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Unstable expression of a soybean gene during seed coat development

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Summary. The *R* gene of soybean is involved in anthocyanin synthesis in the seed coat, and its *r-m* allele conditions a variegated distribution of black spots and/or concentric rings of pigment superimposed on an otherwise brown seed coat. We describe an unusual feature of *r-m* that causes expression at the *R* locus to switch between active and inactive phases both somatically and germinally. Non-heritable somatic changes of the allele produce single plants containing mixtures of seed with different coat colors (black + striped or brown + striped). Heritable changes of the *r-m* allele are manifested in progeny plants which produce all black seed or all brown seed. Surprisingly, subsequent generations from revertant sublines show continued instability of the allele such that brown revertants (*r*/r**) or homozygous black seed revertants (*R*/R**) can give rise to striped or striped + black-seeded plants. Thus, the revertants produced by the *r-m* allele are not stable but interconvert between all three forms (*R**, *r**, and *r-m*) at detectable frequencies. Mutability of the *r-m* allele in a different genetic background has also been found after intercrossing various soybean genotypes.

Key words: Anthocyanin gene – Mutable allele – Variegation – *Glycine max* – Seed coat

Introduction

Significant insights into the control of gene expression and development have been derived from examination of variegated, mosaic, or mottled patterns. The existence of mobile elements was proven by genetic analysis of variegation in maize kernel pigmentation (reviewed in McClintock 1984). Both dramatic and subtle effects of transposable element systems were identified by their interactions with a number of genes in the anthocyanin biosynthetic pathway in maize.

In soybean (*Glycine max*), there are several examples of variegation affecting easily observable phenotypic markers such as leaf color, seed coat color, and flower color (Peterson and Weber 1969; Groose et al. 1988). One of these is found at the *R* locus, an as yet unplaced gene in the anthocyanin biosynthetic pathway that is expressed in the seed coat. The *r-m* allele of this gene produces a striped seed coat with spots and/or concentric rings of black (dark purple) pigment superimposed on an otherwise brown seed coat (Bernard and Weiss 1973). The cell layers of the seed coat are all derived from the non-germinal L1 cell layer (Carlson 1973). Thus, the seed coat is a maternally derived tissue and reflects the genotype of the previous plant generation rather than that of the embryo it surrounds. The embryo (hypocotyl-radical axis and cotyledons) is derived from the L2 cell layer of the inner meristem which gives rise to the germ line of the plant (Groose et al. 1988). Chimeras result if a mutation occurs in one of the progenitor cell layers (L1 or L2) and not the other. In other words, in seed derived from a selfed inbred line, the genotype of the seed coat can differ from that of the embryo if a mutation has occurred in either one of the two progenitor cell lines.

We have examined the inheritance of the *r-m* allele through several generations and found that it exhibits instability in both the somatic and germinal cell layers. Surprisingly, the revertants are not stable but can convert to the other expression forms of the allele at high frequencies. This behavior indicates an unusual feature of

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gene regulation that has not been seen in other unstable alleles in soybean. Although continued instability of germinal revertants is not typical of most plant transposable elements, it is logical to suggest that an insertion sequence or transposable element residing in or near the *r-m* allele may be the reason for the changes in gene expression that occur at this locus.

Materials and methods

Genetic terminology and seed stocks. The *R* gene conditions seed coat color and three alleles have been identified: *R* – black color, *r* – brown color, and *r-m* – a variegated seed coat color of black spots and stripes on brown (referred to as a striped phenotype). *R* is dominant to *r-m* which is dominant to *r* (Bernard and Weiss 1973). Since a brown pigment (possibly a polymer of leucoanthocyanin) accumulates in seed of *r/r* genotype (Bernard and Weiss 1973), it is likely that the *R* locus encodes a structural gene whose activity is needed in the final stages of the pathway, to produce the deep red anthocyanin in developing seed coats that gains a dark purple/black appearance at seed maturation. On the other hand, the dominant *I* gene appears to be regulatory, since it inhibits anthocyanin production in the seed coat resulting in a yellow seed. An allelic form of the locus, *i-i*, restricts pigmentation to the hilum region (point of attachment of seed to pod), and another allelic form *i* permits full expression of either black (*R/R*) or brown (*r/r*) genotypes. The *I* allele is dominant to *i-i* which is dominant to *i*. Thus, brown, black, or striped seed must also carry the recessive *i* allele that permits self color. The standard cultivar Clark is yellow with a black hilum and is homozygous for *i-i* and *R*. The Clark isoline, L72-2040, which is homozygous *r-m/r-m*, was used in this study. It was produced by backcrossing the striped-seeded inbred line PI 91.073 (*r-m/r-m*) for five generations to a brown-seeded Clark isoline homozygous for the *i* and *r* alleles and selecting for the *r-m* allele (R. L. Bernard, personal communication).

A small sample of the L72-2040 seed (*i/i, r-m/r-m*) provided by R. L. Bernard, USDA Soybean Germplasm Collection, University of Illinois, Urbana, was grown in 1983 in Beltsville and progeny were bulk-harvested to increase our stock. In all subsequent plantings in 1985, 1986, and 1987, single plants of this line or its revertant derivatives were harvested and these are designated as RM-83-85, with the appropriate plant numbers representing the first or second generations of selfed progeny. *R** and *r** are used to designate revertant alleles from the *r-m* stock which closely mimic, but may not be identical to, phenotypes produced by standard *R* and *r* alleles. One phenotypic difference between black seed carrying the standard *R* allele and the black-seeded *R** revertants of the *r-m* allele is the presence of a brown hilum on an otherwise black seed. However, for brevity we will refer to the *R** phenotype as black rather than brown hilum on a black seed.

Additional genetic lines used in these studies were T146 (*i/i, r-m/r-m*), Sooty (*i/i, R/R*), and PI 171.428 (*i-i/i-i, r/r*), and these were also obtained from the soybean stock center in Urbana, Illinois. Standard crossing techniques were used as described by Fehr (1980). Additional genetic markers such as flower color were used to verify that the seed produced were hybrid.

Results

Identification of a mutable soybean gene. Three alleles of the *R* locus which determines seed coat color have been described (Bernard and Weiss 1973) and are illustrated in Fig. 1). One allele (*r-m*) produces highly variable spots and stripes similar to variegation produced by the interaction of a transposable element with a gene. In our field plot of 1983, we planted a sample of approximately 30 seed of the isoline, L72-2040, which contains the *r-m* allele in a near isogenic Clark background. After threshing a bulk harvest of the plants, approximately 150 black seed were found mixed in with the striped seed. It seemed unlikely that the black seed were derived from a rogue plant or from an outcross with a plant carrying the *R* allele because, although the seed coat was black, the hilum was brown. The standard *R* allele produces a black hilum and seed coat. However, to eliminate the possibility that the black seed were derived from a stray plant or rare outcross and to verify that mutability of the *r-m* allele was being observed, a large number of striped seed from the 1983 bulk harvest were grown in 1985. When the mature plants were scored for their seed coat phenotype, many variant plants were observed. From a total of 544 plants, 496 (91.2%) produced all striped seed, 29 (5.3%) produced a mixture of black and striped seed 7 (1.3%) produced all black seed, 2 (0.4%) produced a mixture of brown and striped seed, and 10 (1.8%) produced all brown seed.

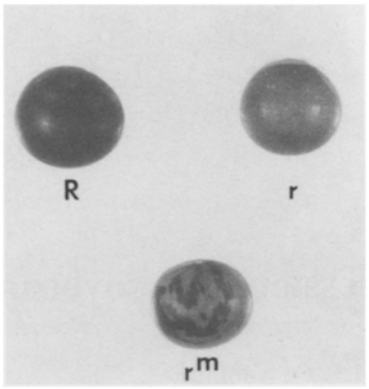

Fig. 1. Seed pigmentation phenotypes produced by three alleles of the *R* locus in soybean (Bernard and Weiss 1973). The *R* allele is dominant to *r-m* which is dominant to *r*. Plants having *R/R* produce black seed, those with *r/r* have brown seed, and *r-m/r-m* plants show a pattern of black spots or streaks on a brown background. Often these occur in a concentric circular pattern and the trait is referred to as a striped phenotype

Soybean is an inbreeding species and the ovules are self-fertilized by pollen shed before the flower opens. However, natural outcrossing can occasionally occur by insect vectors, primarily honeybees; thus, it is necessary to rule out the possibility that variant plants result from rare outcrosses as opposed to mutations. Since the seed coat is maternal, an outcross event or germinal mutation in the 1983 generation would not be reflected in the seed coat surrounding the embryo but would be revealed in the next generation.

Several facts rule out that the variant plants in 1985 arose from rare outcrosses in the previous 1983 generation. (1) The 7 plants that produced all black seed retained the brown color in the hilum region as in the original striped seed. As discussed, this would not be the case if the plants arose from an outcross during the previous generation to an *R*-containing plant since *R* conditions a black hilum and seed coat. (2) There is only one occurrence in the entire soybean germplasm collection of a genetic stock that displays the brown hilum on black seed phenotype. We have never grown this line (T16) in our Beltsville plots, so it would have been impossible for this allele to have been carried by insect vectors during the 1983 season. (3) The two classes of plants with mixtures of seed coat colors cannot be due to outcrosses. Since the seed coat reflects the genotype of the parent plant, the seed from any given plant should be uniform in their seed coat color. Instead, there appears to be instability in expression of the *r-m* allele in the somatic tissues so that it changes frequently from an active expression form (black phenotype) to an inactive state (brown phenotype). (4) When the different classes of revertant plants were compared for other phenotypic markers (flower color, pubescence color, leaf shape, plant height, etc.), they were identical to the original isoline and no segregation in these traits was observed in subsequent generations, as would be expected had they arisen from an outcross to different varieties. (5) Lastly, 8.8% of the 544 *r-m* seed produced variant plants and, as will be presented in Tables 1–4, even much higher rates of mutability in the allele were found in progeny of some individual *r-m* plants or their revertants. These values are much higher than expected for natural outcross events (Jaycox 1970) unless there is partial male sterility in the genetic line used, a situation that does not apply in the present investigations.

Somatic mutability of the r-m allele. Somatic mutability of the *r-m* allele is reflected in those plants which produce mixtures of seed with different seed coat colors (i.e., black + striped or brown + striped). Since the revertants of the *r-m* allele may not be identical to the standard *R* and *r* forms, we designate these derivative alleles as *R** and *r** in order to distinguish them from the standard types. The mutation from *r-m* to *R** or *r** can occur early or late in development and is reflected in the variety of seed mixtures produced by different plants. There appears to be no consistent pattern of somatic mutability. Early events are detected, for example, when an entire branch of a plant produces seed with black coats. Late events are detected when only a few pods on a branch, usually at the end of a branch, produce black seed. Very late events are observed in single seed pods that produce both black + striped seed. The mutation to black can be confined to one or a few branches found at any location on the plant, or it can affect almost the entire plant. On some occasions, the black and striped seed are found scattered randomly on the plant, indicating very late events which occur throughout the plant.

Genetic studies of the unstable r-m allele. The 1985 revertant plants with all black or brown seed were analyzed genetically through two additional generations of self-pollinations. In this way, progeny rows could be analyzed to determine if germinal instability was occurring in addition to the observed somatic instability. Individual plants within the progeny rows were scored directly in the field before harvest. The results are presented in Tables 1 and 2 and can be summarized as follows:

(a) Progeny rows of seed derived from some of the 7 plants that produced all black seed were planted and the mature plants were scored for their seed coat phenotype (Table 1 a). Of 5 plants tested, 3 essentially bred true for the black seed coat and 2 plants segregated 3:1 for black : striped seeds. These segregation patterns indicate germinal mutability of the *r-m* allele. Depending on whether one or both alleles are affected, plants are produced that are either homozygous *R*/R** or heterozygous *R*/r-m*. New, unexpected variant plants also occur at a low frequency in the progeny rows (Table 1 a) and represent continued germinal mutability of the allele. Progeny rows of seed derived from some of the individual plants from Table 1 a were grown for a second generation and again the mature plants were scored for their seed coat phenotype (Table 2 a). These data demonstrate that the homozygous *R*/R**, black-seeded progeny from Table 1 a (RM-83-85-33, e.g.) continue to exhibit a low frequency of mutation to plants with mixed phenotypes of black + striped seed (i.e. RM-83-85-33-4 or RM-83-85-33-5, Table 2 a), indicating that the *R** revertants are not completely stable. Some representatives of the other two plant types, striped and mixed black + striped, also continue to exhibit both somatic and germinal mutability in the second generation. These observations indicate a high degree of instability associated with the *r-m* allele and its mutant derivatives. It is apparent that the mutable allele can convert to one expression form and then back again at a high frequency.

(b) Progeny rows of seed derived from 5 of 10 plants that produced all brown seed were analyzed and each essentially bred true for the brown seed coat (Table 1 b). New variant plants, however, were found in the progeny rows. Seed derived from some of these variant plants were grown for a second generation and again the mature plants were scored for their seed coat phenotypes (Table 2 b). As shown, the black-seeded revertant, RM-83-85-39-2, produced a progeny row that segregated roughly 3:1 for black : brown, indicating that a single mutation event occurred in the parent to produce a heterozygous individual in the progeny (i.e., RM-83-85-39,

Table 1. Segregation in the first generation of progeny plants derived from the mutable seed stock and revertants. Each line represents a single plant of given phenotype derived from the original *r-m* stock. In **d**, each plant contained a mixture of black and striped seed in the percentages indicated. Expected phenotypes and genotypes in **a** and **b** progeny: black ($R^*/r\text{-}m$) → 3 black: 1 striped; or black (R^*/R^*) → all black; brown (r^*/r^*) → all brown

Parent line and phenotype	Total progeny	Seed phenotype of progeny plants			
		striped	black	bl+str	brown
a 100% black					
RM-83-85-30	66	2*	62	2*	
RM-83-85-31	54		54		
RM-83-85-32	60	17	43		
RM-83-85-33	39	1*	35	3*	
RM-83-85-34	61	13	44	4*	
b 100% brown					
RM-83-85-37	66				66
RM-83-85-38	79	1*			78
RM-83-85-39	88		1*		87
RM-83-85-40	111	2*			109
RM-83-85-41	84				84
c 100% striped					
RM-83-85-55	52	52			
RM-83-85-56	39	39			
RM-83-85-57	49	42	1*	6*	
RM-83-85-58	51	51			
RM-83-85-59	41	21	8*	12*	
d Black+striped					
RM-83-85-4					
59% black	33	2	29	2	
41% striped	36	2	26	8	
RM-83-85-8					
84% black	47		47		
16% striped	25	1	24		
RM-83-85-10					
55% black	39	22	11	6	
45% striped	41	22	8	11	
RM-83-85-20					
81% black	38	5	20	13	
19% striped	40	3	21	16	
RM-83-85-23					
58% black	44	9	18	17	
42% striped	47	7	21	19	

* Represent new germinal or somatic reversion events

Table 2. Segregation in the second generation of progeny plants derived from the mutable seed stock and revertants

Parent line and phenotype	Total progeny	Seed phenotype of progeny plants			
		striped	black	bl+str	brown
a From the original 100% black seed stock of Table 1 a					
RM-83-85-30					
-2 (black)	45		45		
-3 (bl+str)	38	2	22	14	
RM-83-85-32					
-3 (striped)	41	39	1	1	
-4 (striped)	57	56		1	
-5 (striped)	41	41			
RM-83-85-33					
-1 (bl+str)	46		42	4	
-2 (bl+str)	49		44	5	
-6 (bl+str)	46		43	3	
-4 (black)	30		29	1	
-5 (black)	31		30	1	
RM-83-85-34					
-1 (black)	41		41		
-2 (black)	35		29	6	
-3 (bl+str)	44		30	14	
-4 (bl+str)	33	2	23	8	
-5 (bl+str)	50	1	48	1	
-6 (bl+str)	49		39	10	
b From the original 100% brown seed stock of Table 1 b					
RM-83-85-39					
-2 (black)	36		23	7	6
RM-83-85-40					
-6 (striped)	9	5			4
-7 (striped)	22	10			11
c From the original 100% striped seed stock of Table 1 c					
RM-83-85-57					
-3 (black)	59		53	6	
-5 (bl+str)	50	24	11	15	
-6 (bl+str)	66	25	19	22	
-9 (bl+str)	35	5	18	12	
RM-83-85-59					
-8 (black)	57		53	4	
-11 (black)	48		45	3	
-6 (bl+str)	45		43	2	
-7 (bl+str)	43	11	8	24	
-10 (bl+str)	37	18	9	10	
-13 (bl+str)	53	12	8	33	

r^*/r^*→RM-83-85-39-2, R^*/r^*). Thus, it appears that the newly derived r^* form of the *r-m* allele is still unstable such that it can mutate to R^* (black seed with brown hilum). Interestingly, some plants with mixed black +striped seed were also found in the progeny row (Table 2 b). This finding indicates again a common tendency for this mutable allele to interconvert between the three alternative forms (R^*, r^*, and *r-m*) both somatically and germinally. Two other striped plants (derived from the brown-seeded RM-83-85-40) produced progeny that segregated for brown and striped phenotypes. It is clear that the conversion of the r^* allele directly to *r-m* can occur as well as r^* directly to R^* as seen above.

(c) From some of the 496 plants that originally produced only striped seed, 2 produced variant plants that also exhibited instability in the next generation (Tables 1 c and 2 c). These data demonstrate the tendency for the *r-m* allele to remain unstable and interconvert to other unstable forms.

(d) Some of the 29 plants which produced a mixture of black and striped seed were harvested and the two different seed types were separated by hand. Separate

progeny rows were planted in order to determine if the black seed represent strictly somatic mutations or if the germinal cell layer is also affected. If the germ line is affected, the black seed should produce plants that either breed true for black or segregate 3:1 for black:striped, while the striped seed should produce plants with seed that continue to exhibits spots and stripes of black color. As an example: RM-83-85-23 produced seed of which 58% were black and 42% were striped. As can be seen (Table 1 d), the separate progeny rows for RM-83-85-23 segregate in a similar pattern. Thus, the black and striped seed types are identical in genotype, although the original seed were phenotypically distinct. Therefore, the mutations that produce black seed on plants with mixed phenotypes are strictly somatic (L1 layer) and have no affect on the germ line (L2 layer). This is true for each example in Table 1 d. However, germinal mutability is occurring in these lines, otherwise they would breed true for striped seed coats. As illustrated previously in Table 1 a, plants that produce all black seed are representative of germ line mutations.

(e) The 2 plants of the 1985 harvest which produced a mixture of brown and striped seed were likewise analyzed by planting separate progeny rows of brown versus striped seed (data not shown). Both of these produced progeny with all striped seed, indicating that the original mutation leading to the mixed phenotype was strictly somatic.

Comparison of germinal mutation frequencies. An estimate of the minimal germinal reversion rates of the *r-m* allele was made, based on the number of pure black-seeded plants in the progeny rows of 11 sibling plants that each exhibited somatic mutations, as evidenced by mixed phenotypes of both black and striped seed on the same plant. Pure black-seeded progeny were produced at rates of 7% to nearly 99% and indicated minimal germinal mutation frequencies of 3.5%–49.5% for the *r-m* allele (Table 3). The very high rates suggest that premeiotic germ cells converted from *r-m* to *R** at a very early stage in plant development in some cases.

Changes in the frequency of r-m instability are not heritable. As seen in Table 1 c, individual plants with the striped seed coats produce revertants at different rates (i.e., compare RM-83-85-57 and RM-83-85-59). To test the possibility that these two lines represent different states of the *r-m* allele that exhibit heritable alterations in the frequency and timing of the mutable event, striped seed from these plants were examined in the next generation for rates of instability of the *r-m* allele (Table 4). For example, RM-83-85-57 produced black-seeded plants at a frequency of 2%, but striped-seeded progeny derived from RM-83-85-57 produced black-seeded plants at frequencies ranging from 0%–65%. Black + striped-seeded

Table 3. Minimal estimates of germinal reversion frequencies of the *r-m* allele. The parent lines represent single plants which each exhibited somatic mutability of the *r-m* allele as evidenced by both black and striped seed on the same plant. The progeny plants which produced an all-black seed set reflect germinal revertants of the *r-m* allele to *R**. Since the black-seeded plants could be of either *R*/r-m* or *R*/R** genotype, the minimal germinal reversion frequency of the *r-m* allele is 1/2 of the percentage of black-seeded progeny plants

Parent line	Progeny producing all black seed	Total progeny	Germinal reversion frequency (%)
RM-83-85-5	4	55	3.5
RM-83-85-10	19	80	12.0
RM-83-85-12	24	67	18.0
RM-83-85-7	36	95	19.0
RM-83-85-23	39	91	21.5
RM-83-85-16	39	80	24.5
RM-83-85-26	39	80	24.5
RM-83-85-20	41	78	26.5
RM-83-85-24	45	79	28.5
RM-83-85-4	55	69	40.0
RM-83-85-8	71	72	49.5

Table 4. Frequencies of instability at the *r-m* allele differ in succeeding generations of selfed progeny

Parent line and phenotype	Seed phenotype of progeny			Frequencies of plant seed color	
	striped	black	bl + str	black (%)	bl + str (%)
RM-83-85-55 (1st generation)					
(striped)	52	0	0	0	0
RM-83-85-55 (2nd generation)					
-1 (striped)	65	0	0	0	0
-2 (striped)	46	0	0	0	0
-3 (striped)	41	0	0	0	0
-4 (striped)	31	0	0	0	0
-5 (striped)	39	0	0	0	0
RM-83-85-57 (1st generation)					
(striped)	42	1	6	2.0	12.2
RM-83-85-57 (2nd generation)					
-1 (striped)	57	0	3	0	5.0
-2 (striped)	15	32	2	65.0	4.1
-4 (striped)	48	2	1	3.9	1.9
-7 (striped)	53	16	12	19.7	14.8
-8 (striped)	12	11	14	29.7	37.8
RM-83-85-59 (1st generation)					
(striped)	21	8	12	19.5	29.2
RM-83-85-59 (2nd generation)					
-1 (striped)	45	11	18	14.8	24.3
-4 (striped)	44	3	7	5.5	13.0
-14 (striped)	35	2	5	4.8	11.9

Table 5. Activation of germinal mutability of the *r-m* allele by cross-fertilization. Variant lines represent single plants found in F_3 populations of crosses with the *r-m* allele as described in the text. All three seed phenotypes indicated were found on the same plant, F1484B1-1-1

Variant line and phenotype	Total progeny	Seed phenotype of progeny plants		
		striped	black	bl+str
F1484B1-1-1				
(black)	61	61		
(striped)	54	53	1	
(brown)	10	9		1
F1884G2-4-1				
(black)	79	19	60	

plants indicate somatic mutability and were observed at a frequency of 12% from RM-83-85-57 and between 2% and 38% in the progeny rows of the striped-seeded plants derived from RM-83-85-57. Thus, no heritability of the mutation frequency is indicated. A similar conclusion is drawn from analysis of the striped-seeded progeny of RM-83-85-59. It is interesting to note that a line that originally showed no mutability of the allele (RM-83-85-55) continues to show this lack of instability in subsequent generations (Table 4).

Activation of germinal mutability of the r-m allele by cross-fertilization. Two other independent occurrences of somatic and germinal mutability of the *r-m* allele have been observed after crossing the *r-m* allele into different genetic backgrounds. In each instance, the instability was detected in the seed produced by a single F3 plant. One cross was between parent lines T146 (striped phenotype and homozygous *i/i, r-m/r-m*) and Sooty (black and homozygous *i/i, R/R*) and approximately 100 F3 seed, all having the striped seed coat phenotype, were grown. One plant (F1484B1-1-1) produced a mixture of 3 seed types with 104 black, 14 brown, and 70 striped seed. To test for germ line mutable events, the seed were separated into the three phenotypic categories and planted the following year. The results are summarized in Table 5. Most of the seed, regardless of seed coat color phenotype, produced plants that yielded striped seed, indicating that the original mutation was somatic. However, two new revertants were recovered, indicating that the mutability of the allele can be passed on through the germ layer. A second cross between lines L72-2040 (striped phenotype and homozygous *i/i, r-m/r-m*) and PI 171.428 (yellow seed brown hilum with homozygous genotype *i-i/i-i, r/r*) was examined in a similar manner and homozygous striped F3 plants *(i/i, r-m/r-m)* were identified by examining segregation patterns in the F3 progeny rows. In one of the progeny rows, a black-seeded plant was found among the expected striped plants. Progeny from this plant segregate in an approximate 3:1 ratio (Table 5). We determined that this plant represents a germinal mutation of one of the *r-m* alleles to *R** based on the presence of a brown rather than a black hilum on the seed and the lack of segregation in several other markers that were known to be homozygous in the parent plant.

Discussion

Reversible changes in gene expression at the R locus. An unusual feature of the *r-m* allele is that revertants are not completely stable but can interconvert between all three forms (*R**, *r-m*, and *r**), each of which has distinctly different effects on expression of the *R* locus. Switching between these forms occurs in germ line progenitor cells (L2 layers) as well as somatic cells (L1). In somatic cells, a switch is manifested by individual plants that have seed of two or more phenotypes, i.e., black and striped or brown and striped. Conversions which occur in premeiotic germ layer cells produce unexpected seed color phenotypes in progeny tests (Tables 1 and 2). The allelic changes sometimes result in both alleles being converted within one generation. For example, plants that were *r-m/r-m* yielded *R*/R** plants directly in the next generation as illustrated by RM-83-85-30, 31, and 33, which are all homozygous black revertants as determined by testing their progeny (Table 1 a). Whether both alleles convert simultaneously or in succession during a lineage of premeiotic cells in unknown. The frequencies of germinal mutability differ between plants (Table 3) and do not appear to be similar in succeeding generations (Table 4). The genetic background may be important in determining whether the *r-m* allele is stably inherited or exhibits instability (Table 5), since germinal instability of the *r-m* allele was observed in the F4 generations resulting from two separate crossing experiments.

Because the molecular mechanism of these changes in gene expression is not known, the choice of terminology can be confusing. Use of the term revertant is clearly warranted, since each expression phase of the allele can be scored and germinal revertants can be distinguished from somatic changes. However, one usually considers revertants to occur from the recessive to the dominant state, but the *r-m* allele can also "revert" or switch to the recessive *r** state as well as to the dominant *R** form. The allele is clearly mutable in its expression but the physical origin of these changes is unknown. By stating that the allele can "switch", "convert", or "cycle" between active and inactive phases, we do not mean to imply any particular molecular mechanism that occurs at the DNA level. The mechanism may involve large structural changes caused by insertions or deletions; alternatively, subtle alterations caused by changes in DNA methylation patterns could lead to different levels of gene expression at

the locus. The fact that the revertants are not completely stable would suggest that changes in structure at the locus are reversible at detectable frequencies.

Any mechanism to explain the behavior of this unstable gene must account for the six observed properties of the *r-m* allele and its revertants, which are summarized as follows: (1) The allele causes gene expression to switch from the recessive expression form (*r**, brown) to the dominant form (*R**, black), or vice versa, in both somatic and germinal tissues. (2) The germinal revertants are not stable but can generate new revertants at high frequencies. (3) Germinal revertants are commonly found to occur in four of the six possible directions, including *r-m* ↔ *R** and *r-m* ↔ *r**. The conversion of an *R** allele directly to *r** has not yet been observed and only one instance of change from *r** directly to *R** has been found. (4) It is possible for both homologous alleles to change expression forms within one generation, as illustrated above (Table 1a), by *r-m/r-m* plants that produced *R*/R** plants in the next generation. (5) The *r-m* allele is inherited as a single gene that is allelic to the standard and stable *R* and *r* expression forms of the locus. (6) *R** revertants do not appear identical to the standard *R* allele in phenotype as they retain a brown hilum, whereas the standard *R* allele has a black hilum as well as a black seed coat.

Comparison of the r-m allele to transposable elements. Although it is unlikely, reversible changes in regulatory regions of the *r-m* allele could lead to the unstable phenotype without involvement of transposable elements or DNA insertions. However, since transposable elements often lead to variegated expression of the affected gene (reviewed in Fedoroff 1983; Nevers et al. 1986; Doring and Starlinger 1986; Vodkin 1989), it is logical to compare the genetic behavior of the *r-m* allele with that of some known transposable element systems in plants. The striking difference between the *r-m* allele and most transposable element systems is that its revertants are not completely stable.

In many cases of variegation caused by maize transposable elements, the somatic and germinal revertants to a dominant phenotype represent element excisions which have restored gene function in a clonal sector of somatic tissues or premeiotic germ cells. In some instances, the mutable phenotype can occasionally be restored by transposition of the element back into the gene to recreate a new mutable phenotype, thus giving a cyclic appearance to the mutants (Orton and Brink 1966; Brink and Williams 1973). Another example of reversible behavior is exhibited by genetic loci under the influence of transposable elements that cycle through active and inactive phases (McClintock 1958, 1962, 1964; Peterson 1966; reviewed in Fedoroff 1983, 1988). Based on the subtle effects that transposable elements can have on gene expression as exemplified by the Spm-dependent and Spm-suppressible alleles of the maize *a1* locus (McClintock 1962; Schwarz-Sommer et al. 1985; Masson et al. 1987; reviewed in Fedoroff 1983), it is possible to envision ways by which two forms (active and inactive) of a single element in the *r-m* allele could have dramatically different effects on expression of the host gene, even in the absence of element excision. Alternatively, the *r-m* allele could consist of a two element system in which a defective element in the *R* locus responds to an autonomous element so closely linked that it is not detected as separable by recombination events. We have examined 1376 F2 progeny from crosses between Sooty (*R/R*) and T146 (*r-m/r-m*) and found a ratio of 1,016 black seed and 360 striped seed, which is consistent with single gene inheritance of the striped phenotype (Chandlee and Vodkin, unpublished results). However, we cannot rule out the presence of an autonomous element within less than 1% recombination distance from the *r-m* allele.

There appears to be a parallel between *r-m* and the behavior of the mutable *bz1-mut* allele at the *bronze1* locus in maize, which conditions finely spotted dark sectors on a pale aleurone background in the presence of an autonomous, unlinked *Mut* transposable element. Revertants of *bz1-mut* to full color are not completely stable and produce mutable progeny at a rate of about 1%. (Rhoades and Dempsy 1982). The mechanism is unknown, but an initial molecular analysis showed that the *bz1-mut* allele and a revertant both contained a 176-bp insertion; thus, no obvious structural change is correlated with the switch in expression at the bronze locus (Walker et al. 1988).

Comparison of the r-m allele to other mutable loci in soybean. In addition to the *r-m* gene, there are two other examples of alleles which produce variegation in soybean. The yellow mutable (*Y18-m*) allele of the *Y18* locus conditions yellow and green variegation in leaves (Peterson and Weber 1969). In addition to somatic sectoring, germinal events also occur such that revertant plants which are fully green (dominant allele) or yellow (recessive allele) are produced. The mutable *W4-m* allele reverts somatically to produce white flowers with purple revertant sectors and germinally to produce progeny that are wild-type with purple flowers (Groose et al. 1988). Reversible behavior similar to that exhibited by the *r-m* allele has not been reported to occur in germinal revertants of the *W4-m* or *Y18-m* mutable alleles. It has been proposed that the mutable nature of the *Y18-m* and *W4-m* alleles is due to the involvement of transposable elements (Peterson and Weber 1969; Groose et al. 1988). As yet, there are no documented instances of movement of a putative element from either of these three genes to other soybean loci. In the present studies of the *r-m* allele in the inbred line L72-2040, we examined a number of

other plant characteristics and found no variability which might have arisen by transposition of an element at the *r-m* allele to another marker.

It is conceivable that transposition activity may be more stringently regulated in a highly inbred species than it is in maize. There is molecular evidence for the presence of a transposable element family in soybean. A stable allele of the soybean lectin gene is inactivated by the presence of an insertion element (Tgm1), which has structural features characteristic of transposable elements (Goldberg et al. 1983; Vodkin et al. 1983) including 13 basepair termini, which are similar to the maize En/Spm element and the Tam1 and Tam2 of snapdragon (Rhodes and Vodkin 1985). Tgm1 is a deletion derivative of much larger (>12 kb) elements, some of which contain an open reading frame with homology to Orf1, an open frame found within an intron of En/Spm (Rhodes and Vodkin 1988). We are currently investigating whether a Tgm-related element is involved in mediating expression of the *r-m* phenotype based on changes of these sequences in Southern blots of *r-m* plants and their revertants.

We plan to isolate the *r-m* allele in order to understand the molecular basis of its instability. Our genetic studies demonstrate that the allele switches in an apparently random manner between active and inactive phases in both somatic and germ line cells. Whatever the molecular nature of this gene, it promises to yield novel information on the function and regulation of gene expression in higher plants that should be applicable to genetic modification of an important crop species by non-traditional approaches.

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