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The Maize Repressor-like Gene *intensifier1* Shares Homology with the *r1/b1* Multigene Family of Transcription Factors and Exhibits Missplicing

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The recessive mutation *intensifier1* of maize apparently causes an overall increase in flavonoid production in the aleurone. The mechanism by which this is achieved is not understood. We have succeeded in cloning the *intensifier1* gene by transposon tagging with *Suppressor-mutator* and found, by sequence analyses, that it shares homology with known transcription factors in the anthocyanin pathway, in particular the *r1/b1* multigene family in maize. Two cDNAs and a genomic clone were completely sequenced, and together they showed that the transcripts were misspliced. The frequency of missplicing was investigated by polymerase chain reaction analyses and sequencing of the individual introns. These studies indicate that very little functional transcript was made. Indeed, missplicing may be a mechanism for reducing the levels of a transcription factor that, when present, acts as a repressor of anthocyanin biosynthesis.

INTRODUCTION

In 1924, A.C. Fraser described a recessive mutation, *intensifier1* (*in1*), that enhanced kernel pigmentation. If all of the other dominant factors required for pigmentation were present, then the homozygous mutant kernels were black, as opposed to the deep purple of wild-type kernels. In the presence of the recessive factor *pr1*, homozygous *in1* kernels were black rather than light red, as they were in *In1*; *pr1* kernels.

The major pigments in wild-type maize kernels are pelargonidin 3-glucoside and cyanidin 3-glucoside. In different backgrounds, the ratio of cyanidin to pelargonidin varies from 25 to 4 (Kirby and Styles, 1970; Chen, 1973; Larson et al., 1986). The effect of the *pr1* mutation is to block flavonoid hydroxylation such that the ratio of cyanidin to pelargonidin is reversed to 0.1 (Kirby and Styles, 1970; Larson et al., 1986). Presumably, a gene product with a duplicate function is able to synthesize the small amount of cyanidin that is still found in mutant kernels. Similarly, the ratios of cyanidin to pelargonidin are 6.6 in *Pr1* and 0.13 in *pr1* kernels that are homozygous for the *in1* mutation (Chen, 1973). Although the amounts of flavonoids present in *in1* endosperms were not reported, we presume that they were higher than in *In1* endosperms.

A recessive mutation of the gene encoding chalcone synthase, *c2*, normally blocks pigment synthesis entirely and leads to colorless kernels. However, in the presence of homozygous *in1*, the kernels are pale colored. Segregation analysis demonstrated that this pigmentation was dependent on the presence of a functional allele at the *white pollen1* (*whp1*) locus (E.H. Coe, informal communication, 1982 Maize Genetics Cooperation Newsletter, Vol. 56, pp. 49 to 50). *whp1* was subsequently shown to encode a duplicate chalcone synthase gene (Franken et al., 1991).

The *bronze1* (*bz1*) gene encodes uridinediphosphoglucose:flavonoid 3-*O*-glucosyltransferase (UFGT), which catalyzes one of the last steps in the anthocyanin biosynthetic pathway (Dooner and Nelson, 1977; Larson and Coe, 1977). The *in1* mutation elevates the level of UFGT twofold (Klein and Nelson, 1983). Therefore, the *in1* mutation appears to control the overall levels of flavonoids rather than to suppress mutant phenotypes directly. One means of achieving this effect would be for the *in1* gene to have a regulatory role. We hypothesize that functional alleles of *in1* repress the synthesis of anthocyanin precursors and that synthesis is derepressed in the presence of mutant *in1* alleles.

In this study, we describe the cloning of the *in1* gene by transposon tagging and show that the gene has homology with a class of *myc* homologs that are involved in the regulation of anthocyanin biosynthesis. The *in1* gene has eight introns; three are seldom processed correctly. If functional alleles of the gene have a repressive effect as hypothesized, then high concentrations of the gene product may be inhibitory to anthocyanin

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biosynthesis. The production of few, correctly processed mRNAs might be a mechanism ensuring that normal regulation is not overcome.

RESULTS

Targeted Tagging of *in1* and Cloning of *in1-m14::Spm*

Targeted mutagenesis using the transposon *Spm* (*Suppressor-mutator*; also known as *Enhancer*) produced transpositions into the *in1* locus, using both linked and unlinked donors. We expected the phenotype of a kernel with the desired mutation to have a black aleurone with reddish pink sectors (*In1-m[utable]; pr1 → In1; pr1*). An example is shown in Figure 1. Table 1 lists the mutants we obtained and the transposon donor stocks that were used. Many of the *in1-m* alleles were obtained by using *opaque2 (o2)-m20::Spm* as the linked transposon donor. The *o2* locus maps ~4 centimorgans (cM) distal to *in1* on the short arm of chromosome 7, and transpositions of *Spm*, like *Activator/Dissociation*, are transposed frequently to nearby locations (McClintock, 1962; Peterson, 1970). However, we were also able to obtain mutations by using three unlinked donors: *In1; pr1; c1-m5::Spm* and *In1; pr1; sh1 bz1 wx1; Spm* and *In1; pr1; Spm*. Genetic crosses with appropriate tester stocks showed that the transposons associated with the locus were either an autonomous *Spm* or a defective *Spm* (*dSpm*). Although it may seem surprising that we obtained a preponderance of *dSpm* insertions, we cannot rule out the possibility that other *dSpm*s were activated and transposed into the target site. These could not be differentiated from defective transpositions from *o2-m20::Spm*. Knowing the nature of the element at the locus allowed us to use segregation analysis to identify a unique 10.6-kb SalI genomic fragment associated with *in1-m14::Spm* that hybridized with an *Spm*

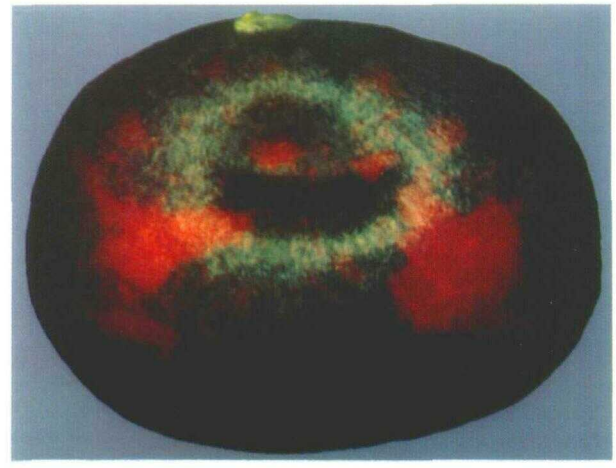

Figure 1. Phenotype of *in1-m14::Spm; pr1* Kernel.

in1-m14::Spm carries an autonomous *Spm* at the locus. The mutant *in1-m14::Spm* aleurone sectors are black; *In1* revertant sectors caused by the excision of *Spm* from the locus are red. The transposon donor used to obtain this mutation was *o2-m20::Spm In1; pr1. o2-m20::Spm* maps 4 cM distal to *in1* on the short arm of chromosome 7.

Table 1. *in1-m* Alleles Obtained from Transposon Mutagenesis by Using *Spm* as the Transposon Donor[a]

Allele No.	Female	Male	Insertion[b]
in1-m1	W22 *in1; pr1*	*c1-m5::Spm; In1; pr1*	*Spm*
in1-m2	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*[c]
in1-m3	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m4	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m5	W22 *in1; pr1*	*In1, pr1; sh1 bz1 wx1; Spm*	*dSpm*
in1-m6	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*Spm*
in1-m7	W22 *in1; pr1*	*In1, pr1; Spm*	*Spm*
in1-m8	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m9	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m10	*o2-m20::Spm In1; pr1*	W22 *In1; pr1*	*dSpm*
in1-m11	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m13	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*dSpm*
in1-m14	W22 *in1; pr1*	*o2-m20::Spm In1; pr1*	*Spm*

[a] Both *c1-m5::Spm* and *o2-m20::Spm* carry an autonomous *Spm* at the locus. In the cases of *in1-5* and *in1-7*, the donor was carrying an unmapped *Spm*. The total number of kernels screened was $>7 \times 10^5$.

[b] The genetic characterization of the transposon at the locus was performed in crosses using the tester *in1; pr1; C1 sh1 bz1 wx1-m8::dSpm; – Spm*.

[c] In these particular *dSpm*s at *in1*, the alleles are stable (nonmutable) in the absence of the autonomous element. Reintroduction of *Spm* by crossing restores mutability.

probe. A junction fragment flanking the *Spm* insertion proved to be a single-copy probe that allowed us to clone an *In1* genomic allele and to map the gene.

Restriction Fragment Length Polymorphism Mapping of an *in1* Fragment

By using the probe derived from *in1-m14::Spm*, we determined an allele distribution of a restriction fragment length polymorphism in the recombinant inbred families CO×Tx and Tx×CM. A comparison of this allele distribution with the Brookhaven National Laboratory Recombinant Inbred Database of mapped markers showed that the probe maps to the position expected for *in1* at 4 cM proximal to *o2* on the short arm of chromosome 7.

cDNA Cloning and Analysis

Three positive clones for *in1* were purified from the λ cDNA library of W22 *In1*, full-color endosperm. One had an insert of only 200 bp and was eliminated from further consideration. The lengths of the inserts in the other two clones were estimated at 2500 and 2300 bp. Clone pBF277, the larger of the two cDNA clones, was selected for sequencing. After completing the sequencing and doing considerable rechecking, we were unable to derive an extended open reading frame (ORF) from this clone. The reason for this became apparent when pBF276, the shorter clone, was sequenced. By comparison, pBF277 was found to contain two additional inserted sequences and had deleted a portion of sequence present in pBF276. These alterations suggested that pBF277 was a partially spliced pre-mRNA transcript. However, it was not until the genomic sequence was completed and careful comparisons were made that the missplicing events in pBF277 became apparent.

pBF276, the cDNA clone believed to represent the correctly spliced transcript, is 2276 bp in length. The 5′ end was later extended an additional 34 bp by the rapid amplification of cDNA ends (RACE) procedure. Computer searches of nucleotide sequence data bases, translated into all six reading frames, revealed high homology with the *r1/b1* multigene family in maize and *delila* from Antirrhinum. Figure 2 shows the amino acid sequence of IN1 compared with B-PERU (Radicella et al., 1991), R-S (R-Self color; Perrot and Cone, 1989), LC (Leaf Color; Ludwig et al., 1989), SN (Scutellar Node; Consonni et al., 1992, 1993), and DEL1 (Antirrhinum Delila; Goodrich et al., 1992). Regions of homology occur at both the N and C termini, positions 1 to 220 and positions 468 to 647, respectively. A common preserved structural feature of all members of this family, including IN1, is the basic helix-loop-helix region (HLH) in the C-terminal portion of the coding sequence. All of the plant MYC proteins shown here lack homology with other members of this family in the N-terminal half of the basic region. The basic HLH region corresponds to the DNA binding protein dimerization domain of MYC oncoproteins. The major difference between IN1 and the other anthocyanin HLH proteins is a region located in the middle of IN1, between amino acids 220 and 467. Near the start of the RACE-extended cDNA there are two in-frame AUGs. The first is 42 bp from the start; the second, 90 bp from the start, is the more likely initiation codon, based on the extensive homology in this region with related sequences of the *r1/b1* family. In addition, the context of the second AUG codon, AGCCATGG, conforms more closely to the consensus eukaryotic start site, A/GXXATGG (Kozak, 1987). This gives an ORF of 685 amino acids.

Genomic Sequence, Introns, and Alternative Splice Sites

The sequence of the genomic clone from the *In1-W22* allele is 8.3 kb. The genomic sequence extends 224 bp upstream of the 5′ end of the cDNA sequence and 389 bp downstream of the 3′ end. Comparison of the genomic sequence with the cDNA sequence of pBF276 shows that there are eight introns. Its splicing pattern is represented by the solid lines of the diagram shown in Figure 3. Also shown are the alternative splicing patterns (dashed lines) associated with the other cDNA clone, pBF277. Intron 2′ contains an alternative 5′ donor site within itself that results in a 115-nucleotide larger exon 2. Intron 5′, on the other hand, can recognize an alternative 3′ acceptor site within exon 6 that deletes the first 88 nucleotides in exon 6. Finally, intron 7 is not spliced out in pBF277. Table 2 lists the lengths of each intron and their alternatives along with their 5′ and 3′ splice junctions and the percentage of AU base composition.

We wanted to determine how frequently these missplicing events occurred in the mRNA population and whether there were any other anomalous splicing events. cDNA was synthesized with reverse transcriptase primed with oligo(dT) and used as a polymerase chain reaction template with primers that flanked the introns (RT-PCR). Under the same amplification conditions, we obtained only full-length products equivalent to unspliced introns when the genomic clone λB137 was used as template for all seven primer pairs. The PCR products are displayed in Figure 4. All bands were cloned and sequenced to verify the identity of the products with expected sizes and to characterize the novel fragments. Table 3, which relates to Figure 4, gives the observed and expected lengths of the PCR products detected by electrophoresis and the estimated amount of product in each band.

We found that the same missplicing events observed in the cDNA clone pBF277 were confirmed. In fact, the alternative splice sites for introns 2 and 5, as well as the unspliced intron 7, seemed to be preferred to the correctly spliced introns. We also found that intron 1 was frequently unspliced. Only correctly spliced intron products were detected for introns 3 and 4 (amplified as a single unit), 6, and 8. Two unexpected bands of 96 and 108 bp also were observed for intron 2. When these

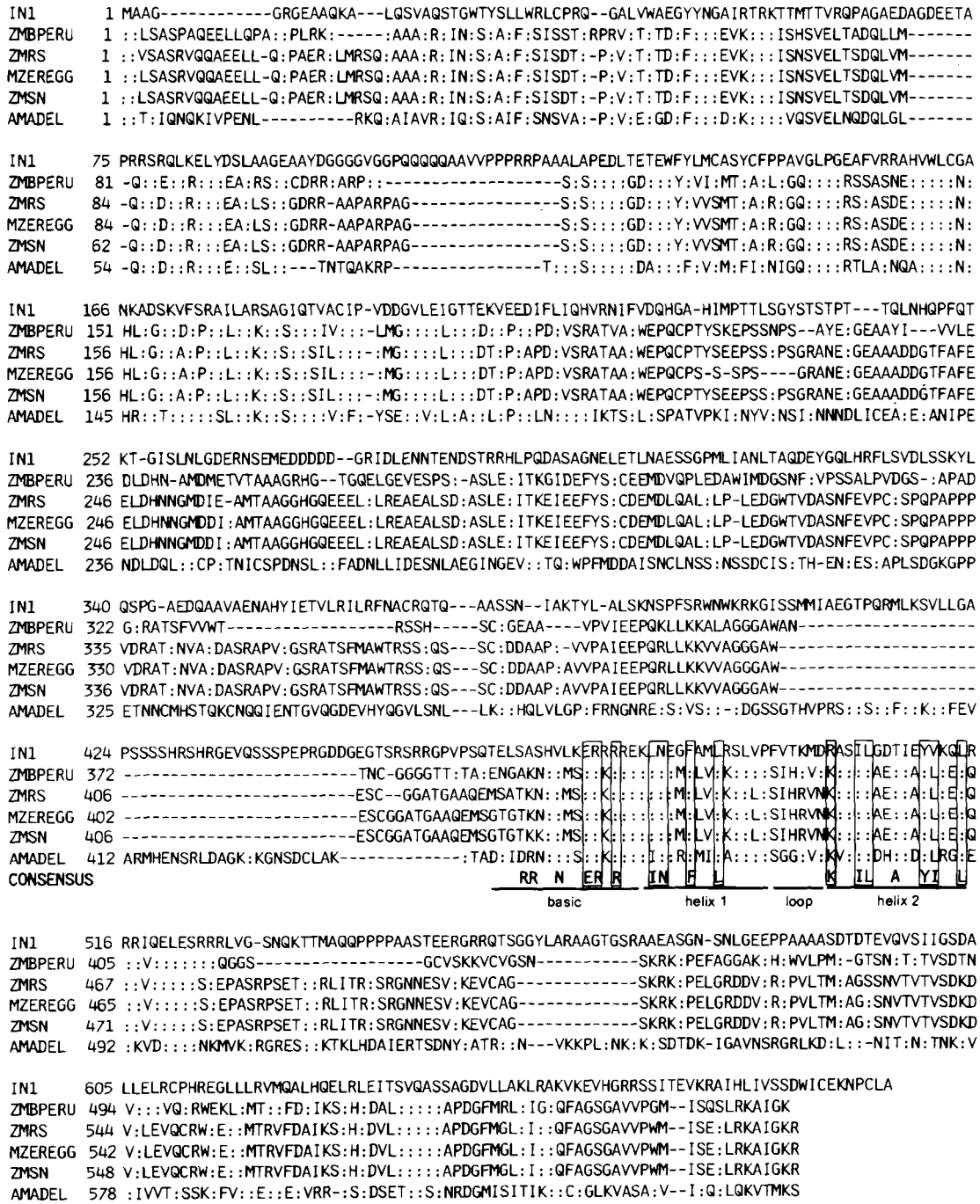

Figure 2. A Comparison of the Amino Acid Sequence of IN1 and Related Anthocyanin Homologs and a Comparison with the MYC Consensus Basic and HLH Regions.

IN1 shares homology in the first 220 amino acids at the N terminus, which includes a part of the region in B1 containing the transcriptional activation domain (up to position 359 in B1-PERU). A unique region of IN1 is found between amino acids 220 and 467. A basic region and adjacent HLH region are found beginning at approximately position 460. The boxes indicate regions of high homology with the MYC consensus sequence from all organisms (Kim et al., 1995). The IN1 amino acid sequence was inferred from the *In1-W22* DNA sequence, which is available from GenBank as accession number U57899. Dashes indicate gaps in the sequences; colons signify identity. IN1, INTENSIFIER1; ZMBPERU, *Zea mays* B1-PERU; ZMRS, *Z. mays* R-SELF COLOR; MZEREGG, MAIZE REGULATORY PROTEIN (or LC1 [RED LEAF COLOR]); ZMSN, *Z. mays* RED SCUTELLAR NODE; AMADEL, *Antirrhinum majus* DELILA.

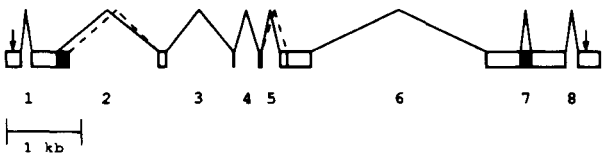

Figure 3. Diagram of the Known Transcribed Region of *in1* Showing Splicing Events.

The size of the transcribed region is 2723 bp. The open boxes represent exons; the filled boxes represent misspliced intron sequences. The solid lines indicate the correct splicing events; the dashed lines indicate the missplicing events. At the 5′ end of intron 2, there is an alternative splice site 115 nucleotides within the intron (filled box). This misspliced intron is called intron 2′. In intron 5, an alternative 3′ splice site 88 nucleotides within the following exon is frequently recognized (open boxed region that is part of the left end of exon 6). This misspliced intron is designated intron 5′. Finally, intron 7 is typically unspliced (filled box). Vertical arrows at the beginning and end of the transcript indicate the positions of the initiation and termination codons. The sizes of the individual exons (open boxes) are as follows: exon 1, 196 bp; exon 2 (open box only), 336 bp; exon 3, 97 bp; exon 4, 15 bp; exon 5, 57 bp; intron 6 (both open boxes together), 365 bp; exon 6 (open box immediately after intron 6), 461 bp; exon 7 (open box immediately before intron 8), 462 bp; exon 8, 272 bp.

were cloned and sequenced, the prominent 96-bp band was shown to contain part of the intron 2 sequence with three closely spaced deletions of 1069, 8, and 213 bp and an unknown 9-bp insertion. The band at 108 bp was a mixture of an unidentified sequence and a sequence similar to the 96-bp band with a duplicated PCR primer sequence at one end. We believe that these two bands of 96 and 108 bp are probably unusual artifacts of PCR amplification. The only other unaccountable product was a faint 184-bp band associated with intron 7. Several attempts to clone this fragment were unsuccessful.

Exon–intron boundaries of *lc1* have been published (Ludwig et al., 1989) and permit comparison with the genomic organization of *in1*. The two genes share homologous sites for six of their eight introns. *lc1* has an intron in the untranslated 5′ leader, which is absent in *in1*, and lacks an intron in the region containing the sixth intron of *in1*. Interestingly, this is the region in which the *in1* ORF lacks homology with the *r1/b1* multigene family.

Site of *Spm* Insertion and Excisions from *in1-m14::Spm*

The junctions of the *Spm* insertion in *in1-m14::Spm* were amplified by PCR and cloned. Sequencing showed that *Spm* had inserted in intron 2 at 109 bp from the 3′ splice site (Figure 5). *Spm* typically causes a 3-bp duplication of target sequence upon insertion (Schwarz-Sommer et al., 1985). In the case of *in1-m14::Spm*, there is a duplication of the bases ACA at the site of insertion.

Rearrangements in the gene correlated with changes in phenotype provide proof that we did not clone an *Spm* insertion into a gene simply linked with *in1*. Excision of *Spm* may leave all, none, or some variant of the 3-bp duplicated sequence behind (Schwarz-Sommer et al., 1985). We selected a number of independent possible revertants of *in1-m14::Spm* and obtained sequences of 11 (Figure 5). Five of the revertants produced clean excisions and six others left mutated sequences.

Effect of the *in1* Mutation on Transcription

Poly(A)$^+$ RNAs from normal and mutant endosperms were examined by gel blot filter hybridization. As shown in Figure 6A, endosperms of *In1; pr1* 30 days after pollination show a light major band at ~2.5 kb and two other minor bands at ~1.3 and 5 kb. In the mutant *in1; pr1* endosperms, there is at least one extremely faint band at 1.3 kb. As a control, the filter was stripped of radioactivity and rehybridized with a probe for *shrunken1 (sh1)*. This is shown in Figure 6B. The comparison indicates that there is very little *In1* mRNA produced in contrast to *sh1* mRNA.

Although no *In1* transcripts could be demonstrated in *in1* poly(A)$^+$ RNA by gel blot filter hybridization, we obtained specific amplification of *In1* sequences when mutant endosperm RNA was assayed by PCR amplification. In fact, the identical splicing patterns were observed for all eight introns for both *In1* and *in1* endosperm mRNA, indicating that a low level of transcription was occurring in the mutant.

Table 2. Characteristics of *in1* Introns

Intron No.[a]	Length (bp)	5′ Splice Site	3′ Splice Site	% AU
1	112	GG/GUACGC	CUCAUCAG/CG	44
2	1332	GG/GUAAAC	CGUUUCAG/GU	59
2′	1217	AG/GUCUCC	(Same)	60
3	851	GG/GUACGC	AAUAUCAG/AG	70
4	292	AG/GUAUUA	UAAUGCAG/AC	69
5	200	AG/GUCAGU	AGUUUCAG/GU	74
5′	288	(Same)	CCUCUCAG/GG	69
6	2325	AG/GUAACA	CCUUCCAG/GG	65
7	113	AG/GUGCGU	CACCGCAG/AU	50
8	143	AG/GUUAGC	UGCUGCAG/GU	58
Consensus[b]		AG/GUAAGU	UUUUGCAG/GU	

[a] Unprimed intron numbers represent correctly spliced introns. Intron 2′ indicates the misspliced intron 2 that recognizes another 5′ splice site; intron 5′ is the alternatively spliced intron 5 that uses another 3′ splice site. Intron 7 is often unspliced.

[b] The consensus sequences for 5′ and 3′ splice sites are shown.

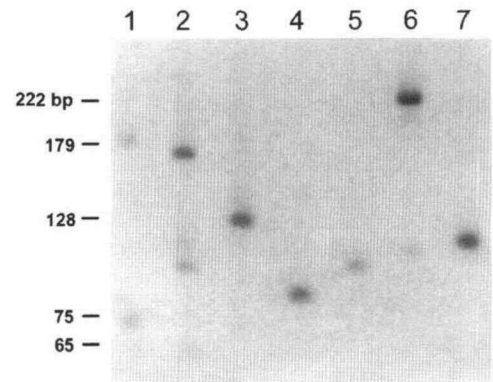

Figure 4. *RT-PCR Products of in1 Introns Using Total Endosperm RNA.*

The PCR products are as follows: lane 1, intron 1; lane 2, intron 2; lane 3, the product spanning introns 3 and 4; lane 4, intron 5; lane 5, intron 6; lane 6, intron 7; lane 7, intron 8. The PCR products were electrophoresed in a 3% MetaPhor gel and stained with ethidium bromide following electrophoresis. Imaging was with a CCD camera, and image reversal was performed electronically. Numbers at left indicate molecular length markers in base pairs.

DISCUSSION

Homology with HLH Transcriptional Regulators

Phenotypically, the effect of the *in1* mutation is the derepression of anthocyanin biosynthesis, which suggests that the wild-type allele encodes a transcription factor that acts as a repressor. Therefore, it was not surprising to find that the sequence of *in1* showed significant homology with a class of *myc* homologs in maize and Antirrhinum that are involved in anthocyanin biosynthesis.

The proteins encoded by these genes share homology with other *myc* homologs in having an abbreviated basic region and a HLH segment (Kim et al., 1995). In c-MYC and related proteins, the basic region has been shown to mediate DNA binding, whereas the HLH region affects oligomerization (Dang, 1991). However, unlike c-MYC, the anthocyanin HLH family of proteins lacks a leucine zipper domain on the C-terminal side of the HLH domain. The leucine zipper domain is sufficient for oligomerization in transcription factors related to the yeast GCN4 protein and is important for heteroduplex formation in the case of c-MYC (Dang, 1991).

IN1 also shares extensive homology with the other anthocyanin HLH proteins in the first 220 amino acids at the N terminus. This is part of a region that has been shown to encompass a transcriptional activation domain in the B1 protein (Goff et al., 1992). Goff and colleagues showed that deletions from the N terminus up to amino acid 359 are deleterious to transcriptional activation by the B1 protein. Surprisingly, the same study indicated that the basic and HLH domains of B1 were dispensable for the activation of a reporter gene fused to an anthocyanin structural gene promoter. They hypothesized that B1 achieved its effect through interaction with another DNA binding protein, namely, C1, which is encoded by a *myb* homolog.

The inhibitory effects of IN1 could be achieved through heterodimerization with other HLH transcription factors with subsequent disruption of functional DNA binding and/or by competition for binding of the activation domains of other transcription factors. For example, Id, a transcription inhibitor from mice, has an HLH region but lacks the basic motif and is able to form heterodimers with at least three HLH transcriptional regulators to inhibit binding to the muscle creatine kinase enhancer and thus inhibit transactivation (Benezra et al., 1990). The HLH protein encoded by Drosophila *extramacrochaetae* appears to exert its negative regulation of the *achaete* promoter in a similar fashion (Martinez et al., 1993). The HLH protein encoded by the *enhancer of split*, on the other hand, contains an intact basic motif and exerts its negative action through heterodimers that bind to a new sequence within the promoter of target genes (Oellers et al., 1994).

In maize, the *r1/b1* family of genes encodes HLH proteins involved in tissue-specific regulation of anthocyanin biosynthesis (Chandler et al., 1989; Ludwig et al., 1989). Although certain alleles of *b1* can function in the seed, *b1* is more typically expressed in vegetative parts of the plant. Its normal seed counterpart is *r1*. Given that there is a poorly organized oligo-

Table 3. Analysis of PCR Products of *int1* Introns Shown in Figure 4

Intron No.	Expected Lengths[a] (bp)	Observed Lengths[b] (bp)	Relative Amounts[c] (%)
1	183 (uns)[d]	184 (uns)	23
	74 (s)[e]	74 (s)	77
2	171 (2′)[f]	171 (2′)	98
		108 (artifact)	
		96 (artifact)	
		56 (s)	2
3 and 4	122 (s)	122 (s)	100
5	172 (s)	174 (s)	48
	84 (5′)[g]	84 (5′)	52
6	99	100	100
7	218 (uns)	220 (uns)	75
		184 (unknown)	
	104 (s)	106 (s)	25
8	113 (s)	113 (s)	100

[a] Expected lengths are the lengths of fragments expected from PCR that represent the correctly processed intron plus the length of primers and supplemental exon sequences.

[b] *Observed lengths are estimated lengths of PCR products detected by electrophoresis in 3% MetaPhor.*

[c] Relative amounts were determined (Sutherland et al., 1987) during the exponential phase of PCR.

[d] uns, unspliced intron product.

[e] s, spliced intron product.

[f] Misspliced intron 2.

[g] Misspliced intron 5.

In1 insertion site	...GTCAAAGC**ACA**AACATTT...
in-m14::Spm insertion	...GTCAAAGC**ACA**-*[Spm]* -**ACA**AACATTT...
in-m14Revertants 2, 3, 7, 12, and *16*	...GTCAAAGC**ACA**AACATTT...
in-m14Revertant 4	...GTCAAAGC**ACG**AACATTT...
in-m14Revertant 5	...GTCAAAGC**ACTG**AACATTT...
in-m14Revertant 6	...GTCAAAGC**AT**AACATTT...
in-m14Revertant 8	...GTCAAAGC**T**AACATTT...
in-m14Revertants 9 and *11*	...GTCAAAGC**AC**AACATTT...

Figure 5. *Spm* Insertion into *in-m14* and Sequences of Revertants as Determined by Cloning of PCR Products.

Insertion of *Spm* is in intron 2 at 109 bp from the 3′ splice site. The 3 bp of target sequence that are duplicated upon *Spm* insertion are shown in boldface. In the case of the revertants that were examined, *in-m14Revertants 2, 3, 7, 12,* and *16* show a clean excision of *Spm*, restoring the original sequence. The other revertants show various permutations (in boldface) at the insertion site when *Spm* excises.

merization domain in the anthocyanin HLH homologs and that the entire region appears to be dispensable for the function of the B1 protein, it seems likely that the IN1 products might exert their effects through competition with the activation domain carried on the R1 protein rather than by interfering with DNA binding. At the N terminus, the presumptive IN1 sequence shares homology with B1 only for the first 220 amino acids. Goff et al. (1992) have proposed that the R1/B1 proteins exert their positive transcriptional effects through association with the PL1 or C1 DNA binding proteins. It is possible that IN1 retains enough similarity to R1 to bind to C1, but its dissimilar amino acid sequence in a portion of the activation domain is sufficient for it to be a competitive inhibitor of the R1 protein.

In our introduction, we mention that the *in1* mutation increases overall the products of the later steps in anthocyanin biosynthesis. The mode of action we envisage for *in1* differs from that of Franken et al. (1991), who proposed that the gene acts at the level of translation. They suggested that the wild-type gene product acts specifically with the *whp1* mRNA either to inhibit its translation or to control the expression of a factor that inhibits the translation of *whp1* mRNA. The homology that IN1 shows with other MYC proteins is consistent with the latter mechanism, but the effect would not be limited to a specific interaction with *whp1* mRNA.

Regulational RNA Processing

We examined the *In1* introns and adjacent sequences for factors that might explain the frequent missplicing and nonsplicing that we detected. Although monocot introns frequently have a higher GC content than dicot introns, the presence of AU-rich stretches and, in particular, uridine-rich tracts just before the 3′ acceptor site characterize well-spliced introns (Goodall and Filipowicz, 1989; Luehrsen and Walbot, 1994a, 1994b). Similarly, adherence to the 5′ and 3′ consensus sequences aids the splicing efficiency of GC-rich introns (Goodall and Filipowicz, 1991). With the exception of intron 1 with 56% GC, none of the *in1* introns is GC rich. All of the introns have a

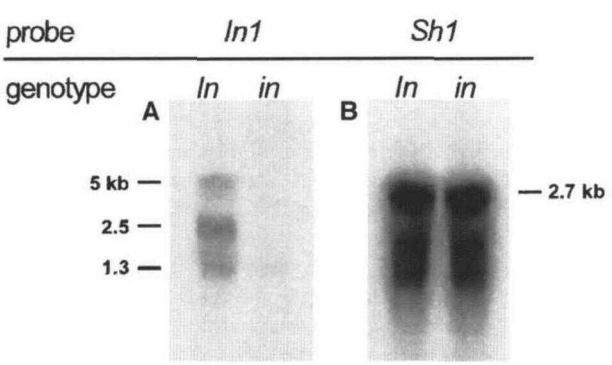

Figure 6. Autoradiography of RNA Gel Blot Hybridizations.

Fifteen micrograms of poly(A)$^+$ RNA of W22 *In1; pr1* and W22 *in1; pr1* from endosperms was loaded in each lane.

(A) The filter was probed with an α-^{32}P-labeled PCR product that selectively amplified a fragment corresponding to the 250–amino acid central region unique to IN1. Exposure was for 3 days at −80°C with an intensifying screen.

(B) As a control, the filter was stripped of radioactivity and rehybridized with an α-^{32}P-labeled probe for *sh1*. Exposure was for 6 hr at room temperature.

Numbers at left and right indicate estimated lengths of bands (in kilobases) based on internal standards.

higher AU content than their surrounding exons. Concerning other compositional characteristics that might bear on missplicing, only intron 7 appears to exhibit unusual features. In addition to having a somewhat lower AU content, intron 7 has no uridines in the 10 nucleotides preceding the 3′ intron boundary. Uridines are usually found in this region and appear to enhance splicing efficiency (Hanley and Schuler, 1988; Lou et al., 1993). Unlike dicots, maize will splice introns that contain large stem and loop structures (Goodall and Filipowicz, 1991). Nevertheless, we performed a search for stem and loop structures in the *In1* sequence. In intron 7, we found only an eight-nucleotide stem with a 16-nucleotide loop starting 48 nucleotides from the 5′ splice site. For introns 2 and 5, no obvious structural or compositional reasons account for the missplicing events. In fact, in intron 5, the more frequently used alternative 3′ splice site deviates more from the 3′ consensus splice site sequence than does the correct site. In addition, there are fewer uridines in the 15 nucleotides preceding the alternative 3′ splice site.

All the missplicing events in *in1* lead to premature translation termination. Misspliced intron 2′ leads to immediate truncation of the polypeptide, whereas misspliced intron 5′ results in the addition of 25 new amino acids before premature termination. Both of these events disrupt the homology shared with the other HLH proteins in the N-terminal region. Lack of splicing of intron 7 destroys the second helix of the HLH region and adds 51 new amino acids before termination. It is therefore unlikely that any of the alternatively spliced transcripts result in translation products with alternative functions. Missplicing, however, could play a regulatory role in modulating the level of functional IN1 protein. The Drosophila *suppressor of white apricot* locus autoregulates its own splicing (Chou et al., 1987). A functional mRNA is the predominant species only during the first few hours of larval development. Thereafter, transcripts with an unspliced intron 1 and/or intron 2 predominate. Chou et al. (1987) have demonstrated that the gene is autoregulated by showing that incorrect splicing was dependent on a functional product of *suppressor of white apricot*. Developmental on/off regulation of gene action through alternative splicing appears to be a common theme in eukaryotes (Bingham et al., 1988).

in1 shares some properties with the aforementioned genes that exhibit regulated splicing. There is a degree of specificity in that four of the eight introns are always correctly spliced, whereas three are usually misspliced, despite having conventional donor and acceptor splice sites. Currently, we have no evidence that the IN1 protein autoregulates its transcription and/or splicing. From the results of the PCR amplifications presented in Table 3, we estimate that <0.2% of the *In1* transcripts are correctly spliced. If the *In1* gene product competes with other transcription factors for gene activation, it would have a very severe effect if all of the mRNAs were correctly spliced. Missplicing may provide an inherent mechanism for dealing with a potent competitor of activation of genes in the anthocyanin biosynthetic pathway.

METHODS

Transposon Tagging

Transposon mutagenesis was performed using the maize transposable element *Suppressor-mutator* (*Spm*). In the crosses, the genotype of the female parent was W22 *in; pr1* and that of the male either *o2-m20::Spm In1; pr1* or *c1-m5::Spm; O2 In1; pr1* or, in one experiment, *O2 In1; pr1; Spm* (autonomous unmapped *Spm*).

o2-m20::Spm seemed a likely, good donor because the *opaque2* (*o2*) locus maps ~4 centimorgans (cM) distal to *intensifier1* (*in1*) on the short arm of chromosome 7 and transpositions are usually to nearby locations (McClintock, 1962; Peterson, 1970). Two other donors were also used to a limited extent, because for reasons that are not clear, we have sometimes failed to get a desired insertion by using a linked transposon (F. Burr and B. Burr, unpublished data). In all, more than 7×10^5 kernels were screened. We expected the *in1-m::Spm* kernel to have a phenotype of a black aleurone (*in1-m/in1; pr1*) with reddish pink revertant sectors (*in1-m revertant/in1; pr1*). We succeeded in isolating 13 confirmed *in1-mutable* (*in1-m*) mutations. Subsequent genetic analyses have shown that the alleles were carrying either an autonomous or a defective *Spm* (*Spm* or *dSpm*).

Genomic Cloning of *in1-m14::Spm*

The mutant *in1-m14::Spm* was selected for cloning because genetic analyses had shown that it contained an autonomous *Spm*. A backcross population of *in1-m14::Spm/in1* × *in1* was classified by phenotype into *in1-m14/in1* and *in1/in1* (1:1) kernels. These were planted, and DNAs were prepared from seedlings of 18 mutable and six stable kernels. Equal amounts of DNA from six plants of the same genotype were pooled, and each pool was digested with the methyl-sensitive enzymes ClaI, EcoRII, FspI, MluI, NaeI, NarI, NruI, PstI, PvuI, PvuII, SacI, SalI, SmaI, SnaI, and XhoI (Bethesda Research Laboratories and New England Biolabs, Beverly, MA). DNA gel blots were probed with an *Spm*-specific probe representing a 450-bp BanII-XbaI fragment from the *dSpm* at *waxy1(wx1)-m8* (Cone et al., 1986). The films of the batch digests were examined for fragments cosegregating with the mutant phenotype that had sizes suitable for cloning in the λ vector. The putative results were subsequently confirmed by DNA gel blot analyses of the individual plants (Cone et al., 1986). In this way, we were able to identify a 10.6-kb SalI fragment that was always present in the *in1-m14::Spm/in1* plants but absent in the *in1/in1* individuals.

Twenty micrograms of *in1-m14::Spm* DNA was digested with SalI and electrophoresed on a 0.6% low melting point agarose (Bethesda Research Laboratories) gel. A small part of the gel that bracketed the 10.6-kb region containing the desired fragment was excised, and the DNA was purified using QIAEX resin (Qiagen Inc., Chatsworth, CA). The DNA fragments were ligated to XbaI- and XhoI-digested λGEM-12 DNA (Promega). Transformants were plated on 22.5 cm^2 NUNC plates, and phage plaques were transferred to nitrocellulose (Schleicher & Schuell). Filters were hybridized using the 450-bp *Spm* probe mentioned above. In this way, several plaques were identified that subsequently were plaque purified. The λ clones were sequenced using a 3′ *Spm* primer, 5′-CAGTAAGAGTGTGGGGTTTTGGCC-3′, which provided sequence extending from the 3′ end of the element into the adjacent genomic region.

The position of the *Spm* insertion in *in1-m14::Spm* was determined

by cloning and sequencing polymerase chain reaction (PCR) products. For the 5′ junction, we used the *In1-W22* primer, 5′-CCGGCTCTATGCTGTTCTGTATTT-3′ (24mer), and the 5′ *Spm* primer, 5′-TGACACTCCTTTGACGTTTTCTTGT-3′ (25mer). For the 3′ junction, the 3′ *Spm* primer, 5′-CAGTAAGAGTGTGGGGTTTTGGCC-3′ (24mer), and the *In1-W22* primer, 5′-ATTGCTCTTGAGAACACCTTGCTG-3′ (24mer), were used. Eleven revertants were sequenced from clones of PCR products by using the primers 5′-CCGGCTCTATGCTGTTCT-GTATTT-3′ (24mer) and 5′-GCTCTCCTTACAAACGCCTC-3′ (20mer), which flank the site of insertion. All of the PCR products were isolated from 1% low melting point agarose with GeneClean (Bio 101, Vista, CA) and cloned into pCR II (TA cloning kit, version B; Invitrogen Corp., San Diego, CA).

Restriction Fragment Length Polymorphism Mapping in Recombinant Inbreds

A 169 bp-fragment spanning the 3′ junction region of the *Spm* insertion was obtained by PCR with the primers 5′-ATTGCTCTTGAGAAC-ACCTTGCTG-3′ (24mer) and 5′-CAGTAAGAGTGTGGGGTTTTGGCC-3′ (24mer). This was used as a hybridization probe to map the putative *in1* gene fragment in the CO×Tx and the T×CM recombinant inbred populations (Burr and Burr, 1991; Burr et al., 1988). The probe identified an EcoRI polymorphism in both families. The allele distribution was compared with the allele distribution of previously mapped alleles in the maize data base maintained at Brookhaven National Laboratory (E.C. Matz, F.A. Burr, and B. Burr, informal communication, 1995 Maize Genetics Cooperation Newsletter, Vol. 69, pp. 257 to 267). Our current data base contains 1876 linked markers.

cDNA and Genomic Cloning of *In1*

Unfortunately, the 169-bp junction fragment described in the restriction fragment length polymorphism mapping did not prove to be a good hybridization probe for screening plaques. Therefore, we used the 169-bp probe to construct a partial restriction map of the λB101 clone from *in1-m14::Spm*. From this map, we identified a 1.15-kb XbaI fragment that was used successfully to probe a cDNA library constructed from W22 *In1; C1; R1* mRNA by K. Cone (University of Missouri, Columbia; Schmidt et al., 1990). Three cDNA clones, λB139, λB140, and λB141, were obtained. These were subcloned into pBluescript II KS+ (Stratagene, La Jolla, CA) as pBF276, pBF277, and pBF278.

Because the cDNA library used was constructed from W22 full-color endosperms, the same inbred was used to clone an 18-kb BamHI genomic fragment from size-selected DNA in λGEM-12 digested with BamHI and XhoI. The genomic λ clone, λB137, was subcloned as either EcoRI fragments of 2.4 and 5.8 kb or SalI fragments of 2.3 and 3.6 kb into pBluescript II KS+.

RNA Analysis

Total RNA was prepared from W22 *In1; pr1* and W22 *in1; pr1* endosperms that had been isolated at 30 days after pollination, frozen in liquid N_2, and stored at −80°C. Poly(A)$^+$ RNA was purified from the total RNA by oligo(dT) cellulose chromatography. Fifteen micrograms of poly(A)$^+$ RNA from each genotype was used for the RNA gel blot hybridizations. The initial experiments used the entire insert of the cDNA clone pBF276. However, because of what we later learned about the homologies of *in1* to *r1* and *b1*, we repeated the RNA gel blot hybridizations, using a 300-bp region from the central unique region of *in1*. The probe was generated by PCR, using the *In1-W22* primer pair 5′-AGC-ACCGAGCAGGACG-3′ (16mer) and 5′-CGCAGGATGAATATGGTCA-3′ (19mer). The PCR product was labeled with α-^{32}P-dATP by random priming. Filters were exposed to Kodak XAR-5 film with Lightning-Plus intensifying screens (Du Pont) at −80°C for 3 to 6 days. Filters were subsequently stripped of radioactivity and reprobed with a labeled fragment of the *shrunken1* (*sh1*) gene (Burr and Burr, 1981). The signal corresponding to *sh1* was found to be so intense that even when exposure was reduced to only 6 hr at room temperature, the autoradiogram appeared overexposed.

Sequencing

Plasmid subclones of the cDNAs were sequenced by the dideoxynucleotide chain termination method by using Sequenase DNA polymerase (United States Biochemical). Genomic subclones and PCR clones were sequenced manually by the dideoxy method with Sequenase, by cycle sequencing with dye terminators or labeled primers on an automated DNA sequencer (model 373A; Applied Biosystems, Inc., Foster City, CA), or, in one instance, by cycle sequencing using dye-labeled primers on an automated DNA sequencer (LI-COR, Inc., Lincoln, NE). In all cases, both strands were sequenced for confirmation. Oligonucleotide primers were selected by using OLIGO, version 5.0 (National Biosciences, Inc., Plymouth, MN) and synthesized by the Genome Facility at Brookhaven National Laboratory. Sequence alignments and editing were done with the Sequencher 2.1 program (Gene Codes Corp., Ann Arbor, MI). Sequence homology searches were conducted with BLAST (Altschul et al., 1990), and sequence alignments were performed with FASTA (Pearson and Lipman, 1988).

Rapid Amplification of cDNA Ends for 5′ Extensions of *In1* mRNA

The rapid amplification of cDNA ends (RACE) procedure (Frohman, 1990) was used to extend the 5′ end of the mRNA. cDNA was synthesized in 25 μL with 1 μg of endosperm RNA at 30 days after pollination, 2.5 pmol of a primer near the 5′ end of the mRNA, 5′-TGCCGCCTT-GTTGAATC-3′ (17mer), 1× first strand buffer (Bethesda Research Laboratories), 0.4 mM deoxynucleotide triphosphates, 10 mM DTT, and 100 units of modified Moloney murine leukemia virus reverse transcriptase SuperScript II (Bethesda Research Laboratories) for 30 min at 42°C. The reverse transcriptase products were purified on GlassMax columns (Bethesda Research Laboratories) and tailed with poly(dA). The tailing reaction mixture contained 1× terminal deoxynucleotide transferase buffer (Bethesda Research Laboratories), 1 mM $MgCl_2$, 0.2 mM deoxyadenosine triphosphate, and six units of terminal deoxynucleotidyl transferase in a total volume of 20 μL and was incubated for 15 min at 37°C. One microliter of the poly(A)-tailed cDNA products was amplified by PCR in a final volume of 50 μL containing 1× PCR buffer (Promega), 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 units of Taq DNA polymerase (Promega), 7% DMSO, 2 pmol of 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′ (37mer) primer, and 25 pmol each of 5′-TAGTAGCCCTCCGCCCAGA-3′ primer (19mer; nested primer 40 bp upstream from reverse transcrip-

tase 18mer primer) and 5′-GGCCACGCGTCGACTAGTA-3′ primer (19mer; subset primer of 37mer poly[dT] primer). The amplification conditions were as follows: step 1, 94°C for 4 min; step 2, six cycles at 91°C for 15 sec, 48°C for 2 min, and 72°C for 4 min (Taq DNA polymerase was added when the temperature reached 48°C in the first cycle); step 3, 30 cycles at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 2 min; and step 4, 72°C for 6 min. The PCR reaction was diluted 1:20 with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. A second PCR reaction of 25-μL final volume contained 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dinucleotide triphosphates, 1.25 units Taq DNA polymerase, 7% DMSO, and 5 pmol each of 5′-GGCCACGTCGACTAGTA-3′ (19mer) and 5′-TGGCTGGGATGGCTACCTG-3′ (19mer; located 173 bp upstream of the previous nested primer). Amplification conditions were as follows: step 1, 94°C for 4 min; step 2, 40 cycles at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 2 min; and step 3, 72°C for 6 min. The products were separated on a 1% low melting agarose gel, and an ~250-bp band was excised, purified with GeneClean, and cloned into pCR II.

The clones were characterized by PCR analyses, using the appropriate *In1-W22* and T7 primers. More than 100 clones carrying amplifiable inserts were obtained, and the 10 longest were sequenced. However, the longest insert added only 34 bp to the 5′ end of the cDNA.

PCR Analysis and Cloning of Novel *In1* Splice Junctions

Five micrograms of total RNA from W22 *In1; pr1* dissected endosperms 30 days after pollination were converted to cDNA with SuperScript reverse transcriptase (Bethesda Research Laboratories) and subsequently treated with ribonuclease H (Bethesda Research Laboratories). For each intron listed in Table 3, approximately one-seventieth of the reaction was amplified with Taq DNA polymerase (Promega) in the presence of 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dinucleotide triphosphates, 1.0 μM primers, and 5% DMSO. The primer pairs used for each intron are given in Table 4. Amplification conditions were as follows: step 1, 94°C for 4 min; step 2, 40 cycles at 95°C for 15 sec, 57°C for 30 sec (introns 1 and 7) or 55°C for 30 sec (introns 2, 3 and 4, 5, 6, and 8), and 72°C for 2 min; and step 3, 72°C for 6 min. For quantitative PCR, aliquots were withdrawn after the extension step in step 2 (72°C for 2 min) at 24, 26, 28, 30, 32, and 34 cycles. The products were separated on a 3% MetaPhor gel (FMC Bioproducts, Rockland, ME) at 6 V/cm, stained with ethidium bromide, and visualized by surface UV illumination. The gel was imaged with a CCD camera (Sutherland et al., 1987), and image reversal was performed electronically. The sizes of bands were estimated, and the amounts of each product were quantified (Sutherland et al., 1987). Relative amounts were estimated during exponential synthesis by measuring the peak sizes and dividing by the molecular length. In the instances where unexpected products were observed, PCR synthesis was repeated, and the products were separated on a 1% low melting temperature agarose gel, purified with QIAEX resin, and cloned into pCR II.

Table 4. Oligonucleotide Primer Pairs Used for Amplification of *In1-W22* Introns

Intron No.	Forward (5′→3′)	Reverse (5′→3′)
1	GTGGACGTACAGCCCCTC (19mer)	TAGTAGCCCTCCGCCCAGA (19mer)
2	TCCTACTGCTTCCCTCCT (18mer)	GCTCTCCTTACAAACGCCTC (20mer)
3 and 4[a]	GAGGCGTTTGTAAGGAGAGC (20mer)	CGACTGGAATGCATGCTACT (20mer)
5	GGTGTCCTGGAAATTGGAACT (21mer)	TTGTCTGGAATGGCTGATGAT (21mer)
6	CGCAGGATGAATATGGT (17mer)	CCGCTACTGCTGCTTGATC (19mer)
7	GCCAGCCATGTCCTCAAG (18mer)	GATCGACGCCCTGTCCAT (18mer)
8	TGGCGACGTGCTACTTGC (18mer)	CCAGTCTGATGAAACGATGA (20mer)

[a] Introns 3 and 4 had to be amplified together because the intervening exon is only 15 bp and could not provide a suitable primer sequence.

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NOTE ADDED IN PROOF

Regretfully, we overlooked the following paper: **Reddy, A.R., and Peterson, P.A.** (1978). The action of the intensifier (*IN*) gene in flavonoid production in aleurone tissue of maize. Can. J. Genet. Cytol. **20,** 337–347. This paper shows that *in1* greatly increases the quantity of anthocyanins, 3-deoxy anthocyanins, and leucoanthocyanidins.