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Genetics

RNA splicing permits expression of a maize gene with a defective Suppressor-mutator transposable element insertion in an exon

(bronze-1 gene/splice-site sequence/intron/*Zea mays*)

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ABSTRACT **The *bz-m13CS9* allele of the bronze-1 gene in maize contains a 902-base-pair defective Suppressor-mutator (*dSpm*) transposable element in the second exon. Nevertheless, 40–50% of the enzymatic activity conditioned by a nonmutant allele at the bronze-1 locus is routinely recovered in crude extracts prepared from plants carrying *bz-m13CS9* in the absence of an autonomous Suppressor-mutator element. Analyses of RNAs produced by such plants show that transcription proceeds through the *dSpm*. The *dSpm* sequence of the messenger RNA precursor is then removed by RNA splicing using the donor site of the single bronze-1 intron and an acceptor site within the inverted terminal repeat of the *dSpm*. This results in a messenger RNA with the proper reading frame that could produce a functional enzyme. These data demonstrate that this *dSpm* insertion in an exon of a structural gene has produced a functional allele with a novel intron consisting, in part, of the *dSpm*. This mechanism appears to allow *dSpm* elements to reduce the impact of their insertions on gene expression.**

The maize defective Suppressor-mutator (*dSpm*) transposable elements are nonautonomous members of the Suppressor-mutator (*Spm*; also known as Enhancer-Inhibitor, *En-I*) family (1–3). They can transpose or excise only in the presence of an autonomous *Spm* element. One of the interesting features of *dSpm* elements, first noted by McClintock (2, 4), is that association of these elements with structural genes in some cases gives rise to nonmutant phenotypes in the absence of *Spm*. In order to understand the underlying mechanism of this phenomenon, we have analyzed an allele of this type at the maize bronze-1 locus.

The bronze-1 locus is one of the many loci involved in the anthocyanin biosynthetic pathway of maize. The gene encodes UDP-glucose:flavonoid O^3-D-glucosyltransferase (UFGTase, EC 2.4.1.91; refs. 5 and 6). Recessive alleles at the locus result in the bronze coloration of the aleurone layer and the brown coloration of many other plant parts as compared with the wild-type purple coloration. The mutant allele analyzed in the present study has a *dSpm* insertion in *Bz*, a nonmutant allele at the bronze-1 locus (7). This mutant allele has been designated *bz-m13CS9* (abbreviated as *CS9*). In the presence of an active *Spm*, expression of *CS9* is suppressed in most cells except for infrequent revertant cell lineages. As a result, one observes small colored sectors on a bronze background. In the absence of an active *Spm*, *CS9* conditions full anthocyanin pigmentation.

In this paper, we report that *CS9* conditions a relatively high level of UFGTase activity in the absence of *Spm* despite the presence of the *dSpm* element in the second exon. Analyses of RNAs produced by *CS9* in the absence of *Spm* show that the *dSpm* element contains an acceptor site for RNA splicing within its inverted terminal repeat. This enables RNA splicing to efficiently remove the *dSpm* sequence from the mRNA precursor, allowing for gene function.

MATERIALS AND METHODS

Plant Materials. The isolation of the *CS9* and *Bz'-3* alleles has been described (7, 8). *CS9* arose by a large internal deletion of the *dSpm* element in the initial insertion mutation *bz-m13* (7). The *bz-m13* allele, in turn, resulted from a 2.2-kilobase (kb) *dSpm* insertion into a progenitor *Bz* allele (9). This progenitor *Bz* allele has been designated *Bz-McC2*, since sequence analyses have indicated that *Bz-McC2* is different from previously identified *Bz* alleles (ref. 10; J.W.S., unpublished data). *Bz'-3* is a revertant allele of *bz-m13* that arose by the precise excision of the *dSpm* and the host duplication (11). *Bz'-3* conditions UFGTase activity equal to *Bz-McC2*. Since *Bz-McC2* was not in an appropriate background when the present study was carried out, the *Bz'-3* allele was used as the wild-type control. In addition to other color factors, *CS9* and *Bz'-3* carry dominant alleles at the *b* (booster) and *pl* (purple plant) loci, which are necessary for maximal *Bz* expression in husk tissue.

Protein Analysis. To prepare crude extracts, samples of husk tissue were harvested 15 days after silk emergence, pulverized in liquid N_2, and extracted for 1 hr in a buffer (4 ml per gram of tissue), consisting of 100 mM Hepes (pH 7.5), 100 mM NaCl, and 20 mM 2-mercaptoethanol, in which AG1-X2 ion-exchange resin (250 mg/ml) was suspended. After centrifugation (10,000 × *g* for 10 min), aliquots of the supernatant fractions were assayed for UFGTase activity as described by Klein and Nelson (12). UFGTase activity in husk tissue used for RNA preparation was as follows: *Bz'-3*, 5.37 units per gram fresh weight; *CS9*, 2.19 units per gram fresh weight (41% of *Bz'-3*). A unit of UFGTase activity is defined as one μmol of product (isoquercitrin) formed per hour. Thermal stability assays of ammonium sulfate-fractionated UFGTase were conducted as described by Dooner and Nelson (13). We found that UFGTases isolated from husk tissue are significantly more stable than those isolated from endosperm tissue (14). The reason for this tissue-specific difference in thermal stability is not currently understood.

RNA Blot Hybridization Analysis. Total cellular RNA was extracted as described by Furtek (10) from the husk tissue of plants homozygous for the specified alleles at the bronze-1 locus in the absence of *Spm*. Husks were harvested 15 days after silk emergence. RNA samples (7 μg) enriched for the polyadenylylated [poly(A)$^+$] RNA fraction were fractionated

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Abbreviations: *Spm*, Suppressor-mutator; *dSpm*, defective Suppressor-mutator; UFGTase, UDP-glucose:flavonoid O^3-D-glucosyltransferase; pre-mRNA, mRNA precursor; *Bz*, nonmutant allele at the bronze-1 locus.

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in 1.0% agarose/2.2 M formaldehyde gels, transferred to nitrocellulose filters, and probed with ^{32}P-labeled DNA probes (15).

Genomic Cloning. The genomic cloning of the *Bz'-3* allele has been described (7). The *CS9* allele was cloned by partial *Bgl* II digestion of genomic DNA and ligation to *Bam*HI arms of bacteriophage λEMBL3 (16). Recombinant phages were selected by plaque hybridization to the plasmid pMBzP17, which contains a unique 2.1-kb DNA fragment from a *Bz* allele (10).

Complementary DNA (cDNA) Cloning. Double-stranded cDNA was made as described by Furtek (10). Since only the sequence of the cDNA around the intron and the *dSpm* insertion was necessary, the double-stranded cDNA was digested with restriction enzymes (*Xma* I and *Sst* I) that have cleavage sites outside of this region (for the structure of *CS9* see Figs. 1 and 2*a*). The digested cDNA was ligated to pUC18 plasmid that had been digested with the same restriction enzymes. Recombinant plasmids containing partial cDNAs of *CS9* RNAs were selected by colony hybridization to the probe spanning the internal transcription unit of the bronze-1 gene. The probe was isolated from the plasmid pMBzP17 (10).

DNA Sequencing. DNA sequence was determined by the dideoxy chain-termination procedure (17) using [α-[^{35}S]thio]-dATP (Amersham).

RESULTS

DNA sequence analysis of the *CS9* genomic clone shows that the *dSpm* element of *CS9* is 902 base pairs (bp) long and is inserted in the second exon 38 bp downstream from the single intron of the bronze-1 gene (Figs. 1 and 2*b*). Forty to fifty percent of the wild-type (*Bz'-3*) level of UFGTase activity is routinely recovered in crude extracts prepared from the husk tissue of plants homozygous for *CS9* in the absence of *Spm*.

Blot hybridization analyses of the poly(A)$^+$ RNAs isolated from the husk tissue of plants homozygous for *CS9* in the absence of *Spm* reveals two bands of bronze-1-specific RNAs (Fig. 1). One has a size (about 1.8 kb) similar to that of the *Bz'-3* mRNA, and the other is larger. These RNAs hybridize to probes from *Bz* sequences both 5' and 3' to the *dSpm* insertion site. However, only the larger RNA also hybridizes to the *dSpm* probe. Its size is consistent with that expected from the presence of the *dSpm* sequence in the unspliced mRNA precursor (pre-mRNA). The 1.8-kb RNA hybridizes to *Bz* probes but not to the *dSpm* probe. It is much more abundant than the larger RNA. These results suggest that in the absence of *Spm*, transcription proceeds through the *dSpm*, and that most or all of the *dSpm* sequence is removed from the pre-mRNA.

Sequence comparison of the *CS9* genomic DNA clone and a cDNA clone representing a *CS9* RNA shows that this RNA does not contain the intron, the sequence between the intron and the *dSpm*, or the *dSpm* sequence except for the terminal two nucleotides (TG) at the 3' end (Fig. 2). We therefore suggest that this cDNA represents a 1.8-kb RNA. The sequence removed from the pre-mRNA ends with an AG dinucleotide within the 13-bp inverted terminal repeat of the *dSpm* (Figs. 2*b* and 3*a*). The border sequences of the portion removed from the pre-mRNA thus conform to the GT/AG rule of pre-mRNA splicing (23, 24). In addition, the inverted terminal repeat contains a sequence that conforms to the consensus sequences of acceptor sites for pre-mRNA splicing (18, 19), as shown in Fig. 3*a*. These data indicate that the *dSpm* sequence is removed by pre-mRNA splicing using the donor site of the bronze-1 intron and the acceptor site within the inverted terminal repeat of the *dSpm*.

Results from the cDNA analysis indicate that the 1.8-kb RNA represented by the cDNA is a processed mRNA. The sequence between the intron and the *dSpm* is not present in this mRNA. To test for splicing that would produce a 1.8-kb mRNA or mRNAs containing the sequence between the intron and the *dSpm*, we probed a blot of electrophoretically fractionated RNA with a DNA fragment including a portion of the intron and the 38 nucleotides between the intron and the *dSpm* (for the probe see the upper diagram of Fig. 4). One possible way to produce a 1.8-kb mRNA containing the 38 nucleotides would be the independent removal of the bronze-1 intron and the *dSpm* sequence as shown in the lower diagram of Fig. 4. If this hypothetical splicing event were to occur, some of the 1.8-kb mRNA produced by *CS9* should hybridize to the probe. The *Bz'-3* mRNA should also hybridize to the probe, since it contains the 38 nucleotides. The probe hybridized to the *Bz'-3* mRNA as expected but not to the 1.8-kb mRNA of *CS9* (Fig. 4). This suggests that there is no splicing event that produces a detectable level of a 1.8-kb mRNA containing the 38 nucleotides.

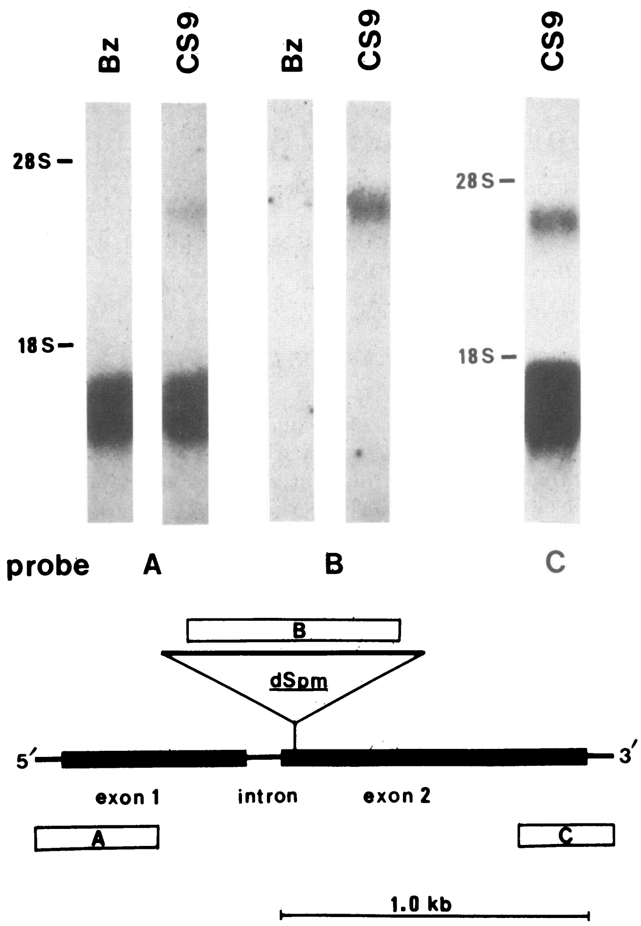

FIG. 1. Blot hybridization analyses of poly(A)$^+$ RNAs with *Bz* and *dSpm* probes. Poly(A)$^+$ RNAs were isolated from the husk tissue of plants homozygous for the indicated alleles at the bronze-1 locus in the absence of *Spm*; Bz refers to *Bz'-3*. Poly(A)$^+$ RNAs were fractionated in formaldehyde-containing 1% agarose gels, transferred to nitrocellulose filters, and probed with the indicated ^{32}P-labeled DNA probes. The diagram below the autoradiogram describes the DNA structure of *CS9*. Probes are indicated by open boxes; probe A contains a *Bz* sequence 5' to the *dSpm*, probe B contains most of the *dSpm*, and probe C contains a 3' *Bz* sequence. Blots probed with A and B were made by fractionating the poly(A)$^+$ RNAs of *Bz'-3* and *CS9* in duplicate lanes in the same gel, transferring to a nitrocellulose filter, and dividing the filter into two parts each containing the RNAs from the two genotypes. Ribosomal RNAs (28S and 18S) provided size markers.

The RNA blot analysis shown in Fig. 4 also suggests that the larger RNA of *CS9* is the unspliced pre-mRNA. The RNA blot

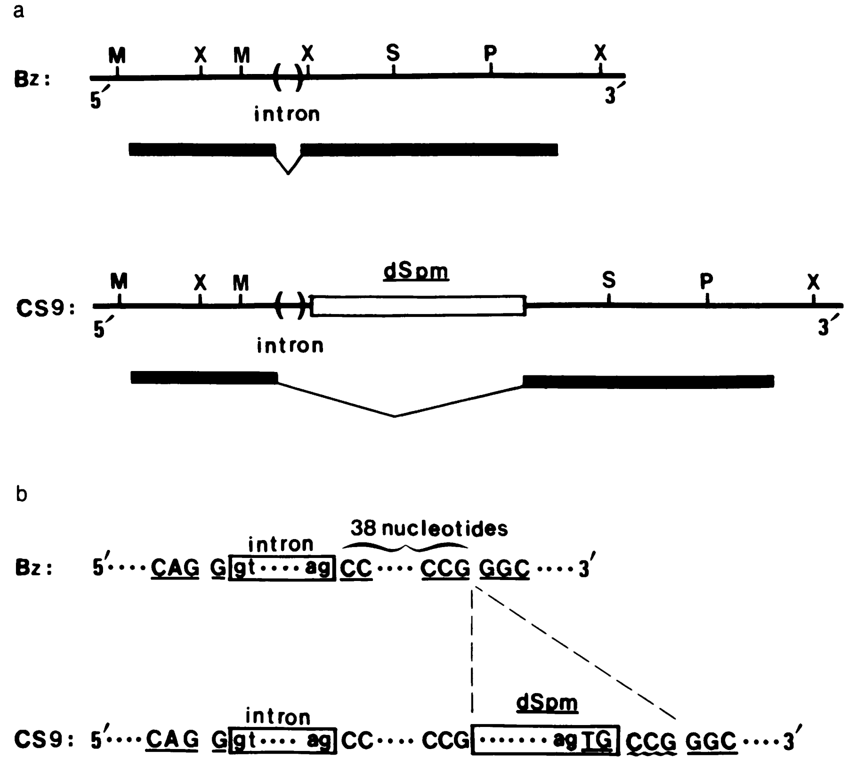

FIG. 2. (*a*) Structure of the *Bz* mRNA and the 1.8-kb mRNA of *CS9*. Bz refers to *Bz′-3*. Heavy lines below the restriction maps indicate the structure of mRNAs with splices between exons. The *Bz′-3* mRNA structure is based on the *Bz* mRNA structure given in ref. 10. Restriction enzymes: M, *Mlu* I; X, *Xma* I; S, *Sst* I; P, *Pst* I. *CS9* has lost the *Xma* I site upon the insertion of the *dSpm*. (*b*) Partial DNA sequences of *Bz′-3* and *CS9* around the intron and the *dSpm* insertion. Bz refers to *Bz′-3*. The underlined trinucleotides represent codons in the reading frame of the *Bz′-3* mRNA or the 1.8-kb mRNA of *CS9*. The reading frame of the *Bz′-3* mRNA is based on that of the *Bz* mRNA given in ref. 10. The trinucleotide (CCG) duplicated upon the *dSpm* insertion is indicated by the wavy line. Lowercase letters indicate the donor and acceptor site sequences (gt and ag, respectively).

probed with the 5′ *Bz* sequence (Fig. 1) indicates that the level of the larger RNA is much lower than that of the *Bz′-3* mRNA. Accordingly, the larger RNA should show a much reduced signal as compared with that of the *Bz′-3* mRNA on the blot of Fig. 4 if its signal were solely due to the presence of the 38 nucleotides between the intron and the *dSpm*. However, the larger RNA shows a signal similar to that of the *Bz′-3* mRNA, suggesting that the signal by this RNA is not only due to the 38 nucleotides but also due to the intron left unspliced. The presence of the unspliced pre-mRNA might imply that the splicing of *CS9* RNA is less efficient than that of *Bz′-3* RNA.

a

Eukaryotic consensus	$\genfrac{}{}{0pt}{}{TTTTTTTTTTT}{CCCCCCCCCCC}N\genfrac{}{}{0pt}{}{C}{T}AG/G$
Plant consensus	$TTT\genfrac{}{}{0pt}{}{T}{R}TT\genfrac{}{}{0pt}{}{TTTT}{RRRR}TG\genfrac{}{}{0pt}{}{C}{T}AG/G$
dSpm	$GACG\underline{TTTTCTTGTAG/TG}$
bronze-1 intron	TTCCATCGTTCGCAG/C

b

Spm	TTTTCTTGTAGTG
Tam1	TTTTGTTGTAGTG
Tgm1	$TTTT\genfrac{}{}{0pt}{}{G}{C}TAATAGTG$

FIG. 3. (*a*) Comparison of the acceptor-site sequence of the *dSpm* in *CS9* with the consensus sequences for eukaryotic (18) and plant (19) acceptor sites. The 13-bp inverted terminal repeat of the *dSpm* is underlined. The acceptor-site sequence of the bronze-1 intron is shown for reference. R = A or G; N = A, C, G, or T; slash (/) = the intron–exon junction. Where two bases are shown in the same position, the upper base is observed more frequently. (*b*) Sequence comparison of 13-bp inverted terminal repeats of *Tam1*, a transposable element of snapdragon (20), and *Tgm1*, a presumed transposable element of soybean (21), with that of *Spm* (22, 33). The fifth nucleotide of *Tgm1* is G in one terminus and C in the other.

The 1.8-kb mRNA revealed by the cDNA does not contain the 38 nucleotides between the intron and the *dSpm* but does include the two nucleotides (TG) from the 3′ end of the *dSpm* and the trinucleotide (CCG) duplicated upon the insertion of the *dSpm* (Fig. 2*b*). This results in a net loss of 33 nucleotides and maintains the proper reading frame. Translation of this mRNA could thus produce a polypeptide for a functional UFGTase if the loss of 11 amino acids and change of alanine (GCC) to valine (GTG) does not destroy UFGTase activity (Fig. 2*b*). Thermal-stability profiles indicate that *CS9* husk UFGTase activity has a half-life at 55°C of 6.5 min as compared with 42 min for *Bz′-3* husk UFGTase activity. This suggests that *CS9* UFGTase protein is altered as compared with wild-type UFGTase protein. In addition, no detectable level of the 1.8-kb mRNA contains the 38 nucleotides between the intron and the *dSpm*. These lines of evidence suggest that the 1.8-kb mRNA represented by the cDNA is responsible for the functional UFGTase encoded by *CS9*.

DISCUSSION

Several authors have proposed that some introns could be the remnants of transposable-element insertions (26–28) despite the claim that no known transposable element has splice-site sequences at its borders (29). The observation that the intron of the yeast mitochondrial 21S rRNA gene encodes enzymes

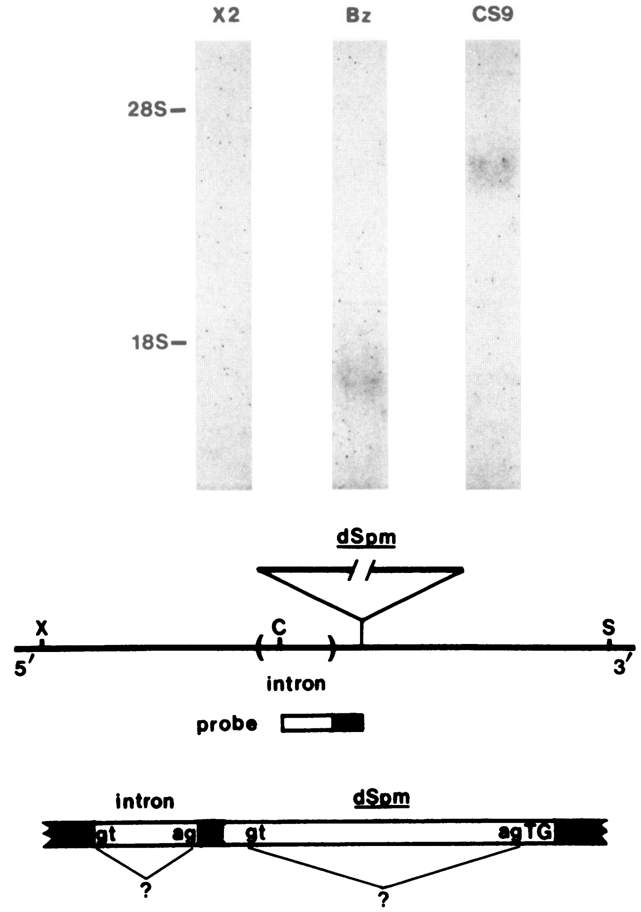

FIG. 4. Blot hybridization analysis of poly(A)$^+$ RNAs to test for the presence of a 1.8-kb *CS9* mRNA or mRNAs containing the sequence between the intron and the *dSpm*. Experimental procedures and genotypes were the same as those of Fig. 1. The probe includes a portion of the intron (open region of the bar representing the probe) and the 38 nucleotides between the intron and the *dSpm* element (filled region of the bar). Poly(A)$^+$ RNA from a deletion stock (*bz-x2*; ref. 25) lacking the bronze-1 locus (indicated by X2) was included as a negative control for the intron sequence. The lower diagram illustrates the splicing event being tested, in which the intron and the *dSpm* are removed independently. Restriction enzymes: X, *Xma* I; C, *Sca* I; S, *Sst* I.

necessary for its own splicing has suggested a relationship between introns and transposable elements (30, 31). The present study clearly shows that a *dSpm* transposable element in maize contains an acceptor site for pre-mRNA splicing within its inverted terminal repeat. Insertion of this element into an exon has resulted in a splicing event that uses this acceptor site. Some acceptor sites in maize might thus have been introduced by *dSpm* or *Spm* element insertions followed by the loss or divergence of sequences required for transposition.

In the absence of *Spm*, the acceptor-site sequence within the inverted terminal repeat of the *dSpm* of *CS9* enables RNA splicing to remove the *dSpm* sequence from the pre-mRNA, allowing for *Bz* expression. However, expression would not be expected if the *dSpm* were inserted in the opposite orientation. *CS9* has resulted from an internal deletion of the *dSpm* element of *bz-m13* without changing the insertion position (7). The *dSpm* of *bz-m13* is virtually the same as the *Spm-I8* element of *wx-m8* (an insertion mutation of the maize waxy gene), but the two elements are inserted in the opposite orientation in terms of transcription of the two genes (ref. 32; J.W.S., unpublished data). Thus, the *dSpm* of *CS9* is oriented in the direction opposite to that of the *dSpm* in *wx-m8*. In the absence of *Spm*, *wx-m8* produces chimeric mRNAs that are prematurely polyadenylylated due to the polyadenylylation signals within the element (32). Presumably, *Bz* mRNAs would also be prematurely polyadenylylated if the *dSpm* of *CS9* were inserted in the opposite orientation, provided that the deletion which produced the *dSpm* in *CS9* from the *dSpm* in *bz-m13* did not remove the polyadenylylation signals.

The insertion position of the *dSpm* is also important for the expression of *CS9*. The 1.8-kb mRNA of *CS9* represented by the cDNA does not contain the 38 nucleotides (12 codons and 2 extra nucleotides) between the intron and the *dSpm*. Thus, the reading frame of the 1.8-kb mRNA would be shifted if the 2 nucleotides from the 3′ end of the *dSpm* were not added. Accordingly, the number of nucleotides between an intron and a *dSpm* insertion must represent a multiple of 3 and an addition of 2 (i.e., $3N + 2$) in order to maintain the proper reading frame of a spliced mRNA. In addition, a loss of or change in amino acids (determined by insertion position) may destroy protein function even though the proper reading frame is maintained. Therefore, the expression of a gene with a *dSpm* insertion in an exon depends on the orientation and insertion position of the element. Moreover, we do not know how far 3′ to the intron a *dSpm* can be inserted and still be removed from a pre-mRNA by the type of splicing event observed with *CS9*. Variation in the length of the intron preceding this *dSpm* might also affect the splicing event observed. The consequences on *Bz* expression of a *dSpm* insertion 5′ to the single intron are also unknown.

Our results demonstrate that a transposable-element insertion in an exon of a structural gene has produced a functional allele with a modified intron consisting in part of the transposable element. As shown in Fig. 3*b*, the DNA sequences of 13-bp inverted terminal repeats of *Tam1*, a transposable element in snapdragon (20), and *Tgm1*, a presumed transposable element in soybean (21), are very similar to that of *Spm* (22, 33). These two elements might also provide an acceptor site when inserted into exons in the same fashion as the *dSpm* of *CS9*. Perhaps, as discussed by Doolittle and Sapienza (28), this structural feature allows these transposable elements in widely divergent plant species to take advantage of RNA splicing to reduce the impact of their insertions on gene expression.

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