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Analysis of Maize *Brittle-1* Alleles and a Defective *Suppressor-Mutator*-Induced Mutable Allele

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A mutant allele of the maize *brittle-1* (*bt1*) locus, *brittle-1-mutable* (*bt1-m*), was shown genetically and molecularly to result from the insertion of a defective *Suppressor-mutator* (*dSpm*) transposable element. An *Spm*-hybridizing restriction enzyme fragment, which cosegregates with the *bt1-m* allele and is absent from wild-type revertants of *bt1-m*, was identified and cloned. Non-*Spm* portions of it were used as probes to identify wild-type (*Bt1*) cDNAs in an endosperm library. The 4.3-kb *bt1-m* genomic clone contains a 3.3-kb *dSpm*, which is inserted in an exon and is composed of *Spm* termini flanking non-*Spm* sequences. RNA gel blot analyses, using a cloned *Bt1* cDNA probe, indicated that *Bt1* mRNA is present in the endosperm of developing kernels and is absent from embryo or leaf tissues. Several transcripts are produced by *bt1-m*. The deduced translation product from a 1.7-kb *Bt1* cDNA clone has an apparent plastid transit peptide at its amino terminus and sequence similarity to several mitochondrial inner-envelope translocator proteins, suggesting a possible role in amyloplast membrane transport.

INTRODUCTION

Although a number of enzymatic functions involved in carbohydrate metabolism have been identified in maize endosperm tissue (Echeverria et al., 1988), the exact pathway or pathways by which photosynthate from the plant is converted into starch in endosperm amyloplasts is not completely known. The identification of the enzymatic defect associated with mutations at loci that affect the quality or quantity of starch has helped elucidate some of the critical steps in amyloplast starch synthesis. Examples include the *waxy* gene, which encodes a starch granule-bound glucosyltransferase responsible for the synthesis of the amylose fraction of starch (Nelson and Rines, 1962), and the *shrunken-2* and *brittle-2* genes, which encode subunits of ADPglucose pyrophosphorylase (Tsai and Nelson, 1966; Bae et al., 1990; Bhave et al., 1990; Preiss et al., 1990).

The *brittle-1* (*bt1*) locus of maize was identified in 1926 by mutations that severely decreased the amount of starch deposition in the endosperm (Mangelsdorf, 1926; Wentz, 1926). The reduced starch synthesis results in kernels with a collapsed, angular appearance at maturity. Although *bt1* mutant kernels are low in a starch granule-bound phospho-oligosaccharide synthase, the primary function of the *Bt1* gene product is not known. (D. Pan and O.E. Nelson, Jr., 1985, The deficiency of a starch granule-bound enzyme phospho-oligosaccharide synthase in developing *bt1* endosperms. Maize Genetics Cooperative News Letter, Department of Agronomy and U.S. Department of Agriculture, University of Missouri, Columbia, Vol. 59, pp. 105–106.).

Transposable elements have become useful tools for the isolation of molecular clones of "tagged" genes by using cloned transposable element sequences as probes (Fedoroff et al., 1984a). Several members of the maize transposable element *Suppressor-mutator* (*Spm*) family (also known as *Enhancer* [*En*]) have been molecularly cloned (Fedoroff et al., 1984b; Schwarz-Sommer et al., 1984; Pereira et al., 1985; Masson et al., 1987). In turn, mutable alleles of the *colorless-1* and *opaque-2* loci, with autonomous *Spm* inserts, have been cloned using *Spm* probes (Cone et al., 1986; Schmidt et al., 1987).

We report here the identification of an allele of *bt1* that results from a defective *Spm* (*dSpm*) insertion and the use of this allele to obtain genomic and cDNA clones representing the *bt1* locus. The results of sequence analysis of a wild-type cDNA clone suggest possible functions for the *Bt1* gene product.

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RESULTS

The *brittle-1-mutable* (*bt1-m*) Allele

The *bt1-m* allele was first identified on an ear segregating for kernels with a "blistered" appearance obtained by Dr. C.R. Burnham (University of Minnesota, St. Paul) from the Northrup King Company (Minneapolis, MN). These unique kernels, in turn, gave rise to ears produced by self-pollination that also segregated for wild-type and stable *bt1* phenotypes. Tests with stocks provided by B. McClintock (Carnegie Institution of Washington; Cold Spring Harbor Laboratory, NY) and P.A. Peterson (Iowa State University, Ames) indicated that a defective element of the *Spm/En* transposable element family (Peterson, 1953; McClintock, 1954) is present at the *bt1* locus in the *bt1-m* line (R.L. Phillips, unpublished observations). In the absence of an autonomous *Spm* in the genome, the *dSpm* in *bt1-m* does not transpose and, therefore, the endosperm has a stable "brittle" phenotype without revertant sectors, as shown in Figure 1. When *Spm* is introduced, the *dSpm* in *bt1-m* can be activated to transpose during endosperm development, giving rise to the distinctive mosaic phenotype (Figure 1). Autonomous *Spm* can also act on the *dSpm* before or during gametogenesis, producing revertant alleles (*Bt1′*) that condition a normal kernel phenotype (Phillips et al., 1991; data not shown).

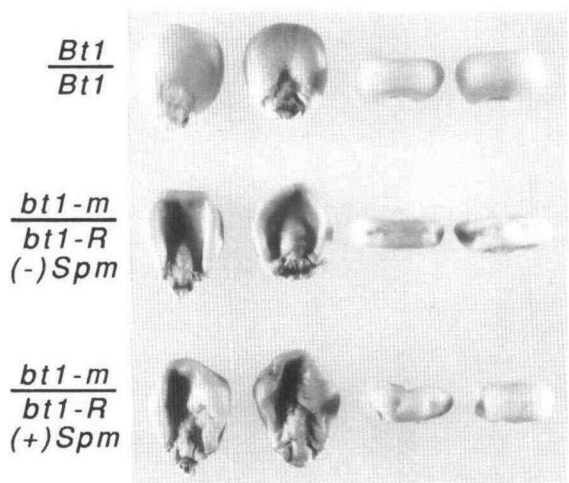

Figure 1. Phenotypes Conferred by *bt1-m*.

Mature kernels viewed from either the crown or abgerminal side. Top row (*Bt1*/*Bt1*) is the normal kernel phenotype conditioned by the dominant, wild-type *Bt1* allele from the inbred line W64A. The bottom two rows are kernels heterozygous for *bt1-m* and *bt1-R* that lack (–) or contain (+) the autonomous *Spm* sequence in the genome.

bt1-m Genomic DNA and *Bt1* cDNA Clones

The presence of a *dSpm* insert in the *bt1-m* gene provided an opportunity to isolate a *Bt1* fragment using a cloned *Spm* element as a probe. A major problem with using *Spm* (or other maize transposable elements) as probes for cloning genomic DNA is the presence of multiple copies of *Spm* sequences in the maize genome (Cone et al., 1988). If methylation-sensitive restriction enzymes are used to digest maize DNA, most copies of *Spm*-related sequences are in regions that are poorly digested (Cone et al., 1986; Schmidt et al., 1987). Presumably, many of these transposable element copies are in highly methylated regions of DNA (Shapiro, 1976; Gruenbaum et al., 1981). Because the desired *Spm* or *dSpm* element is inserted into a gene and is likely to be unmethylated, it may be digested to a smaller fragment that can be separated from the bulk of the *Spm*-hybridizing fragments.

Several restriction enzymes were used in an initial screen to identify an *Spm*-hybridizing restriction fragment associated with *bt1-m*. DNA gel blots of digests with the enzyme SalI resulted in a high molecular weight smear of *Spm*-hybridizing fragments with discrete 4.3-, 6-, and 9-kb bands present in *bt1-m*. Four wild-type revertants of *bt1-m* (*Bt1′-1*, *Bt1′-2*, *Bt1′-3*, and *Bt1′-4*) all contained the 6- and 9-kb fragments but lacked the 4.3-kb fragment. Examples of these DNA gel blot analyses are shown in Figure 2A.

The presence of the 4.3-kb restriction fragment in *bt1-m* and its absence from the *Bt1′* alleles suggest that this *Spm*-hybridizing fragment is derived from *bt1-m*. Further evidence was obtained by analyzing the cosegregation of the fragment with the *bt1-m* allele in an F_2 family segregating for *bt1-m* and *Bt1* alleles. In 25 F_2 progeny, there was complete correspondence of the 4.3-kb fragment and the *bt1-m* allele. Neither the 6- nor 9-kb fragment showed this linkage. The results for three *Bt1′* alleles and 10 F_2 progeny are shown in Figure 2A.

The SalI-digested DNA from *bt1-m* was enriched for fragments less than 10 kb and cloned into the XhoI site of λZAP (Short et al., 1988; Methods). The subgenomic library was screened with the *dSpm* probe, and three clones with identical inserts were isolated. The structure of the cloned SalI fragment is shown in Figure 3.

Two restriction fragments, which were from opposite ends of the *bt1-m* genomic clone and lacked *Spm* sequences (Acc125 and Acc550, Figure 3), were used as probes on a wild-type *Bt1*, endosperm cDNA library. Of 200 clones identified, four were purified and analyzed: pBtcDNA1.1, pBtcDNA1.2, pBtcDNA1.3, and pBtcDNA1.7. The longest cDNA clone, pBtcDNA1.7, contains a 1.7-kb insert.

Tests were made to verify that the *bt1-m* genomic clones and the *Bt1* cDNA clones were derived from the *bt1* locus. First, the pBtcDNA1.1 clone was used to probe DNA gel blots of SalI- or KpnI-digested DNA from plants in the

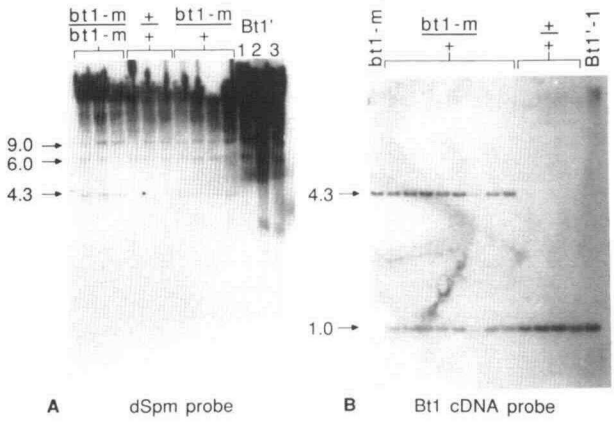

Figure 2. DNA Gel Blot Analysis.

Genomic DNA from individual plants in a segregating F_2 family (from self-pollination of a *bt1-m/Bt1* heterozygote) or from wild-type revertants (*Bt1'*) of *bt1-m* digested with SalI. The genotype of each individual is indicated above the autoradiograph. The (+) denotes wild-type *Bt1* from W64A. The length in kilobase pairs is indicated on the left in each panel.

(A) *dSpm* probe. The *Bt1'* alleles are all homozygous.

(B) *Bt1* cDNA probe. The far left and right lanes contain DNA pooled from several plants homozygous for either *bt1-m* or *Bt1'-1*.

family segregating for the *bt1-m* allele and from the plants homozygous for wild-type revertants of *bt1-m*. Figure 2B shows the results of a DNA gel blot with SalI. The results are consistent with the expectations for the *bt1* locus probe. Plants homozygous for *bt1-m* or heterozygous for *bt1-m* and the wild-type *Bt1* allele of W64A contained a 4.3-kb fragment, as seen earlier with the *Spm* probe and as expected for *bt1-m*. In heterozygotes, there was a second hybridizing fragment of approximately 1 kb. The homozygous wild-type segregants and samples homozygous for the *Bt1'-1* allele contained only the 1-kb fragment. The difference in SalI fragment length between *bt1-m* (4.3 kb) and the *Bt1'* alleles (1 kb) is consistent with the length of the *dSpm* determined from sequencing the *bt1-m* clones (see below).

Comparable results were obtained using KpnI (data not shown). An ~11-kb KpnI fragment was detected by a *Bt1* cDNA probe on DNA gel blots of homozygous *bt1-m* samples, and an ~8-kb fragment was detected in samples of four independently isolated *Bt1'* alleles. In a segregating F_2 family, heterozygous *bt1-m/Bt1* samples contained both fragments, whereas homozygous *Bt1* samples had only the 8-kb band.

These results indicated that the isolated genomic and cDNA clones represent sequences from the *bt1* locus. Sequence analysis of the genomic and cDNA clones confirmed that they are derived from the same sequences and that the *bt1-m* genomic clone contained an insertion with *Spm* sequences (see below). Finally, RNA gel blot analyses of *Bt1* alleles indicated that these clones hybridized to RNA with the tissue-specific distribution expected for the *Bt1* gene product (see below).

bt1-m dSpm Sequence

The *bt1-m* genomic clone is composed of *Bt1* sequences flanking a *dSpm* insertion (Figure 3). The *bt1-m dSpm* is unlike other characterized *dSpm* elements (Banks et al., 1985; Gierl et al., 1985; Schiefelbein et al., 1985; Schwarz-Sommer et al., 1985a; Masson et al., 1987; Cuypers et al., 1988) in that it is not a simple deletion derivative of autonomous *Spm*. Rather, as shown in Figure 4, the 3326-bp sequence is a composite of 219 bp from the 5′ end of *Spm* and 1392 bp of the 3′ end of *Spm* surrounding 1714 bp of the non-*Spm* sequence. There are 101 differences within the 1611-bp *Spm* sequence (Figure 4), including nine deletions or insertions of single base pairs, 57 transitions, and 35 transversions, relative to an autonomous member of the *Spm/En* family (Pereira et al., 1986). One polymorphism near the 3′ terminus reduces the *bt1-m dSpm* inverted terminal repeats to only 12 bp, versus 13 bp for *Spm* and other *dSpm* elements (Schwarz-Sommer et al., 1984; Pereira et al., 1986; Kim et al., 1987; Masson et al., 1987).

The insertion of the *dSpm* in *bt1-m* is in an exon, 131 bp from the 3′ end of the *bt1-m* genomic clone. Typical of *Spm* insertions (Schwarz-Sommer et al., 1984; Pereira et al., 1986), there is a 3-bp target site duplication flanking the *bt1-m dSpm* insertion. The location of the *dSpm* target site duplication is indicated in the cDNA sequence in Figure 5.

Within the non-*Spm* region of *bt1-m dSpm* are four short regions of *Spm*-related sequences (Figure 4). The first three (Figure 4, positions 363 to 384, 386 to 405, and 411

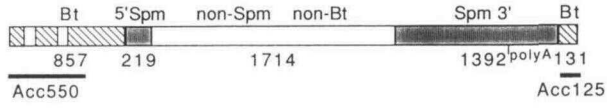

Figure 3. Map of the *bt1-m* 4.3-kb SalI Genomic Clone.

The map is based on the complete DNA sequence determined from the clone and comparison with *Bt1* cDNA clones. The map is split into regions of *bt1* (diagonals, with two small open boxes indicating introns) and *Spm*-related sequences (shaded boxes) and includes a large non-*Bt1*, non-*Spm* region (open box between shaded regions). The length, in base pairs, of each region is indicated below the map, as is the position of the poly(A) site utilized in transcripts from autonomous *Spm* elements. The locations of the two restriction enzyme fragments used as probes to screen the cDNA library for *Bt1* clones are also indicated below.

Figure 4. *bt1-m dSpm* Sequence.

The *dSpm* sequence is arranged 5′ to 3′ relative to the *bt1-m* transcriptional orientation. Uppercase letters designate the *Spm* sequences; lowercase letters indicate the non-*Spm* sequences. Nucleotide differences from an autonomous *Spm* sequence (*En-1*, Pereira et al., 1986) are indicated below the *dSpm* sequence. Also indicated are terminal inverted repeats (overlined with arrows), repeats of the SRR (underlined with half arrows), an imperfect triplication in the non-*Spm* sequence (dashed underline), regions within the non-*Spm* sequence that are related to the *Spm* (solid underline, see text for description), and the position within the *Spm* sequence of the start and poly(A) sites of autonomous *Spm* transcripts.

to 430) are related to sequences found in the *Spm* "GC-rich" region (Gierl et al., 1985; Pereira et al., 1986). The second and third of these form an imperfect direct repeat, with 18 of 20 matches, of a sequence that is also found as imperfect direct repeats in the GC-rich region. The fourth region is at the 3′ end of the non-*Spm* sequence (position 1918 to 1937). This 20-bp sequence matches, at 19 positions, a sequence in autonomous *Spm* (position 6788 to 6807, Pereira et al., 1986) that is 108 bp upstream of the position where the 3′ segment of *bt1-m dSpm* diverges from *Spm* (Figure 4, position 1934; Pereira et al., 1986, position 6896).

Near the 5′ end of the non-*Spm* sequences of *bt1-m dSpm* is an imperfect direct triplication of a 24-bp sequence, within which is a 9-bp sequence (TCTCCTACC) that is perfectly matched in the three repeats (Figure 4).

As noted for some sites of *Spm* element insertion (Masson et al., 1991), the *Bt1* sequence near the *dSpm* insertion site has some sequence similarity to the repeat sequence of the *Spm* subterminal repetitive region (SRR) (Figure 5).

mRNA Analysis

RNA gel blot analyses using cloned *Bt1* cDNA probes were undertaken to determine the size and tissue distribution of the mRNA from wild-type *Bt1* alleles and to determine the

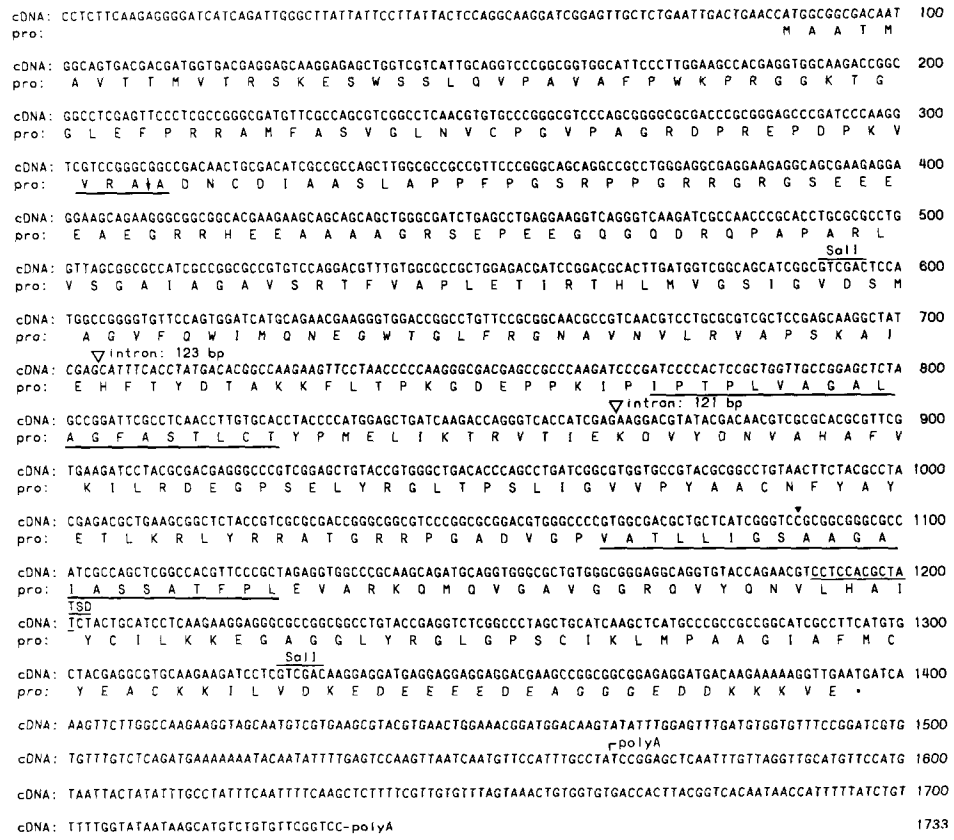

Figure 5. *Bt1* cDNA Sequence.

The pBtcDNA1.7 clone sequence (cDNA) is aligned with the deduced amino acid sequence (pro). SalI sites mark the ends of the sequence covered by the *bt1-m* genomic clone. Also indicated for the nucleotide sequence are the positions and length of introns in the genomic clone (open triangles), position of the *dSpm* target site duplication (overlined, TSD), the sequence near the target site duplication that is related to the consensus repeat of the *Spm* SRR (underlined), a single difference between the genomic clone (G) and the sequence of two cDNA clones (C, filled triangle), and two poly(A) sites found in *Bt1* cDNA clones. Indicated in the amino acid sequence are the 4-amino acid sequence that matches the consensus cleavage site of the transit peptide (underlined) with the putative cleavage site (arrow) and two putative membrane-spanning domains of the protein (underlined).

effects of the *dSpm* insertion on the transcripts from *bt1-m*. Figure 6 shows examples of the RNA analyses.

In samples from the inbred lines W64A and R802, the *Bt1* mRNA migrates as a broad band of approximately 1.7 kb (Figure 6A and data not shown). This is the length of the insert in the cDNA clone pBtcDNA1.7. No *Bt1* mRNA was detected in RNA from seedlings (Figure 6A, lanes 6 and 7 and data not shown). Within kernels, *Bt1* mRNA was detected in the endosperm but not in the embryo (Figure 6B).

There are *Bt1*-hybridizing RNAs of at least five lengths in *bt1-m* kernels. One of these is as abundant as the mRNA in wild-type kernels but is slightly shorter, about 1.5 kb (Figures 6A and 6C). Additional, less abundant, higher molecular weight RNAs were also detected (2.6, 3, 4, and 5 kb, Figure 6C). All but the 5-kb RNA failed to react with the Acc125 probe (Figure 6D). (Longer exposure of this and other blots showed hybridization with the 5-kb band [data not shown].)

RNA from the *bt1-R* reference allele was also analyzed. The nature of the mutation that caused the *bt1-R* allele is not known. The *bt1-R* allele produces an mRNA that is less abundant than the wild-type transcripts in W64A and R802. In addition, the *bt1-R* transcript appears to be slightly larger than that of the wild type (Figure 6A and data not shown).

Two wild-type revertant alleles of *bt1-m* were analyzed and, as expected, were found to produce a single abundant *Bt1* transcript of similar size as the wild-type allele from W64A stocks (Figure 6A, lanes 4 and 5). This result

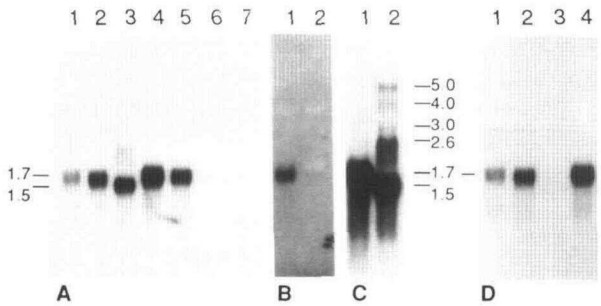

Figure 6. RNA Gel Blot Analyses.

(A) pBtcDNA1.1 probe. Each lane contains 2 μg of poly(A)$^+$ RNA from homozygous plants; autoradiography was for 4 hr. Lanes 1 to 5 represent kernels 18 days after pollination. Samples include *bt1-R* (lane 1), *Bt1* (W64A, lane 2), *bt1-m* (lane 3), *Bt'-1* (lane 4), and *Bt'-2* (lane 5). Lanes 6 and 7 represent seedling shoot RNA from *Bt1* (W64A, lane 6) and *Bt'-1* (lane 7).
(B) Endosperm versus embryo. Each lane contains 3 μg of *Bt1* (W64A) total RNA from kernels 22 days after pollination. The blot was probed with pBtcDNA1.7 and autoradiographed for 23 hr. Lane 1 contains endosperm RNA; lane 2, embryo RNA.
(C) *bt1-m* transcripts. Three micrograms of poly(A)$^+$ RNA from kernels 18 days after pollination were probed with pBtcDNA1.7 and autoradiographed for 18 hr. Lane 1 contains *Bt'-1*; lane 2, *bt1-m*.
(D) Acc125 probe. Sample lanes are as given in **(A)** for lanes 1 to 4. Autoradiography was for 70 hr.
Indicated RNA lengths (kilobases) are based on comparisons to RNA standards.

is consistent with the data from DNA gel blots of the *Bt1'* alleles, which indicate that the *dSpm* is removed in these alleles.

DNA Sequence Analysis

A comparison of the *bt1-m* clone sequence with the cDNA clone pBtcDNA1.7 shows that the genomic SalI clone represents an internal gene fragment that does not include all of the transcribed region (Figure 5). There are 985 bp of *Bt1* sequence in this partial genomic clone, including 740 bp in 3 exons and 2 introns of 123 and 121 bases. Both introns have GT and AG dinucleotides at their respective 5′ and 3′ ends (Breathnach and Chambon, 1981). There is a single base pair difference in the overlapping sequences of the genomic and cDNA clones, which were isolated from different progenitor lines. This nucleotide change does not result in a change of the amino acid encoded at this position (Figure 5). Two sites of poly(A) addition were found in four cDNA clones analyzed (Figure 5).

The *Bt1*-Encoded Polypeptide

The inferred polypeptide sequence translated from the open reading frame in the 1.7-kb *Bt1* cDNA is 447 amino acids long, with a calculated molecular mass of 47 kD (Figure 5). Inspection of the amino terminus reveals a sequence that has characteristics of a plastid transit peptide (Keegstra et al., 1989; von Heijne et al., 1989). The first 75 amino acids of the polypeptide are rich in serine, threonine, alanine, and glycine, have a net positive charge, and terminate in a sequence that matches a consensus sequence found at the transit peptide/mature protein junction of several chloroplast or amyloplast proteins (Gavel and von Heijne, 1990). In the *Bt1*-encoded polypeptide, this sequence is valine-arginine-alanine-alanine (Figure 5). Cleavage at this sequence would yield a polypeptide of 362 amino acids with a calculated molecular mass of 38.6 kD for the "mature" protein.

The amino acid sequence of the putative mature protein was used to search the National Biomedical Research Foundation Protein Identification Resource (NBRF-PIR) (release 25) and European Molecular Biology Organization Swiss-Prot (release 14) protein sequence data bases for similar proteins (Lipman and Pearson, 1985). A family of mitochondrial proteins was identified with partial sequence similarity to the *Bt1*-encoded protein. These proteins include ADP/ATP carrier proteins (AACP; also known as adenylate translocator) from yeast, Neurospora, human, bovine, and maize mitochondria (Aquila et al., 1982; Arends and Sebald, 1984; Baker and Leaver, 1985; Adrian et al., 1986; Battini et al., 1987; Houldsworth and Attardi, 1988; Lawson and Douglas, 1988; Cozens et al., 1989); mitochondria uncoupling protein of rat, mouse, and hamster brown fat (Aquila et al., 1985; Bouillaud et al., 1986; Ridley et al., 1986; Kozak et al., 1988); and a phosphate carrier protein from bovine and rat mitochondria (Runswick et al., 1987; Ferreira et al., 1989). These proteins form a small family of related proteins that function in the transport of solutes across the mitochondrial inner-envelope membrane (Aquila et al., 1985; Runswick et al., 1987). The level of similarity among these proteins is about 20% to 30% between the translocator types, and from 50% to 90% between species for proteins with the same solute specificity (Lawson and Douglas, 1988). Similarly, the putative *Bt1*-encoded protein shows about 20% sequence identity with these mitochondrial proteins, including the maize AACP (data not shown). The protein with greatest similarity to the *Bt1*-encoded protein is a yeast AACP (Lawson and Douglas, 1988), which is identical for 24% of the amino acids and shares an additional 29% of the amino acids that are conservative replacements (Dayhoff, 1978; Lipman and Pearson, 1985). The computer-based alignment of the *Bt1*-encoded polypeptide with the yeast AACP is shown in Figure 7.

Because these proteins with sequence similarity to *Bt1* are all membrane associated, the *Bt1*-encoded polypeptide

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Bt1     ADNCDIAASLAPPFPGSRPPGRRGRGSEEEEAEGRRHEEAAAAGRSEPEEG
                                        :A: :    :A:A :.E::
AACP                                1 MSSNAQVKTPLPPAPAPKKESNFL

Bt1     QGQDRQPAPARLVSGAIAGAVSRTFVAPLETIRTHLMVGSIGVDSMAGVFQ
         :    :A :   A :     ::   :       :: : F:
AACP    IDFLMGGVSAAVAKTAASPIERVKLLIQNQDEMLKQGTLDRKYAGILDCFK

Bt1     WIMQNEGWTGLFRGNAVNVLRVAPSKAIEHFTY-DTAKKFLTPKGDEPPKI
        :   :EG  :: RGN: NV:R  P::A: :F:: D  K   :  K :E
AACP    RTATQEGVISFWRGNTANVIRYFPTQAL-NFAFKDKIKAMFGFKKEEGYAK

Bt1     PIPTPLVAGALAGFASTLCTYPMELIKTRVTIE--------KDVYDNVAHA
         ::  L :G: AG  S L  Y::: :TR:: :          :: : :
AACP    WFAGNLASGGAAGALSLLFVYSLDYARTRLAADSKSSKKGGARQFNGLIDV

Bt1     FVKILRDEGPSELYRGLTPSLIGVVPYAACNFYAYETLKRLYRRATGRRPG
        : K L: :G : LYRG: PS::G:V Y :  F  Y::LK L    TG   G
AACP    YKKTLKSDGVAGLYRGFLPSVVGIVVYRGLYFGMYDSLKPLL--LTGSLEG

Bt1     ADVGPVATLLIGSAAGAIASSATFPLEVARKQMQVGAVGGRQVYQNVLHAI
        :    :A::L:G    : AS: ::PL:  R::M : :  : : Y:  :: :
AACP    S---FLASFLLGWVVTTGASTCSYPLDTVRRRMMMTSGQAVK-YDGAFDCL

Bt1     YCILKKEGAGGLYRGLGPSCIKLMPAAGIAFMCYEACKKILVDKEDEEEED
          I:  EG G:L::G G:: :: :::AG:  M Y:  : IL :K
AACP    RKIVAAEGVGSLFKGCGANILRGVAGAGVISM-YDQLQMILFGKKFK 318

Bt1     EAGGGKKKVEX 362
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Figure 7. Amino Acid Sequence Comparison between *Bt1* and AACP.

The deduced amino acid sequence encoded by *Bt1*, starting at the putative transit peptide cleavage site (Figure 5), is aligned with the yeast AACP (Lawson and Douglas, 1988). Identical amino acids are written between the aligned sequences, and conservative amino acid differences are indicated by colons (Dayhoff, 1978; Lipman and Pearson, 1985).

sequence was also searched for potential membrane-spanning domains (Kyte and Doolittle, 1982; Eisenberg et al., 1984). Two putative membrane-spanning domains were found (Figure 5).

DISCUSSION

bt1-m Genomic and *Bt1* cDNA Molecular Clones

The *bt1-m* allele was first identified because of its somatic instability. This instability results from the insertion of a *dSpm* transposable element which, in the presence of autonomous *Spm*, will excise during endosperm development and produce clones of cells with restored *Bt1* function (Figure 1). The germinal instability of *bt1-m* is demonstrated by the production of wild-type revertant derivatives in the presence of autonomous *Spm*. In four cases studied by DNA gel blot analysis, the reversion to the wild type is accompanied by the excision of the *dSpm*. Presumably, inexact germinal excision of the *dSpm* would also generate stable, mutant alleles (Schwarz-Sommer et al., 1985b), but we have not tested for these events.

We have used the *bt1-m* allele to obtain molecular clones of the *bt1* locus by an approach outlined by Cone et al. (1988). The identification of the correct *Spm*-containing restriction fragment and the proof that the isolated clones represent the *bt1* locus was based on the genetic linkage of the genomic fragment with the *bt1-m* allele and the disappearance of the restriction fragment harboring the *dSpm* insertion in four wild-type revertants of *bt1-m*. In addition, the mRNA identified using the cloned cDNA as a probe is altered in lines harboring either *bt1-m* or *bt1-R* alleles and is expressed in the expected tissue-specific manner in lines with wild-type *Bt1* alleles, i.e., the mRNA is found only in the endosperm, and not in either the embryo or seedlings. This is similar to the tissue distribution of *waxy* gene transcripts (Klösgen et al., 1986).

bt1-m dSpm Structure

Although there are similarities between the *dSpm* in the *bt1-m* clone and *dSpm* isolated from other maize genes, such as a 3-bp target site duplication and sequence similarity at both ends of the *dSpm*, there are several differences. Approximately 6.7 kb of internal *Spm* sequence have been deleted from *bt1-m dSpm*, and ~1.7 kb of non-*Spm* sequence have been inserted just downstream of the SRR at the 5′ end of the *dSpm*. Although *dSpm* structures with insertions of non-*Spm* sequence have not been described, defective transposable elements that were apparently produced by a combination of deletion and insertion are known for the *Activator/Dissociation* (*Ac/Ds*) transposable element family (*Ds1*, Sutton et al., 1984; *Ds2*, Merckelbach et al., 1986; *waxyB4 Ds*, Varagona and Wessler, 1990). The maize *mutator* (*Mu*) family of elements is characterized by several elements that share the approximately 220-bp terminal inverted repeat but have different internal sequences (Barker et al., 1984; Schnable et al., 1989; Talbert et al., 1989; Qin and Ellingboe, 1990).

At the junction of non-*Spm* and *Spm* sequences is a short sequence also found 108 bp farther upstream in autonomous *Spm* (position 1918 to 1937 of the *bt1-m dSpm*, Figure 4). The sequence TGCA is found at the end of this short sequence and at the beginning of the 3′ *Spm* block. This arrangement suggests that sometime during the genesis of the *bt1-m dSpm* there was a deletion of the sequences between the TGCA direct repeats.

The *Spm* sequences within the *bt1-m dSpm* differ from an autonomous *Spm/En* element by 6% (Figure 4) (Pereira et al., 1986). This is much higher than between two autonomous *Spm* elements,which differ by less than 0.15% (Masson et al., 1987). The degree of divergence of the *bt1-m dSpm* from the autonomous *Spm* may reflect the length of time since they diverged and less selective pressure to maintain the sequence of a defective element. A number of polymorphisms may be allowed in defective elements with minimal effects on their response to an autonomous element. The defective *Ds1* elements of the

Ac element family differ from each other by 6% to 13% (Schiefelbein et al., 1988).

One notable change in *bt1-m dSpm* is a T-to-G mutation at the inside end of the 3′ terminal inverted repeat (Figure 4). This reduces the length of the inverted repeats to 12 bp from the 13 bp found in other *Spm* and *dSpm* elements (Schwarz-Sommer et al., 1984; Pereira et al., 1986). A derivative of *bronze-mutable 13*, which has a 2.2-kb *dSpm* insertion, contains a 2-bp deletion at the outside end of the 3′ terminal inverted repeat sequence. In this allele, *bz-m13CS6*, the frequency of somatic and germinal excision is reduced compared to the progenitor allele with the intact terminal inverted repeat (Schiefelbein et al., 1985; Raboy et al., 1989).

In *bt1-m* both of the SRR are present, although there is approximately 7% sequence divergence relative to the autonomous *Spm* sequences within the repeats of the SRR. Analysis of the transposition frequency of *dSpm* elements with internal deletions suggests that, in addition to the SRR, the GC-rich region is also necessary in *cis* for efficient excision (Masson et al., 1987). Although the *bt1-m dSpm* lacks the primary sequence of the GC-rich region, it does contain patches of related sequence, including an imperfect duplication of sequences found as repeated sequences within the GC-rich region (Figure 4).

The size and frequency of revertant sectors in developing endosperm and the production of wild-type revertants of *bt1-m* indicate that the *bt1-m dSpm* is competent for transposition in the presence of autonomous *Spm*. Because there are multiple differences between the *bt1-m dSpm* and other *dSpm* elements, and because the various *dSpm* elements are not located in the same locus, it is not possible directly to compare somatic or germinal excision frequencies of the *bt1-m dSpm* with other *dSpm* elements and deduce the quantitative effect on transposition of a particular sequence alteration in the *bt1-m dSpm*.

Masson and coworkers have noted that there is sequence similarity between some sites of the *Spm* insertion and the 12-bp repeat sequence of the SRR (Masson et al., 1991). The sequence at the site of the *dSpm* insertion in *bt1-m* matches the sequence of the consensus repeat at seven of 12 positions. In addition, the *bt1-m* site matches the *Spm* insertion site in *a1-m2* (an allele of the *anthocyaninless-1* locus) at eight of 12 positions, with 11 of 12 bases being found in common with either the *a1-m2* site or with one of the SRR individual repeats.

bt1-m Transcript Analysis

Several transcripts are produced by the *bt1-m* allele (Figure 6C). One is similar in abundance to the wild-type 1.7-kb *Bt1* mRNA but is shorter (~1.5 kb). Other transcripts are longer and less abundant. All but the longest of these RNAs fail to hybridize to the Acc125 probe from downstream of the *dSpm* insertion. Two mechanisms may account for the production of the *bt1-m* transcripts. Use of the *Spm* poly(A) addition site, which is present in the *Bt1* transcriptional orientation (Figures 3 and 4), would produce transcripts that do not extend beyond the insert. The introduction of the *dSpm* sequences within *bt1-m* primary transcripts could present alternative splice sites or activate cryptic splice sites within the *Bt1* sequences. Examples of apparent termination at a poly(A) site within an element and alternative splicing reactions involving *dSpm* element insertions have been described previously (Gierl et al., 1985; Kim et al., 1987; Raboy et al., 1989).

Bt1 DNA Clones and the Deduced Amino Acid Sequence

Comparison of the genomic and cDNA clones indicates that the genomic SalI fragment does not contain the entire gene sequence. There are two introns within the genomic SalI fragment, and the *dSpm* insertion is in an exon (it is not known whether there are other introns in the transcribed regions outside of the cloned genomic fragment). As expected for plant genes (Dean et al., 1986), there is more than one site of poly(A) addition used in *Bt1* transcripts (Figure 5).

The polypeptide deduced from translation of the long open reading frame of pBtcDNA1.7 is 447 amino acids long with a molecular mass of 47 kD. A 75-amino acid sequence at the amino terminus appears to encode a plastid transit peptide. The presence of the putative transit peptide suggests that the *Bt1*-encoded protein is imported into the amyloplasts of endosperm cells. This is not unexpected for a protein involved in starch synthesis.

There is an obvious need for the uptake of large amounts of carbon and a source of metabolic energy for starch synthesis in amyloplasts. Current knowledge of the pathway for starch synthesis in maize endosperm amyloplasts indicates that the primary substrate for glucose incorporation is ADPglucose (ADPG), and the primary pathway for production of ADPG is by way of ADPG pyrophosphorylase, an amyloplast enzyme (Echeverria et al., 1990), subunits of which are encoded by the *shrunken-2* and *bt2* loci (Bae et al., 1990; Bhave et al., 1990). According to this model, there must be a source of both ATP and glucose 1-phosphate for the production of ADPG inside the plastid. Recent experiments suggest, however, that ADPG itself may be translocated into amyloplasts, following synthesis in the cytoplasm (Pozueta-Romero et al., 1991).

Amino acid sequence similarity between *Bt1* and the AACP proteins suggests that the *Bt1* gene product functions as an adenylate translocator in amyloplasts. Cultured sycamore cells contain amyloplast proteins that are immunologically related to the *Neurospora crassa* mitochondrial adenylate translocator (Ngernprasirtsiri et al., 1989). Because the *Bt1*-encoded polypeptide also shows similarity to translocator proteins with other solute

specificities, further experiments are necessary to define the precise function of the *Bt1* gene. The deduced *Bt1*-encoded polypeptide appears to be unrelated to the chloroplast phosphate translocator (Flugge et al., 1989; data not shown).

The isolation of a genomic clone of the *bt1* locus by way of a *dSpm*-tagged allele and the subsequent isolation of wild-type *Bt1* cDNA clones have provided insight into the possible role of the *Bt1* gene in endosperm starch synthesis. Further experiments are under way to determine the intracellular location of the *Bt1*-encoded protein and its possible role as an amyloplast translocator protein.

METHODS

Genetic Stocks

The mutant alleles *bt1-R* (the recessive, reference allele) and *bt1-m* were maintained in either a W64A background or a W64A/OH43 hybrid background. Wild-type *Bt1* alleles were those present in the inbred line W64A or in a stock of the inbred line R802 containing a mutation of the *opaque-2* gene (Aukerman et al., 1991). The specific wild-type progenitor of *bt1-m* is no longer available.

Plant Materials and Nucleic Acid Isolation

DNA was isolated either from leaves of mature plants or from 2-week-old seedlings, using a modification of the procedure of Shure et al. (1983).

Tissues for RNA isolation were quick frozen in liquid nitrogen and stored at −70°C. Kernels were harvested at 18 or 22 days after pollination as indicated. In some experiments, the endosperm and embryos were separated before freezing. Seedling shoot tissue was obtained from 3-week-old plants grown in the greenhouse. Total RNA was isolated according to Cone et al. (1986). Poly(A)$^+$ RNA enrichment using oligo(dT)-cellulose was done according to Maniatis et al. (1982).

Blot Hybridizations

Probes for DNA gel blot, RNA gel blot, and plaque lift hybridizations were generated by random-primed labeling of isolated restriction fragments according to Feinberg and Vogelstein (1984) to specific activities of ~10^9 dpm/μg. The *dSpm* probe fragment was obtained by AluI digestion of DNA from a clone of *bz-m13* (see Raboy et al., 1989). Acc125 and Acc550 are fragments of the *bt1-m* genomic clone (Figure 3). The *Bt1* cDNA probes were made using the EcoRI insert fragment of pBtcDNA1.1 or pBtcDNA1.7.

DNA gel blot analysis was performed with approximately 10 μg of DNA digested with an excess of restriction enzyme. Gel treatment and blotting onto nitrocellulose (Schleicher & Schuell, Keene, NH) or nylon (Pall Biodyne, ICN Biomedicals, Irvine, CA) membrane were done as described by Maniatis et al. (1982). Hybridizations were done in a buffer containing 400 mM NaCl, 100 mM $NaPO_4$, pH 6.8, 20 mM sodium pyrophosphate, 5 mM EDTA, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 100 μg/mL single-stranded salmon sperm DNA, and either 10% dextran sulfate or 10% polyethylene glycol. Incubations were done overnight at 65°C, and final washes were done in 15 mM $NaPO_4$, pH 6.8, 5 mM sodium pyrophosphate, 5 mM EDTA, and 0.5% SDS, at 65°C.

RNA gel blot analyses were done as described by Lissemore et al. (1987). Hybridizations were done overnight at 42°C. Final washes were done at 68°C for the cDNA probes and 60°C for the Acc125 probe in a buffer containing 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.25 mM EDTA, 1.25 mM $NaPO_4$, pH 6.8, and 0.5% SDS.

Plaque lifts were performed according to Benton and Davis (1977). Hybridizations and washes were done as for DNA gel blots except that the NaCl was left out of the hybridization buffer.

Genomic Clone Isolation

DNA from seedlings homozygous for the *bt1-m* allele was digested with SalI and size fractionated by glycerol gradient velocity sedimentation. Fractions containing fragments less than 10 kb, including the 4.3-kb *Spm*-hybridizing fragment, were pooled and used to construct a subgenomic library by ligation with XhoI-digested, alkaline phosphatase-treated λZAP DNA (Short et al., 1988). Ligated DNA was packaged using Gigapack Gold (Stratagene, La Jolla, CA). A total of 1 × 10^5 recombinant phage were screened on *Escherichia coli* BB4. Fourteen positive clones were isolated and plaque purified. Three clones had 4.3-kb insertions and were rescued as pBluescript SK− plasmids using M13 helper phage (Short et al., 1988). These plasmid clones were used for subsequent restriction mapping, probe preparation, and sequence determination.

cDNA Clone Isolation

The cDNA library was prepared from endosperm RNA isolated 22 days after pollination from inbred line R802 (homozygous for a wild-type *Bt1* allele) by Karen Cone (University of Missouri, Columbia) and provided by Bob Schmidt (Aukerman et al., 1991). A portion of an amplified library was screened with the Acc125 probe and a second portion was screened with Acc550. Of approximately 200 positive clones, four were purified and their inserts were subcloned into pBluescriptII KS+ (Stratagene) or pGEM3Z (Promega, Madison, WI) for further analysis.

Sequence Analyses

Genomic clones with the SalI fragment in opposite orientations within the pBluescript SK− vector were used to generate a series of exonuclease III deletion clones (ExoIII/Mung Bean Nuclease Kit, Stratagene). Single-stranded templates were generated by rescue with helper phage (Short et al., 1988) and sequenced using modified T7 polymerase, ^{35}S-dATP, and vector-specific primers (Sequenase Sequencing Kit, United States Biochemical, Cleveland, OH). The complete, overlapping sequence was determined for both strands. The cDNA clone pBtcDNA1.7 was also

completely sequenced using the same approach. DNA sequence analysis was performed using software from DNASTAR, Inc. (Madison, WI). Both the wild-type *Bt1* cDNA sequence (acquisition No. M79333) and the *bt1-m dSpm* sequence (acquisition No. M79334) have been entered in the GenBank DNA sequence data base.

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