gametes were tested and six near-colorless and weakly pigmenting mutants were recovered. The mutants were reported by McWhirter to resemble phenotypically the near-colorless aleurone mutants recovered directly from R^{st} by mutation. The change of R^{st} to R^{sc} is apparently independent of the R component that conditions near-colorless aleurone, and if the aleurone pigmenting action of an R^{sc} mutant is lost by a subsequent mutation the phenotype characteristic of the near-colorless aleurone component is expressed.

If stippled kernels are examined under magnification, some can be found that have areas of weak aleurone pigmentation in addition to the strongly pigmented spots characteristic of the stippled phenotype. The pigmentation in such areas is phenotypically similar to that of the near-colorless aleurone mutants. The near-colorless aleurone phenotype could be present on all stippled kernels but masked to a greater or lesser extent by the strongly pigmented spots conditioned by R^{st}.

Mutations to near-colorless would then result from loss of R^{st} action and the consequent revealing of the near-colorless phenotype.

If the stippled phenotype were dependent on two components it would be analogous to other gene controlling systems in maize. For example, R^{st} might be composed of one component potentially capable of mutating to self-color (structural gene) but doing so only in the presence of a second component (controlling element). The similarity between such a hypothetical structure and certain of the Ds—Ac, and P^{RR}—Mp and a_1—Dt gene controlling systems is self evident. Based on such a hypothesized structure for R^{st} and on the assumption that near-colorless mutants are one of the crossover products of the separation of the two components, several attempts were made to resynthesize the R^{st} allele. Two R alleles were considered as possible carriers of an R^{st} component complementary to one carried by the near-colorless mutants: R^{sc} mutants, and R^g mutants isolated from R^r R^{st} heterozygotes. Two R^{sc} mutants were tested in heterozygotes with each of three near-colorless aleurone, red plant mutants, and the total number of kernels scored was 65,975. Two R^g mutants were tested in heterozygotes with each of four near-colorless aleurone mutants, two with red and two with green plant color, and the total number of kernels scored was 193,110. No instances of R^{st} resynthesis were found in the heterozygous combinations tested.

The $R^{st} \cdot R^g$ compound allele apparently resulted from incorporation, by crossing over, of R^{st} and the seed color component from R^r, S in STADLER's terms, together on the same chromosome. The nine mutants isolated from the stocks marked both proximally and distally received the proximal marker from the R^{st} chromosome and the distal marker from the R^r chromosome, a combination of markers reciprocal to that of the $+ M^{st}$ seed color mutants. The $R^{st} \cdot R^g$ mutants have been examined for mutations from green to red plant and none have been found. As near as can be determined, the expressions of the two components are unchanged, R^{st} producing darkly pigmented spots and paramutant R^g producing very light mottled in one dose and mostly fully colored kernels in two and three doses.

Stippled was found to be as unstable when heterozygous with near-colorless aleurone mutants as when homozygous, and its instability was reduced significantly in heterozygotes with r^g (Table 3). The mutation enhancing action of the near-colorless mutants duplicated the effect of another R^{st} allele. This would seem to indicate that the crossover in R^r R^{st} plants that produces the near-colorless mutants also separates the mutation enhancing component of R^{st} from the stippled phenotype, but the validity of such an interpretation is questionable when the data in Table 2 are considered that show R^{st} to be just as unstable when heterozygous with R^r as when homozygous or heterozygous with a near-colorless aleurone mutant. The frequency of R^{sc} mutations in R^r R^{st} heterozygotes was 40/18,930 (21.1×10^{-4}, pooled data) which is not statistically significantly different from that observed in homozygotes or heterozygotes with near-colorless aleurone mutants. The mutation-enhancing action of the near-colorless mutants apparently could have been received from either R^r or R^{st}.

SUMMARY

Maize plants heterozygous R^r (self-colored aleurone, red plant) and R^{st} (stippled aleurone, green plant), and marked proximally and distally with outside genetic markers, were tested for mutations to or toward colorless aleurone, and for the association of such mutations with recombination between the outside markers. Forty-three mutations to colorless or near-colorless aleurone were isolated; nonmutant kernels totaled 50,515 self-colored and 49,446 stippled. Thirty-seven mutants were recombinant for the outside markers, and 35 of these received the proximal marker from the R^r chromosome and the distal marker from the R^{st} chromosome. The 37 crossover mutants fell into three classes based on aleurone phenotype and plant color: (1) near-colorless aleurone, green plant; (2) near-colorless aleurone, red plant; (3) colorless aleurone, red plant. Some mutants exhibited certain exceptional characteristics: one was lethal when homozygous; one (and possibly three others) was unstable for seed color and mutated to fully colored; six were unstable for plant color, mutating from green to red.

Additional mutants were isolated by germinating the self-colored kernels from the original matings and selecting exceptional seedlings without anthocyanin pigment. Three classes of mutants were isolated: (1) self-colored aleurone mutants (R^{sc}) from R^{st}; (2) plant color mutants (R^g) from R^r; and (3) compound mutants composed of R^{st} and the seed color component from R^r ($R^{st} \cdot R^g$). The mutants in the latter group were always associated with recombination between the outside markers.

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