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Bronze-2 Gene Expression and Intron Splicing Patterns in Cells and Tissues of *Zea mays* L.[1]

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ABSTRACT

A large fraction of the transcripts of the *Bronze-2* (*Bz2*) gene of maize (*Zea mays* L.) are unspliced in purple husk tissues. The accumulation of unspliced messages could have destructive potential if the intron-bearing mRNAs are translated into aberrant proteins. Our initial studies suggested that both genetic and physiological factors may influence the degree of splicing failure. Nuclear background rather than *cis*-sequence effects is shown to contribute to the genetic component. The accumulation of unspliced message does not appear to be directly influenced by diurnal effects on transcript abundance, by the expression level of the *Bz2* gene, or by thermal stress. We also show that maize cell cultures (Black Mexican Sweet, BMS) can be used to examine the molecular details involved in splicing failure. Much like whole maize plants, the BMS cells excise the *Bz2* intron with varying degrees of efficiency. In contrast with heterologous constructs containing plant introns, splicing of the native *Bz2* intron can appproach 100% in BMS cells. Splicing of transcripts from a marked, introduced gene can be compared to the endogeneous *Bz2* gene facilitating analysis of the impact of sequence changes.

Gene expression in eukaryotic cells requires orchestration of the many biochemical events of transcription initiation, pre-mRNA processing, mRNA transport, and translation; each step is subject to regulatory controls. We focus on RNA processing and the modulation of transcript splicing. There are two general classes of transcription units in eukaryotes (15): a simple transcription unit encodes a single protein product regardless of cell type or developmental cues, and a complex transcription unit encodes multiple protein products by utilizing different coding and noncoding sequences of the same transcript as specified by the regulatory environment of the cell. For example, differential splicing of the first intron of *En/Spm*, a complex maize (*Zea mays* L.) transcription unit, produces from its single transcript the unique *TnpA* and *TnpD* gene products required for transposition of this mobile element (9). In contrast, *Bz2*,[3] a maize gene that acts late in the anthocyanin biosynthetic pathway, is a simple transcription unit; it encodes only a single protein provided the sole intron is appropriately removed. Therefore, it was unexpected that unspliced *Bz2* message represented more than half of the total *Bz2* transcript population in some purple maize husk tissues (21). The finding of unspliced *Bz2* message accumulation suggests failure in intron recognition or excision. Translation of the unspliced message would produce a truncated 13.9-kD protein instead of the 26-kD protein encoded by the accurately spliced transcript because there is an in-frame stop codon in the intron of *Bz2*. We have shown that mutations in the second exon can abolish gene function (19, 21); therefore, if a truncated protein is present, it should not contribute to *Bz2* gene activity.

Although splicing is a complex process involving a multitude of accessory RNA and protein factors, it is considered to be both efficient and accurate because few unspliced or incorrectly spliced mRNAs are detected in animal or fungal cells; the major exception involves cells subjected to extreme stress. When *Drosophila melanogaster* or *Saccharomyces cerevisiae* cells are exposed to a heat shock, splicing fails. The sole introns of the *Drosophila hsp82* pre-mRNA and of the yeast actin pre-mRNA are not removed from these transcripts (30, 31). Protein products from these unspliced transcripts are produced, demonstrating that these unspliced transcripts are present in the cytoplasm and are translatable. Recovery of the cells from the heat shock is coincident with recovery of the normal efficiency and accuracy of the splicing machinery. Because most hsp genes in animals have no introns, it has been proposed by Yost et al. (32) that there has been an evolutionary selection against heat-inducible hsp genes that bear introns because of the disruption of the splicing machinery during heat shock when products from these genes are required. It is interesting that almost all plant hsp genes contain introns (25). Does heat shock produce a different effect on splicing in plants or is splicing failure merely better tolerated by plants?

Incompletely spliced transcripts produced from a number of plant genes have been reported to accumulate in a broad range of plant species (Table I). Transcript splicing of the *hsp70* gene of *Petunia* and the *Gmhsp26-A* gene of *Glycine max* is disrupted when plants or plant tissues are exposed to high concentrations of heavy metals (8, 29). The splicing failure of these transcripts is specific to a few heavy metals and cannot be induced by heat-shock treatments. These results suggest that the splicing efficiency of the heat-shock gene transcripts responds differentially to environmental conditions. Another stress treatment that results in splicing failure is hypoxia; oxygen-deprived maize plants can accumulate up to 10% unspliced *Adh1* RNA (22). However, not all cases

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[3] Abbreviations: *Bz2*, *bronze-2*; hsp, heat-shock protein; BMS, Black Mexican Sweet.

Table I. *Survey of Unspliced Message Accumulation in Plant Tissues*

Species	Gene	Growth Conditions	Citation
Unspliced messages detected by RNA hybridization analysis			
Z. mays	*Bz2*	Field, greenhouse growth chamber	(21)
Z. mays	*Adh1*	Hypoxia	(22)
Z. mays	*P*	Not specified	(13)
Z. mays	*cyppdkZm1* (cytosolic pyruvate orthophosphate dikinase)	Growth chamber	(27)
Z. mays	*TFIID*	Not specified	J. Vogel and M. Freeling (personal communication)
Z. mays	*LC* (*R* homolog)	Not specified	S. Wessler (personal communication)
Brassica oleracea	*srk* (*S*-locus related kinase)	Not specified	(28)
P. hybrida	*hsp70*	$CdCl_2$, 0.05–5 mM	(29)
G. max	*Gmhsp26-A*	$CdCl_2$, 0.25 mM $CuCl_2$, 0.2–1 mM	(8)
Cloned cDNAs containing unspliced intron sequences			
Z. mays	*C1-I*	Not specified	(24)
Z. mays	PLTP (phospholipid transfer protein)	Not specified	(1)
Z. mays	*TFIID*	Not specified	J. Vogel and M. Freeling (personal communication)
Z. mays	*per 5* (cationic peroxidase)	Not specified	(20)
Z. mays	Transcription factor regulating *Sh1*	Not specified	W. Werr (personal communication)
Z. mays	*Mu9* gene A	Not specified	R. J. Hershberger and V. Walbot (personal communication)
Solanum tuberosum	*α-amylase*	Not specified	(14)

of splicing failure in plants involve stress. The maize *P* gene, a locus that regulates pigment accumulation in the pericarp, produces high levels of unspliced message in normal growth conditions (13). Also, cDNAs produced from RNA populations of plants that had received no known stress commonly contain introns (1, 24). In these cases, it is possible that polyadenylated, nuclear RNAs were included in the cDNA libraries, but the frequencies of recovery of cDNAs with introns is high and does not reflect the expected fraction of nuclear RNA in these RNA preparations. Thus, in contrast with the precisely regulated, highly efficient RNA processing pathways in animals and fungi, splicing failure may be a common experience for plants growing in a variable environment.

The extent of splicing failure of *Bz2* transcripts in maize plants suggests that the inhibition of splicing could contribute to quantitative regulation of gene expression of *Bz2* (21). In the initial report of splicing failure of *Bz2* transcripts, the accumulation of unspliced *Bz2* messages was found to be more pronounced in one line of maize, B139, than in the inbred line, W23. The B139 plants were grown in the field and the W23 plants were grown in the greenhouse; therefore, the splicing failure event could have been conditioned by the environmental circumstance in which the B139 plants were grown. Alternatively, there could be a genetic basis for splicing failure, the result of an inherently "poor splicing" *Bz2* allele or a defect in splicing in general in the genetic background of the B139 plants.

To explore the cause(s) of splicing failure in *Bz2* transcripts, we have compared the splicing patterns of B139 and W23 plants grown side by side in the same soils and environments to determine the relative impact of environmental conditions and genetic background on the accumulation of unspliced *Bz2* RNA. Because *Bz2* has a single intron, it is a favorable gene for these studies: all unspliced messages are identical and identifiable as a single molecular species. The splicing behavior of *Bz2* is examined both in mature plants and in seedlings grown in changing environmental circumstances.

Also, to gain an understanding of the mechanisms of splicing failure and the fates of unspliced message in plant cells, we have demonstrated the utility of a maize cell culture system for studying the splicing of *Bz2* RNA and the regulatory role of RNA processing in *Bz2* gene expression.

MATERIALS AND METHODS

Plant Material

Both the standard inbred W23 (*B Pl*) and B139 (our designation of a purple *Mutator* line originally obtained from D.S. Robertson, *B pl*) maize (*Zea mays* L.) stocks contain all the genes required for anthocyanin synthesis. Husk tissues were obtained from plants grown in the Stanford field during the summer of 1990. Growth chamber conditions for seedling tissue preparations were 16 h light/8 h dark, 28°C for 9 d. Heat-stressed seedlings received a 1-h exposure at 41°C after the 9-d growth regimen at 28°C.

Electroporation

Protoplasts were prepared from cultured BMS cells and electroporated with DNA constructs as previously described (10). DNA for electroporation was twice purified by CsCl gradient centrifugation (26). Twenty micrograms each of pR and pC1, plasmids encoding regulatory factors required for anthocyanin expression under the control of the 35S cauliflower mosaic virus promoter (2), were co-porated with 40 μg of pBz2 (21). pBz2 is a genomic clone of the *Bz2* gene including all native regulatory features (from 704 bp upstream to 92 bp downstream of the translation unit). Activated expression from the endogenous and introduced *Bz2* genes was measured by RNase protection analysis.

RNA Preparations

Husk and seedling tissue was harvested, immediately frozen in liquid N_2, and stored at −80°C. For RNA extraction, the tissue was homogenized to a fine powder with a mortar and pestle and then RNA was purified as described (16), except that the first centrifugation was eliminated. Selection of poly(A)$^+$ RNA was by affinity to Hybond mAP paper (Amersham Corp.) according to the manufacturer's instructions. Protoplast RNA was prepared from cells 24 h after electroporation as described in Luehrsen and Walbot (17).

RNase Protection

Because the probe, pSS390 (the internal *Sst*I-*Sst*II fragment of *Bz2*) (21), overlaps the entire sequence of the *Bz2* intron, it protects a 383-bp fragment of unspliced *Bz2* mRNA and two fragments (245 and 60 bp) from spliced *Bz2* mRNA. Labeled probe (5×10^5 cpm [Cerenkov]) was hybridized to 5 to 15 μg of total cellular RNA at 45°C overnight for each protection assay as described (17). Samples were digested sequentially with RNase H (Bethesda Research Laboratories), followed by RNases A and T1. The protected fragments were treated with 0.2% (w/v) SDS and 8 units/mL proteinase K (Bethesda Research Laboratories), extracted with phenol/chloroform, and ethanol precipitated. RNA hybrids were denatured, and the labeled fragments were separated on a 6% (w/v) acrylamide gel. The gels were fixed with 10% (v/v) methanol/10% (v/v) acetic acid and autoradiographed with or without (for densitometry) two intensifying screens for 1 to 15 d. Densitometry of autoradiograms was performed with an LKB laser scanner.

RESULTS

Unspliced *Bz2* mRNA in Field-Grown Plants

The accumulation of unspliced *Bz2* mRNA was initially found to be more pronounced in field-grown B139 plants than in greenhouse-grown W23 plants, leaving unresolved whether splicing failure resulted from either the genetic or the environmental differences between these plants. To define the relevant differences, B139 and W23 plants were grown side by side in the field; plants grown in the same greenhouse were also compared. RNA samples collected from husk tissue at similar developmental stages (prior to silking) were examined by RNase protection analysis. Unspliced and spliced *Bz2* transcripts are distinguished in this assay by hybridization to a labeled antisense RNA probe, pSS390, that overlaps the 78-bp intron, as diagrammed in Figure 1. Unspliced *Bz2* RNA is protected by the full-length probe and survives the RNase treatments intact, yielding a 383-bp protected fragment upon acrylamide gel separation. Spliced *Bz2* mRNA is protected only by the exon sequences of the labeled probe, and the looped-out intron of the pSS390 probe is digested by the RNase treatments. Two protected fragments of 245-bp (exon 1) and 60-bp (exon 2) are produced; the smaller fragment was resolved by the gel system (21) but is not included in the figures.

In tissue harvested from greenhouse-grown plants, the accumulation of unspliced message is similar in both the B139 and W23 lines (Fig. 1). Approximately 5% of the *Bz2* mRNA is detected in the unspliced form for both tissue sources. In contrast, B139 plants grown simultaneously with and adjacent to W23 plants in the field accumulate significantly greater relative amounts of unspliced message, 18% of total *Bz2* mRNA, as compared with spliced *Bz2* mRNA, whereas less than 6% of *Bz2* mRNA is unspliced in the W23 plants (Fig. 1). A repetition of this experiment showed that W23 plants consistently accumulate ≤ 5 to 10% unspliced *Bz2* message, whereas the levels of unspliced message in field-grown B139 plants range from 18 to 60% of the total *Bz2* message (21). These data indicate that there may be a genetic predisposition for *Bz2* splicing failure in the B139 line that is exhibited in field-grown plants. We also interpret these results as evidence that unspliced transcripts are stable enough to accumulate in maize.

To address the possibility of local, *cis*-sequence effects on splicing behavior in B139 and W23 plants, it was important to compare the nucleotide sequences of the intron and surrounding exon sequence of the two *Bz2* alleles to determine if base changes might explain the differences in splicing failure. Specific sequences can be important in determining the splicing efficiency of a particular intron; for instance, the splice junction must have a GU at the 5′ border and an AG at the 3′ border for accurate intron recognition and exon

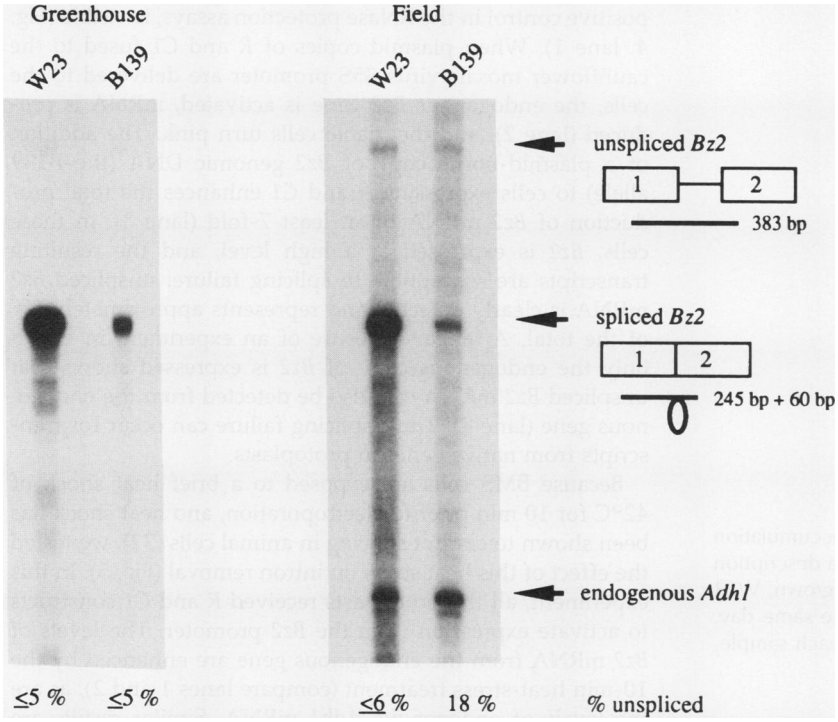

Figure 1. RNase protections of RNA samples from husks of two lines of maize (B139 and W23) with the intron-spanning *Bz2* probe, pSS390, shows that the relative levels of unspliced *Bz2* transcripts are different in field-grown tissue of these two lines but not in greenhouse-grown tissue. Protected fragments (indicated by arrows) of unspliced and spliced *Bz2* transcripts are 383 bp and 245 bp, respectively. The 152-bp *Adh1* protected fragment (18) was used as an internal standard in field-grown samples.

joining (3). Additional longer sequence motifs at the intron/exon borders and in the internal regions of the introns are known to be required for efficient splicing in yeast and mammalian introns, and it is expected that longer sequence motifs also influence splicing efficiency and accuracy in plants (12). Polymerase chain reaction amplification was used to obtain the W23 intron of *Bz2* and two cloned representatives were sequenced (data not shown). These sequences precisely matched the cloned *Bz2* gene of B139 in the intron region and in the 20 bases of surrounding upstream and downsteam exon sequence (21). In addition, RNase protection analysis is sensitive enough to detect single mismatched bases at U, C, and G residues and none have been detected in the transcripts with these and other probes (data not shown). Hence, the two genes appear to be very similar, if not identical. In addition, the only splicing mutants identified that disrupt intron removal result from mutations in the first six bases of the 5′ splice site consensus sequences, the last two bases of the 3′ splice site sequences, or within the intron (23); it is unlikely that distant upstream or downsteam gene regions would affect *Bz2* splicing efficiency in the B139 line.

Not only is there a significant difference in accumulation of unspliced *Bz2* mRNA in B139 versus W23 plants in field-grown plants, there is also a significant difference in the absolute levels of *Bz2* message; the message level is much lower in B139 tissue, and these husk tissues are paler purple than the W23 tissues. To examine the possibility that the amount of unspliced message in a particular genotype is related to absolute levels of *Bz2* mRNA, husk tissue samples were extracted from field-grown W23 plants throughout the day (Fig. 2). Absolute levels of *Bz2* mRNA showed a diurnal fluctuation. The level of spliced (or "mature") message was low in the morning (10 AM), increased by approximately 6-fold at midday (2 PM), and decreased in the early evening (6 PM). The tissue was harvested on a slightly overcast day that created a very consistent thermal and moisture environment for the plants; the temperature was always between 21 and 24°C. This is important because heat stress has been shown to disrupt splicing in *Drosophila* and yeast cells (30, 31). Despite the fluctuations in *Bz2* mRNA throughout the day, the ratio of unspliced/spliced mRNA was always the same in W23. This experiment was repeated, including samples throughout the evening and night, and comparable results were obtained (data not shown). The accumulation of unspliced message does not appear to be related either to absolute mRNA abundance or to the time of day.

Unspliced *Bz2* Message in Seedlings

There are so many variables that could affect the physiological status of a field-grown plant that it is difficult to establish a causal relationship between environmental conditions and intron splicing efficiency. To simplify the problem, we used chamber-grown maize seedlings to assay the production of unspliced *Bz2* mRNA. RNA samples were extracted from seedling coleoptile and mesocotyl tissue following 9 d at 28°C (Fig. 3, lanes 1 and 2). Although unspliced *Bz2* mRNA levels are low (≤10%, for both the W23 and B139 genotypes), unspliced message is clearly accumulated in W23 plants grown under nonstress conditions (lane 2). Unfortunately, the levels of *Bz2* mRNA are too low in seedling tissue from the B139 line to detect reliably the low levels of unspliced message (lane 1). RNA was also prepared from seedlings exposed to a 1-h treatment at 41°C following the 9-d constant 28°C growth regimen (lanes 3 and 4). Cooper and Ho (6) reported that maize seedlings exposed to 40°C tem-

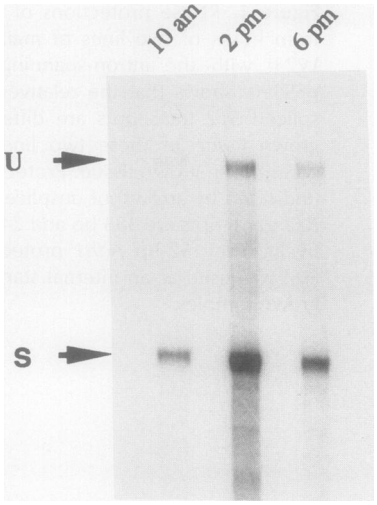

Figure 2. Spliced (S) and unspliced (U) *Bz2* message accumulation was detected by RNase protections (refer to Fig 1. for a description of probes used) using 15-μg RNA samples from field-grown, W23 husk tissue extracted at 10 AM, 2 PM, and 6 PM on the same day. Unspliced *Bz2* message accumulation is the same in each sample, representing 16% the level of spliced message.

peratures for longer than 20 min rapidly initiate a heat-shock response as exhibited by the production of the hsps and a decline in normal protein synthesis. Because *Bz2* is not a heat-shock gene, the heat-stress treatment dramatically diminishes the levels of *Bz2* transcript (i.e. in W23 seedlings grown at 41°C, the level of mature *Bz2* message is 10% of the amount seen in plants grown at 28°C, lanes 2 and 4). Unspliced message does not predominate at 41°C in these tissues (levels of unspliced *Bz2* message greater than 15 to 20% of the total *Bz2* mRNA would be detected by this assay, lanes 3 and 4), indicating that thermal stress does not significantly disrupt the splicing of *Bz2* in maize seedlings.

Unspliced *Bz2* mRNA in Plant Cells

Because a deeper examination of the physiological aspects of splicing failure is limited by our fragmentary knowledge of the molecular requirements for splicing in plants, we initiated a study of splicing failure using maize cell cultures. The most common maize cell culture line, BMS, is colorless and does not express anthocyanin structural genes. To study splicing of the native *Bz2* gene in these cultures, the anthocyanin pathway must be activated. Bodeau and Walbot (2) discovered that BMS cells could be conditioned to express anthocyanin structural genes if the two regulatory genes, *R* and *C1*, are delivered to the cells. Within 24 h, approximately 95 to 100% of the viable cells turn pink as a result of activated expression from the endogenous anthocyanin structural genes, including *Bz2*. We confirm that pR and pC1 activate *Bz2* transcription from both the chromosomal locus and a plasmid gene copy (Fig. 4).

RNA samples prepared from electroporated protoplasts were analyzed by the RNase protection assay. In control cells electroporated with no plasmid DNA, no *Bz2* mRNA is produced, although the endogenous *Adh1* RNA, included as a positive control in the RNase protection assays, is visible (Fig. 4, lane 1). When plasmid copies of *R* and *C1* fused to the cauliflower mosaic virus-35S promoter are delivered to the cells, the endogenous *Bz2* gene is activated, mRNA is produced (lane 2), and the viable cells turn pink. The addition of a plasmid-borne copy of *Bz2* genomic DNA (the B139 allele) to cells expressing *R* and *C1* enhances the total production of *Bz2* mRNA by at least 7-fold (lane 3). In these cells, *Bz2* is expressed at a high level, and the resulting transcripts are susceptible to splicing failure: unspliced *Bz2* mRNA is clearly detected and represents approximately 5% of the total. A longer exposure of an experiment in which only the endogenous copy of *Bz2* is expressed shows that unspliced *Bz2* mRNA can also be detected from the endogenous gene (lane 4). Thus, splicing failure can occur for transcripts from native genes in protoplasts.

Because BMS cells are exposed to a brief heat shock of 42°C for 10 min prior to electroporation, and heat shock has been shown to disrupt splicing in animal cells (32), we tested the effect of this heat stress on intron removal (Fig. 5). In this experiment, all the protoplasts received *R* and *C1* constructs to activate expression from the *Bz2* promoter. The levels of *Bz2* mRNA from the endogenous gene are enhanced by the 10-min heat-stress treatment (compare lanes 1 and 2), as are the levels of endogenous *Adh1* mRNA. Similar results are obtained when a plasmid-borne copy of *Bz2* is included (lanes 3 and 4). No unspliced *Bz2* message was detected in any of the samples from this batch of protoplasts. Therefore, the heat-stress treatment prior to electroporation appears to have a general stimulatory effect on RNA production and does not dramatically upset the splicing machinery of the protoplasts. These data are consistent with our finding (Fig. 3) that

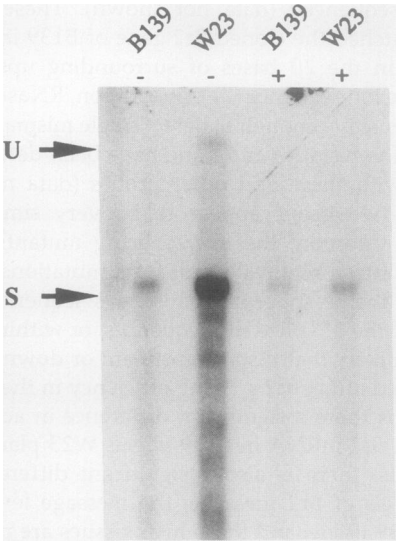

Figure 3. Unspliced *Bz2* transcripts (U) were observed in RNA samples from W23 seedlings grown in a growth chamber for 9 d (lane 2), but overall levels of *Bz2* RNA were too low in B139 seedlings to verify the production of unspliced message (lane 1) using RNase protections (refer to Fig 1. for a description of probes used). A 1-h treatment (+) of 41°C depressed the levels of spliced (S) and unspliced (U) *Bz2* message in both lines.

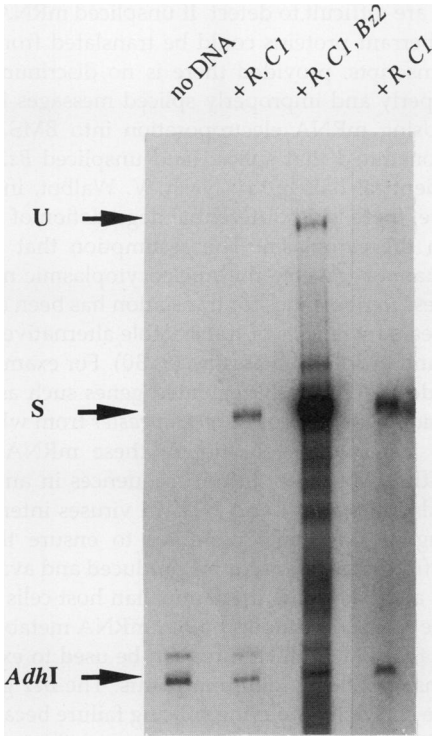

Figure 4. The accumulation of *Bz2* mRNA in BMS protoplasts requires the presence of *R* and *C1* as detected by RNase protections (refer to Fig 1. for a description of probes used). Endogenous *Bz2* mRNA is absent in cells that are not expressing *R* and *C1* (compare no DNA lane with +*R*, *C1* lane). Unspliced *Bz2* transcripts are accumulated in protoplasts co-porated with *R*, *C1*, and a plasmid copy of *Bz2*, pBz2. A longer exposure of a comparable experiment shows that unspliced *Bz2* message (U) is also produced from the endogenous, BMS, copy of the *Bz2* gene (far right lane).

thermal stress in maize seedlings does not cause the accumulation of unspliced *Bz2* message. Because *Drosophila* and yeast cells experience a near total disruption of spliceosome activity following a heat-shock treatment so that essentially no spliced, mature message accumulates, it can be inferred that the disturbance, if any, of the plant cell-splicing machinery by this level of heat stress is very mild by comparison.

Although protoplasts are stripped of their cell wall and might be considered to be highly stressed, previous studies of pre-mRNA splicing of introduced heterologous gene constructs show that accurate, but often inefficient, splicing does occur (11, 12, 17, 18). In contrast, the splicing of *Bz2* mRNA can approach 100% efficiency in BMS cells (Fig. 5). These data suggest that the range of splicing efficiency found for the *Bz2* gene in protoplasts (up to 100%) is similar to the range found in intact plants. It is interesting to note that the splicing efficiency for *Bz2* mRNA in these protoplasts is consistently higher than has been found for any of the chimeric gene constructs examined in this laboratory or others. In previous studies, constructs created using combinations of endogenous and heterologous gene fragments with native (17, 18) and synthetic (11, 12) introns have been used to define the sequences required for splicing. In most cases, the efficiency of splicing is 40 to 70%, lower than would be expected for a "normal" intron. Despite splicing inefficiency for these constructs, the relative importance of intron mutations can be confirmed by measuring the increase or decrease in the efficiency of splicing compared with rigorously defined controls.

DISCUSSION

Because intron removal is a required step in the maturation of many transcripts, the detection of abundant unspliced mRNA in plants is surprising. We have demonstrated that unspliced *Bz2* mRNA accumulates in mature maize plants (Fig. 1 and ref. 21), in seedlings grown in a growth chamber (Fig. 3), and in BMS cells (Fig. 4). All of the mature RNA detected is accurately spliced, indicating that the *Bz2* introns and exons do not contain cryptic splice junctions. Because the relative proportion of unspliced to spliced message is variable, we propose that the tendency to accumulate especially high levels of unspliced *Bz2* mRNA can result from a genetic predisposition or environmental cues or a combination of both.

For example, although levels of unspliced *Bz2* mRNA are

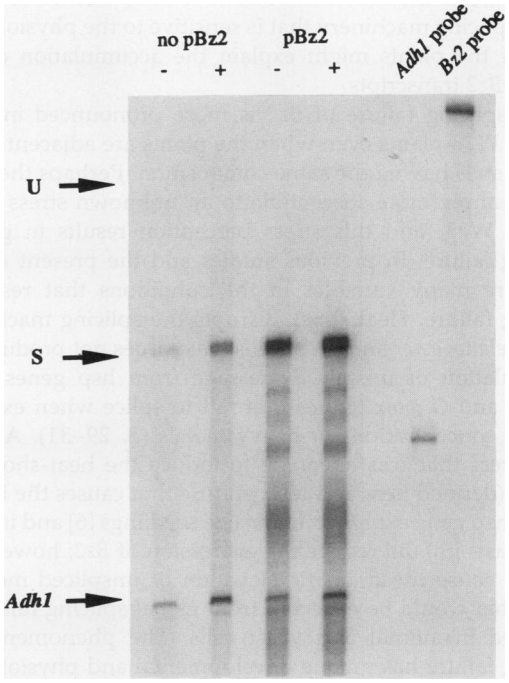

Figure 5. Splicing of *Bz2* transcripts can be very efficient in electroporated BMS protoplasts. In this experiment, RNase protections (refer to Fig 1. for a description of probes used) of RNA samples from protoplasts electroporated with *R* and *C1* (lanes 1 and 2) or with *R*, *C1*, and *Bz2* (lanes 3 and 4) accumulate no unspliced *Bz2* message (U) despite a 10-min 42°C heat-stress treatment (+ lanes) prior to DNA delivery; cells represented in (−) lanes received no heat stress. The antisense RNA probes for *Bz2* (pSS390, 492 nucleotides) and *Adh1* (pAdh1, 202 nucleotides [17]) are shown in the far right lanes.

similar in husk tissues from B139 and W23 plants grown in the greenhouse, husk tissues from the B139 plants grown in the field consistently accumulate 18 to 60% unspliced *Bz2* mRNA whereas W23 plants accumulate less, approximately 6% of the total *Bz2* mRNA. There could be several explanations for the predisposition of B139 plants to splicing failure. One possibility is that these plants are more susceptible to an unknown variable (such as metal poisoning) present in the field. Alternatively, the high accumulation of unspliced *Bz2* message could be related to the lower overall expression of *Bz2* in the paler purple B139 plants: the regulatory alleles known to control anthocyanin production in B139 and W23 plants are *B pl* and *B Pl*, respectively. Third, the high accumulation of unspliced message in the B139 plants could result from a posttranscriptional regulatory mechanism that affects the rate or efficiency of the splicing reaction required to produce mature *Bz2* mRNA. A fourth possibility, that *cis*-acting nucleotide substitutions affect splicing, appears unlikely because the W23 and B139 introns and immediate flanking sequences are identical.

Our data indicate that the ratio of unspliced to spliced *Bz2* is independent of mRNA amount per se. Plants sampled throughout a diurnal cycle contain maximal *Bz2* mRNA at midday followed by lower levels as the evening approaches. Because unspliced/spliced *Bz2* message ratios are consistent throughout a diurnal cycle, the levels of unspliced RNA are not related to the absolute levels of the *Bz2* mRNA. A defect in the splicing machinery that is sensitive to the physiological state of the plants might explain the accumulation of unspliced *Bz2* transcripts.

The splicing failure of *Bz2* is more pronounced in B139 than in W23 plants even when the plants are adjacent in the field in soils having the same composition. Perhaps the B139 line is simply more susceptible to an unknown stress factor than is W23, and this stress perception results in greater splicing failure. In previous studies and the present report, there are many variables in the conditions that result in splicing failure. Heat shock disrupts the splicing machinery in *D. melanogaster* and yeast cells, yet it does not produce the accumulation of unspliced message from hsp genes in *P. hybrida* and *G. max* tissues that fail to splice when exposed to high concentrations of heavy metals (8, 29–31). A 41°C heat stress that was adequate to induce the heat-shock response (defined here as a temperature that causes the induction of hsp gene expression in maize seedlings [6] and in BMS protoplasts [5]) did reduce the expression of *Bz2*; however, it did not cause the dramatic elevation of unspliced message levels that would be expected from global splicing failure as observed in animal and yeast cells. The phenomenon of splicing failure has unique developmental and physiological correlates in each organism studied. The detection of unspliced message in seedlings grown in a growth chamber (Fig. 3) may present an opportunity for exploring physiological parameters in a controlled environment that affect splicing behavior in plants.

At present, the examples of splicing failure appear to reflect a loss of efficiency of a normal process rather than a regulated event. The accumulation of even 5% unspliced mRNA in a cell, a typical amount for *Bz2* in plant tissues, is significant in comparison with fungi and animals, in which unspliced transcripts are difficult to detect. If unspliced mRNA exits the nucleus, aberrant proteins could be translated from the unspliced transcripts, provided there is no discrimination between properly and improperly spliced messages in the cytoplasm. Using mRNA electroporation into BMS cells, we have demonstrated that spliced and unspliced *Bz2* mRNAs have an identical half-life (J. Nash, V. Walbot, in preparation); hence, there is no differential degradation of unspliced mRNAs in the cytoplasm. The assumption that unspliced messages cannot traverse the nucleocytoplasmic membrane to find access to ribosomes for translation has been disproven in recent years by reports of translatable alternatively spliced messages and unspliced messages (9, 30). For example, there are many developmentally regulated genes such as the sex-determination gene, *dsx*, of *D. melanogaster* from which transcripts are translated even though these mRNAs include sequences that would be "intron" sequences in another cell type (4). Also, the HIV-I and HTLV-I viruses interfere with the splicing of their own messages to ensure that some unspliced, full-length transcript is produced and available for translation and packaging in mammalian host cells (7).

As a base for future studies on *Bz2* mRNA metabolism, we show here that BMS cell cultures can be used to explore the mechanisms of splicing failure in plants. The *Bz2* gene is an appropriate choice for studying splicing failure because it has only one intron and this intron is susceptible to splicing failure in some conditions. Splicing failure would be more difficult to assess in a gene such as *Adh1*, which has nine introns and could produce hundreds of different unspliced message species of varying sizes. BMS cell cultures accumulate unspliced *Bz2* messages from endogenous alleles (Fig.4, lane 4) and from introduced genes (Fig. 4, lane 3). Sometimes, as is true for mature plants, these cell cultures can splice *Bz2* transcripts with nearly 100% efficiency (Fig. 5). A criticism of using BMS cells to study splicing behavior has been that low splicing efficiencies are observed. From our examination of the splicing behavior of the endogenous and introduced

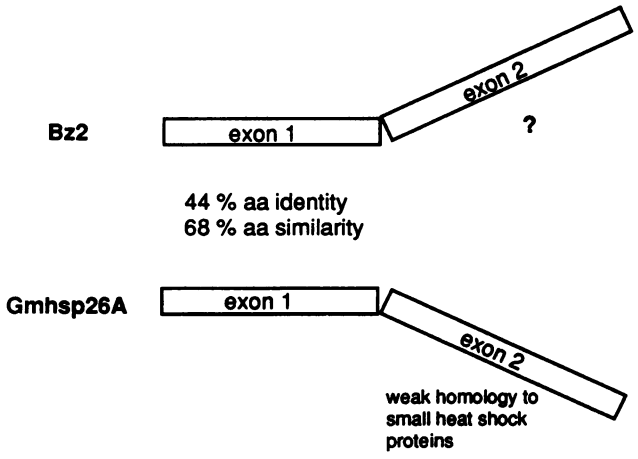

Figure 6. Protein sequence comparison of *Bz2* and *Gmhsp26-A*. Amino acid sequence similarity is indicated for exon 1, whereas exon 2 shares no homology. The weak homology of *Gmhsp26-A* to animal, but not plant, small hsp genes is 29% (8). *Bz2* has no significant homology to any hsp, nor is it heat inducible (Fig. 3).

Bz2 gene in BMS cells, it appears that the low splicing efficiencies in previous studies may have resulted from the use of chimeric gene constructs rather than being a property of the cell cultures, per se. In fact, a mild heat-stress treatment of 42°C for 10 min prior to electroporation did not disrupt the accuracy or near-perfect splicing efficiency of transcripts subsequently produced by the protoplasts (Fig. 5). The BMS cell culture system will thus be useful in future studies to examine the production and fate of unspliced mRNAs in maize cells.

Another question is whether transcripts subject to splicing failure share common features. In comparing *Bz2* to the genes listed in Table I, we discovered that the structure of the *Gmhsp26-A* gene is very similar to that of *Bz2* (Fig. 6). Both transcripts contain a single, centrally located intron and encode a putative 26-kD protein. The first 85 amino acids of exon 1 share a 44% identity and a 68% similarity. Both introns contain an in-frame stop codon; unspliced transcripts would result in similarly sized translation products of approximately 14 kD. The second exons are not similar at the level of DNA or protein sequence. Although the structural similarity of the first exon region of these proteins is very high, the function of neither protein has been determined, and it remains unknown whether these transcripts share structural features related to the tendency toward splicing failure.

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