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Biochemical consequences of the insertion of a suppressor-mutator (*Spm*) receptor at the bronze-1 locus in maize

[anthocyanin/transposable elements/inhibitor, enhancer (*I, En*)/UDPglucose:flavonol 3-*O*-glucosyltransferase]

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ABSTRACT **Transposition of a receptor element (*Rs*) for the suppressor-mutator (*Spm*) has generated the first *Spm*-controlled mutable allele of the bronze-1 (*bz*) locus in maize (*Zea mays* L.). In the absence of *Spm*, *bz-m13* conditions full anthocyanin pigmentation in the aleurone but has a markedly decreased level compared to wild type of the gene product, UDPglucose:flavonol 3-*O*-glucosyltransferase (UFGT; EC 2.4.1.91). This UFGT activity appears to be qualitatively similar to the wild-type enzyme. Throughout endosperm development, the amount of a crossreactive material far exceeds the amount of detectable UFGT activity. This and other observations suggest that this insertion of *Rs* leads to the production of two polypeptides from the *bz-m13* allele.**

The action of transposable elements was first recognized in maize more than three decades ago through the intuitive and elegant genetic studies of McClintock and others (1–3). Since then, transposable elements have been the subject of extensive investigations in bacteria, yeast, and *Drosophila* (4, 5). Interest in transposable elements in eukaryotes has been heightened recently by the realization that they can be used in a strategy to identify and clone different genes (transposon tagging) and as a vector for transformation in higher eukaryotes (6).

McClintock (3) first used the term "controlling elements" to describe the mobile genetic elements in maize. This term denotes the ability of these elements to modify gene expression when an element becomes associated with a specific locus (7–9). Workers in our laboratory have been interested in the biochemical basis for changes in gene expression associated with the insertion of controlling elements. This information, coupled with knowledge of specific changes in gene structure at the DNA level, will provide insight into the molecular basis of the action of controlling elements and the regulation of gene expression in higher eukaryotes.

The wild-type allele at the bronze gene (*bz*) locus is required for anthocyanin pigmentation in the aleurone (purple or red color) and in other plant tissue. The *Bz* allele is the structural gene for the enzyme UDPglucose:flavonol 3-*O*-glucosyltransferase (UFGT; EC 2.4.1.91; refs. 10 and 11). The enzyme is active as a monomer of ≈50,000 daltons (12, 13) and mediates glucosylation at the 3-hydroxyl group of both anthocyanidins and the corresponding flavonoids (14). The enzyme accumulates in developing *Bz* kernels and reaches its highest level in mature dried seeds (12).

Specific and distinct changes in *Bz* gene expression were associated with various dissociation (*Ds*)-controlled *bz* mutables (12, 15). Several independent insertions of a *Ds* element are at the *bz* locus. In these nonautonomous two-element systems, each allele conditions a stable phenotype, distinct from that of the progenitor *Bz* allele, in the absence of a signal from the *trans*-acting regulatory element *Ac* (*Activator*). The mutable alleles *bz-m1*, *bz-m2* (derivative I), and *bz-m2* (derivative II) of the *Ac/Ds* system produce bronze kernels that have no UFGT activity and no detectable protein that reacts with anti-UFGT antibodies. Kernels homozygous for *Bz*-weak mutable (*Bz-wm*), also in the *Ac/Ds* system, exhibit considerable anthocyanin pigmentation. Low levels of UFGT activity, which correspond to the amount of a crossreactive material (CRM), can be detected in developing kernels if assayed directly after harvest. The UFGT activity of *Bz-wm* endosperm is considerably more heat labile (half-life at 55°C, 0.7 min) than the enzyme encoded by its progenitor allele, *Bz-Mc* (half-life at 55°C, 5 min). These data suggest that the *Bz-wm*-encoded enzyme differs qualitatively from that of *Bz*.

The *Ds*-controlled mutable allele *bz-m4* produces dark bronze seeds. The UFGT activity of *bz-m4* seeds is qualitatively similar to that of the *Bz* enzyme (heat lability, electrophoretic mobility; see refs. 12 and 16). However, most of the enzyme in *bz-m4* is produced early in seed development and in the internal cells of the endosperm tissue, whereas anthocyanin synthesis and UFGT accumulation are associated with the outermost endosperm layer, the aleurone, in *Bz/−/−* seeds. Thus, the pattern of gene regulation is modified in this mutable allele.

The complex regulatory properties of the suppressor-mutator (*Spm*) inhibitor, enhancer (*I, En*) system have been the subject of extensive genetic investigation (17, 18), but no information has been available about the biochemical changes in gene function associated with *Spm* elements (1). The readily recognized phenotypes produced by mutable alleles of the *Bz* gene facilitate their isolation. This allowed detection of the initial kernel in which a receptor (*Rs*) of the *Spm* gene-control system had been inserted at the *Bz* locus in a germ-line cell of one of the parent plants known to contain elements of the *Spm* system. This modified *Bz* locus is designated *bz-m13* (unpublished data). In the absence of an active *Spm*, *bz-m13* supports the development of a normal amount of anthocyanin in the aleurone layer of the kernel. If an active *Spm* is present elsewhere in the genome, its *Sp* (suppressor) component, effective in *trans*, suppresses gene expression. A bronze phenotype results. If the *Spm* element has an early acting *m* (mutator) component, its *trans*-acting product induces locus modifications (either excision of the insertion or internal rearrangements), both somatic and germinal, that alter the subsequent expression and the stability of that gene. It is the *trans*-acting suppression of gene function that most clearly distinguishes the *Spm/Rs* and the *Ac/Ds* systems. In the latter, when *Ac* is present, clones of intermediate to wild-type cells are superimposed on a mutant

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Abbreviations: UFGT, UDPglucose:flavonol 3-*O*-glucosyltransferase; *bz*, bronze gene; *Spm*, suppressor-mutator; *Rs*, receptor for *Spm*; CRM, crossreactive material; DAP, days after pollination; *Ds*, dissociation element; *Ac*, activator element.

background identical to the phenotype of the *Ds*-mutable in the absence of the regulatory element. By contrast, *Sp* suppresses any residual gene function at the *Rs*-controlled locus.

This paper describes the consequences of insertion of a *Rs* element into a *Bz* allele. In the absence of *Spm*, *Rs* causes a significant decrease of apparently normal UFGT activity throughout seed development and the production of another polypeptide that crossreacts with antibodies against UFGT.

MATERIALS AND METHODS

Plant Materials. A two-element *Spm*-controlled *bz*-mutable allele, *bz-m13*, was selected (unpublished observations). The receptor element for *Spm*, designated *Rs*, has transposed to a *Bz* allele. The reference number—e.g., *bz-m13-R4*—identifies sublines of *bz-m13* tracing to 13 progeny plants of the plant in which *bz-m13* was identified. In each subline, isolates lacking *Spm* were extracted.

For biochemical studies, kernels homozygous for *bz-m13* and lacking *Spm* were used; these have colored nonvariegated phenotypes. Comparisons were made with seeds homozygous for (*i*) *bz-R*, the null reference allele; (*ii*) *Bz-Mc*, the wild-type progenitor allele; or (*iii*) *Bz-W22*, another wild-type isoallele. *Bz-Mc* and *Bz-W22* encode UFGT polypeptides, which are distinguishable based on their thermal stability (H. K. Dooner, personal communication). All stocks were homozygous for the wild-type alleles of complementary color loci (*A1*, *A2*, *Bz2*, *C1*, *C2*, and *R*).

Unless noted otherwise, all references to *bz-m13* in this paper mean homozygous plants without the regulatory element *Spm*.

Measurement of Enzymatic Activity. Samples were prepared as described (19), except that phenylmethylsulfonyl fluoride was omitted from the extraction buffer. Samples of 20–50 kernels were used routinely for each preparation. When possible, kernels were pooled from two or more ears of the same genotype to minimize plant-to-plant variation. The term "mature dried kernels" refers to samples from ears that were allowed to develop for at least 55 days after pollination, and then harvested and dried to ≈10% moisture content.

UFGT activity was measured as described by Dooner and Nelson (10, 12) with some modifications: each 50-μl reaction mixture contained 500 nmol of Hepes buffer (pH 8.2), 310 nmol of $CaCl_2$, 250 nmol of 2-mercaptoethanol, and 3% polyethyleneglycol 6000 with 100 nmol of UDP]U-^{14}C]glucose (specific activity, 375 nCi/nmol or 750 nCi/nmol; 1 Ci = 37 GBq) and 250 nmol of quercetin (dissolved in 5 μl of ethyleneglycol monomethyl ether). Under these assay conditions, two other flavonoid glucosyltransferases were detected in the extracts. These enzymes appear to glucosylate the 3′- and 7-hydroxyls of quercetin and were previously identified in embryos of *bz-R* (20). They represent a small proportion (≈10%) of the UDP-glucosyltransferase activity in *Bz-W22* or *Bz-Mc* samples but are more serious contaminants (60–70% of total transferase activity) in extracts prepared from *bz-m13* and some of its derivatives. Accordingly, the assay procedure was modified to account for these competing activities, and UFGT activity for the different genotypes was determined in the following manner: the initial rate of incorporation of [^{14}C]glucose into ethyl acetate-extractable material was measured. This is equivalent to the total flavonoid-dependent glucosyltransferase activity. Subsequently, the distribution of label, in isoquercitrin and the other glucosylated flavonoids, was determined by separating the products by paper chromatography as described by Dooner and Nelson (10). Finally, UFGT activity was calculated as the proportion of total glucosyltransferase activity representing isoquercitrin formation. A unit of enzyme activity is defined as the synthesis of 1 μmol of isoquercitrin per hr.

Whenever the UFGT activity in the crude extract was low (<0.05 unit per endosperm), the sample was concentrated at least 10-fold and UFGT activity was assayed again: extracts were precipitated by ammonium sulfate (30–60% of saturation), and the precipitate was dissolved and dialyzed overnight against 10 mM Hepes, pH 7.5/10 mM NaCl/10% glycerol and either 1 mM dithiothreitol or 10 mM 2-mercaptoethanol. Under these conditions, at least 80% of UFGT activity was recovered routinely from crude extracts of *Bz* kernels. Wherever mutant lines with apparently low levels of UFGT activity were to be compared to *Bz* material, the latter was also subjected to the ammonium sulfate precipitation.

Thermal Lability. The thermal lability of UFGT from *Bz-Mc*, *Bz-W22*, and *bz-m13* was determined as described by Dooner and Nelson (12) with modifications described above.

Preparation of Antisera. Partially purified UFGT was prepared according to the method of Fedoroff and Mauvais (13) with the following modifications: the pericarp and germ were removed from mature kernels (*Bz-W22*). These were ground in a Wiley mill (40 mesh) and extracted in 100 mM Hepes, pH 7.5/100 mM NaCl/1 mM dithiothreitol with the ion exchange resin AG 1-X2 (30 mg/ml, 3 ml of buffer per g of tissue). The extract was taken through three successive chromatography steps: (*i*) DEAE-cellulose, (*ii*) Affi-Gel Blue (Bio-Rad), and (*iii*) CM-cellulose. The resulting preparation had five bands on a 12% $NaDodSO_4$/polyacrylamide gel stained by the silver technique (21). This material was used to inoculate two rabbits. One rabbit produced antibody against UFGT. Antibody activity was determined by an immune-inactivation assay as described (12), except that formalin-treated *Staphylococcus aureus* cells were used to enhance precipitation of antigen–antibody complexes.

Measurement of CRM. A standard amount of wild-type UFGT (from *Bz-W22*) was titrated with the rabbit serum (12). The volume of serum that precipitates 95–100% of the UFGT activity was determined. This amount of serum was challenged with extracts from *bz-m13* and other genotypes, and the supernatant fractions from these reactions were subsequently used to precipitate the wild-type UFGT standard. This measures the ability of the test sample to remove anti-UFGT antibodies from the serum. CRM is proportional to the amount of UFGT activity remaining in solution after the second precipitation.

Anthocyanin Content. Whole kernels were ground in a Wiley mill (60-mesh screen), then extracted overnight in the dark in 0.1% HCl/methanol (1 kernel per ml). The samples were clarified by low-speed centrifugation. Extracts were diluted at least 1:10 in the acidified methanol. Combined cyanin and pelargonin content was estimated from the absorbance at 530 nm (22, 23).

RESULTS

Spm acts on *bz-m13* to suppress gene expression and to cause transposition or rearrangements restoring *Bz* function, giving rise to clones of pigmented cells on a bronze background in the aleurone tissue and other plant parts. In the absence of *Spm*, *bz-m13* endosperms develop normal *Bz* pigmentation. This was verified by measuring the anthocyanin content of mature kernels of both genotypes: the combined absorbances of cyanin and pelargonin (A_{530nm}) in acidified methanol extracts were 2.33 for *bz-m13* and 2.08 for *Bz-Mc* (relative units per endosperm, three replicates each).

We were interested to know how the inserted *Rs* element in the absence of *Spm* modified the synthesis and accumulation of UFGT in comparison with the progenitor allele *Bz-Mc*. The

Table 1. UFGT activity in mature endosperm

Allele	Specific activity	% *Bz* activity
Bz-Mc	1.39	100
bz-m13-R4	6.95×10^{-3}	0.5
bz-R	1.65×10^{-4}	0.01

Specific activity is shown as enzyme unit(s) per endosperm.

amount of UFGT in mature dried kernels was decreased in *bz-m13* to 0.5% the amount in wild-type *Bz-Mc* (Table 1). The differences in enzyme activity between the two alleles were of similar magnitude in samples grown the previous summer. However, the amount of UFGT in kernels for a given ear was influenced by the length of the growing season and by the general healthiness of the plant. Other isolates of *bz-m13* (R5 and R3) were also fully pigmented and had much decreased UFGT activity at maturity.

The decrease of UFGT activity in *bz-m13* could, by analogy to previous investigations of *Ds*-induced mutables at the *bz* locus (12), be the result of (*i*) synthesis of an altered product in *bz-m13* endosperms that was less stable than the wild-type enzyme, or (*ii*) due to an altered pattern of gene expression during development in *bz-m13* kernels as compared to wild type. To distinguish between these possibilities we measured the thermal lability of UFGT from *bz-m13* and *Bz-Mc* extracts. The enzymes were prepared as ammonium sulfate concentrates (30–60% saturation) of protein extracted from mature kernels. The thermal lability of both preparations was similar (Fig. 1). The $t_{1/2}$ at 55°C was 7 min for *bz-m13* enzyme and 8 min for that of *Bz*. The $t_{1/2}$ for different preparations of the two genotypes ranged between 5 and 8 min. Under these assay conditions the thermal lability of UFGTs from two wild-type isoalleles, *Bz-Mc* and *Bz-W22*, was quite distinct ($t_{1/2}$ at 55°C, 5 and 12 min, respectively). This agrees with previous observations (H. K. Dooner, personal communication). The data suggest that UFGT activity in mature kernels of *bz-m13* may be ascribable to a protein identical to that found in *Bz-Mc* endosperm.

If *bz-m13* had an altered pattern of gene expression, it might synthesize UFGT at some earlier stage of development, and enzyme activity might decline as the kernel matured. We harvested ears, homozygous for *Bz-Mc* or for *bz-m13*, at 4-day intervals from 14 to 50 days after pollination (DAP); by 50 DAP the kernels had progressed to the drying stages of maturation (Fig. 2). These fresh ears were used to prepare ammonium sulfate concentrates (30–60% saturation) of extractable proteins, and after overnight dialysis, the UFGT activity was determined for each sample.

UFGT could be detected readily in *Bz-Mc* kernels at 14 DAP. By 18 days, the crown of the aleurone was faintly pigmented as was seen in *bz-m13* kernels. However, the *in vitro* level of UFGT from *bz-m13* was <1/10th that found in *Bz-Mc* extracts (Fig. 2). UFGT in *Bz-Mc* endosperms continued to increase linearly during kernel development, reaching its highest level in mature dried kernels (Table 1). Irregularities in the trend were probably due to plant-to-plant variations and to the periodically cold temperature in September 1982 while this study was in progress. The crowns of the kernels of both genotypes were fully pigmented by 30 DAP; color spread toward the base of the kernel between 40 and 50 days. In marked contrast to *Bz*, the amount of UFGT in *bz-m13* kernels falls to trace levels by 26 DAP. UFGT activity in *bz-m13* endosperms remained near the limit of detection in the *in vitro* assay (0.1 milliunit per endosperm) until the later stages of development when activity increases as the kernels begin to dry in the field. These results were similar to a preliminary study of UFGT accumulation in greenhouse-grown *bz-m13* kernels.

Although the patterns of UFGT accumulation in *bz-m13* and *Bz* endosperms were distinctly different, there were no differences in the anthocyanin accumulation between the two lines throughout development (data not shown).

Samples prepared from *bz-m13* endosperms for the time-course study were also assayed for the presence of protein reacting with antibodies prepared against wild-type UFGT (Fig. 3). From 14 DAP there were substantial amounts of CRM in *bz-m13* extracts. The level of CRM reached a plateau around

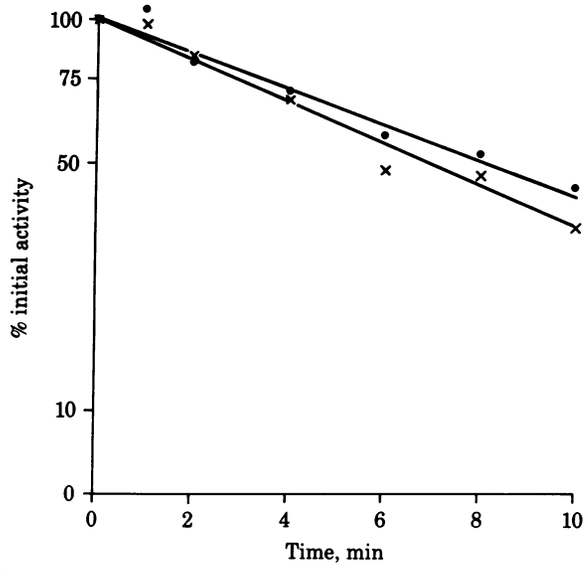

FIG. 1. Thermal stability of UFGT from *Bz-Mc* (●) and *bz-m13* (×) kernels. Aliquots of the two preparations were incubated at 55°C for the times indicated. The amount of UFGT activity remaining (as compared to the unheated controls) was determined.

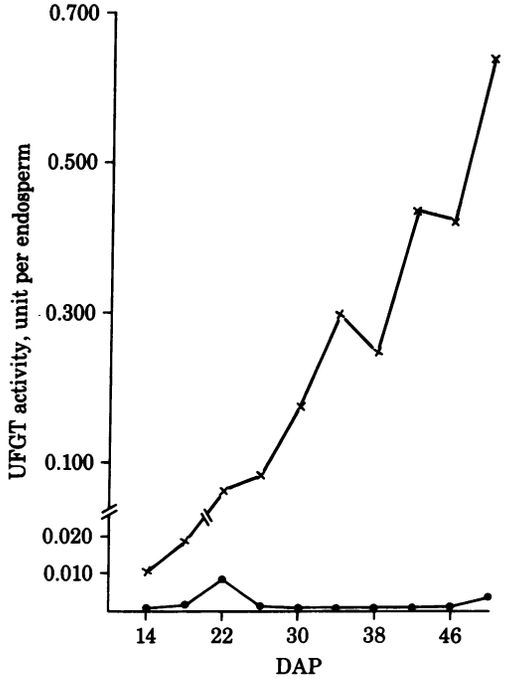

FIG. 2. UFGT activity during endosperm development in *Bz-Mc* (×) and *bz-m13* (●). Fifty-kernel samples from separate ears were harvested at 4-day intervals, extracted, and concentrated by ammonium sulfate precipitation (30–60% saturation). After overnight dialysis, the amount of UFGT activity in each sample was determined.

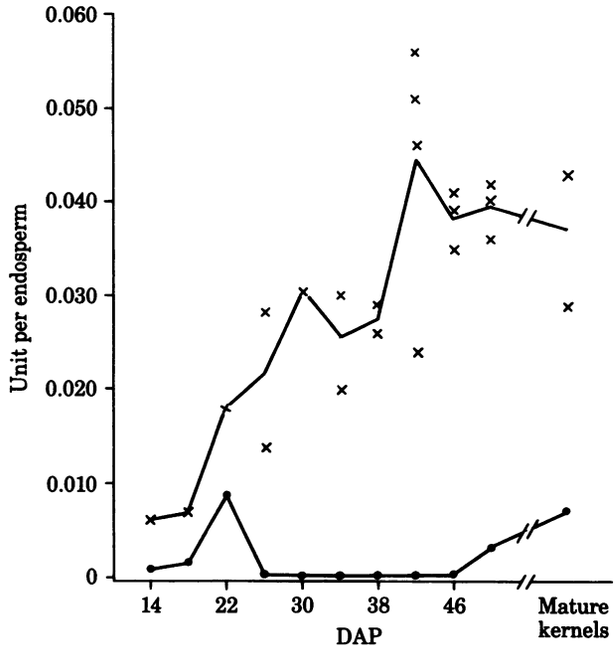

FIG. 3. Accumulation of UFGT activity (●) and CRM (×) in *bz-m13* kernels during development. CRM was measured in samples prepared either from kernels processed immediately after harvest and then frozen or from ears frozen at different stages of development.

46 DAP and was slightly decreased in dried kernels.

Dooner and Nelson (12) have previously shown that the stable *bz-R* allele produced no UFGT activity in mature kernels and produced no detectable CRM. To verify that the CRM in *bz-m13* kernels was characteristic of that allele and not an artifact of the indirect assay technique, protein extracts were prepared simultaneously from frozen *bz-m13* and *bz-R* kernels from ears collected at 26, 34, and 42 DAP and from dried kernels of both genotypes. No CRM was detected for any of the *bz-R* sample (as compared to a buffer control). Substantial amounts of CRM were found in *bz-m13* samples that had trace or small amounts of UFGT activity *in vitro*.

DISCUSSION

The insertion of *Rs* in a *Bz* allele that produced *bz-m13* decreased the synthesis of extractable UFGT activity in *bz-m13* kernels. This modification of gene expression is not reflected in the phenotype of *bz-m13* kernels lacking *Spm*; they are indistinguishable from *Bz* in appearance and in anthocyanin content.

UFGT mediates one of the final steps in anthocyanin biosynthesis and is probably not a control point for the flow of carbon through this synthetic pathway. UFGT accumulation is proportional to the number of copies of the wild-type allele *Bz* (10), although one copy of *Bz* is sufficient to condition full anthocyanin pigmentation, as is one copy of *bz-m13*. This suggests that in *Bz* kernels the enzyme is in considerable excess of that necessary to condition the wild-type phenotype. Thus a low level of UFGT activity in *bz-m13* endosperm may be sufficient for normal anthocyanin synthesis. In *Drosophila*, some revertants (ry^+) of the rosy (*ry*) locus, the structural gene for xanthine dehydrogenase, produce <1% enzyme found in ry^+ flies, and this quantity is still sufficient to condition normal eye color (24).

The unique feature of *bz-m13*, in comparison with the *Ds*-mutable alleles of *Bz* is that the extractable UFGT activity in this *Rs*-controlled mutable is substantially less than the amount of CRM at any time during development. At least two different models could account for this result. In the first model, insertion of *Rs* causes the UFGT polypeptide produced by the *bz-m13* allele to have altered kinetic properties with a much slower V_{max}. This is still adequate to glucosylate anthocyanins *in vivo* but is too low to be detected enzymatically *in vitro*, except at the later stages of development when large amounts of the polypeptide have accumulated. Two observations argue against this model. The heat lability of the UFGT activity from *bz-m13* kernels is similar to that of the enzyme produced by the wild-type allele; a structural modification of the protein altering its V_{max} might also be expected to modify its thermal stability. Second, the developmental profile for CRM accumulation in *bz-m13* endosperm is different from that of the accumulation of measurable UFGT activity: CRM increases gradually until the later stages of kernel development, when the absolute amount of CRM levels off and then falls slightly in mature seeds (Fig. 3). In contrast, UFGT activity can readily be detected from 14 to 22 DAP and then not again until 46 DAP. These data suggest that the UFGT activity measured *in vitro* is not ascribable to a catalytically ineffective protein.

We favor a second hypothesis regarding the underlying molecular events associated with the changes in gene expression seen in *bz-m13* endosperms: this *Rs* insertion conditions the synthesis of two polypeptides. The first is similar to the wild-type UFGT but is produced in far lower amounts than by the *Bz* allele. A second polypeptide(s) corresponding to the excess CRM is also synthesized from the *Bz* locus in *bz-m13* kernels. The pattern of CRM accumulation throughout development is similar to that of UFGT activity in *Bz-Mc* endosperms. It is not yet clear whether the second polypeptide is enzymatically active *in vivo* but not *in vitro* or how it is modified in comparison with the wild-type gene product.

Insertion of *Rs* in a 5′ noncoding sequence or an intervening sequence might lead to a decrease in normal transcription, production of additional transcripts from a new promoter, or, if *Rs* itself is transcribed, lead to errors in processing or translation in the gene product. The dissimilarities in the developmental profiles between UFGT activity and CRM in *bz-m13* might tend to favor a two transcript model, each independently regulated. Alternatively, insertion of *Rs* near the 3′ end of the gene might cause premature termination of transcription or translation, while allowing for the synthesis of a low level of full length mRNAs or polypeptides.

The effects of insertion of transposable elements have been examined in *Escherichia coli*, yeast, and *Drosophila* (4). In some instances, insertions of the element IS2 in one of two possible orientations between a promoter and a structural gene still allow gene function or cause constitutive expression at that locus (25). Saedler and co-workers (25) originally postulated that IS2 functioned as a mobile promoter, however, it is now clear that IS2 does not contain DNA sequences that would be compatible with that model (26, 27). In contrast, Tn*10* has been shown genetically to provide a promoter for transcription of adjacent sequences (28). The ROAM mutations of yeast (*R*egulated *O*verproducing *A*lleles responding to *M*ating type; Ty insertions) have been shown to respond to *trans*-acting signals in which the regulation of gene function is mediated through the inserted element (29–31). Strains carrying different Ty elements at precisely the same site relative to the *His4* locus were either His^+, His^-, or cold-sensitive His^+ (32). However, for those cases that have been analyzed in detail, the transcripts beginning within the Ty element do not extend into the flanking gene (33). Thus the regulation of adjacent genes does not, necessarily, result from promoters and transcriptional read through from the Ty elements. The mechanisms by which *Rs* modifies expression of adjacent genes may be equally complex.

Nevers and Saedler (34) proposed a working model for the organization of *Spm* and its receptor. It has not yet been possible to test many aspects of that model. However, in its simplest form, it suggests that insertion of *Rs* between the wild-type promoter and the structural gene leads to a decrease in the rate of transcription through the gene. *Rs* then functions as a terminator sequence (of transcription) responding to a signal from *Sp*. These aspects of their model are not incompatible with the observations we have made regarding *bz-m13*. However, in addition to their model, one would have to postulate that *Rs* interferes with RNA processing or translation (or both) to generate two polypeptides.

To determine whether the changes in gene expression observed in *bz-m13* are characteristic of a *Rs* insertion, it will be necessary to examine other *Rs*-controlled mutable alleles at *bz* or other loci that are amenable to biochemical and molecular studies.

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