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 (Btl) 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 Btl cDNA probe, indicated that Btl 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 Btl 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; Schmid 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.
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).

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 Sail 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 F2 family segregating for bt1-m and Bt1 alleles. In 25 F2 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 F2 progeny are shown in Figure 2A.

The Sail-digested DNA from bt1-m was enriched for fragments less than 10 kb and cloned into the Xhol 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 Sail 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 Sail- or KpnI-digested DNA from plants in the
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 Sall. 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 Btl 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 Btl'-1 allele contained only the 1-kb fragment. The difference in Sall fragment length between bt1-m (4.3 kb) and the Btl'- 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 Btl' alleles. In a segregating F2 family, heterozygous bt1-m/Bt1 samples contained both fragments, whereas homozygous Btl' 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 dSpms (Banks et al., 1985; Gierl et al., 1985; Schiebelin et al., 1985; Schwarz-Sommer et al., 1985a; Masson et al., 1987; Cuyper 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 1932 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 dSpms (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 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 sequence. Also indicated are terminal inverted repeats (overlined with arrows), repeats of the SRR sequence near the dSpm 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 sequence.
The pBtcDNAl.7 clone sequence (cDNA) is aligned with the deduced amino acid sequence (pro). Sal1 sites mark the ends of the sequence. Two cDNA clones (C, filled triangle), and two poly(A) sites found in covered related to the consensus repeat of the Btl mRNA migrates as a broad band btl-m.

amino acid sequence that matches the consensus cleavage site of the transit peptide (underlined) with the putative cleavage site (arrow) clone (open triangles), position of the and two putative membrane-spanning domains of the protein (underlined).

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

In samples from the inbred lines W64A and R802, the Btl 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 Btl mRNA was detected in RNA from seedlings (Figure 6A, lanes 6 and 7 and data not shown). Within kernels, Btl mRNA was detected in the endosperm but not in the embryo (Figure 6B).

There are Btl-hybridizing RNAs of at least five lengths in btl-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 btl-R reference allele was also analyzed. The nature of the mutation that caused the btl-R allele is not known. The allele produces an mRNA that is less abundant than the wild-type transcripts in W64A and R802. In addition, the btl-m transcript appears to be slightly larger than that of the wild type (Figure 6A and data not shown).

Two wild-type revertant alleles of btl-m were analyzed and, as expected, were found to produce a single abundant Btl transcript of similar size as the wild-type allele from W64A stocks (Figure 6A, lanes 4 and 5). This result
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 Sall 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); mitochrondia 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
The deduced amino acid sequence encoded by tive amino acid differences are indicated by colons (Dayhoff, 1978; AACP. acids are written between the aligned sequences, and conserva-

Figure 7. Amino Acid Sequence Comparison between Btl and AAC

The deduced amino acid sequence encoded by Btl, starting at the putative transit peptide cleavage site (Figure 5), is aligned with the yeast AAC (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).

DISCUSSION

btl-m Genomic and Btl cDNA Molecular Clones

The btl-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 Btl function (Figure 1). The germinal instability of btl-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 btl-m allele to obtain molecular clones of the btl 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 btl locus was based on the genetic linkage of the genomic fragment with the btl-m allele and the disappearance of the restriction fragment harboring the dSpm insertion in four wild-type revertants of btl-m. In addition, the mRNA identified using the cloned cDNA as a probe is altered in lines harboring either btl-m or btl-R alleles and is expressed in the expected tissue-specific manner in lines with wild-type Btl 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).

btl-m dSpm Structure

Despite the similarities between the dSpm in the btl-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 btl-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 mutant (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 SRR sequences is a potential membrane-spanning domain (Kyte and Doolittle, 1982; Eisenberg et al., 1984). Two putative membrane-spanning domains were found (Figure 5).
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 germinial 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 germinial 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 4C). 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 transcripational 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 Sal1 fragment does not contain the entire gene sequence. There are two introns within the genomic Sal1 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 btl 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 btl-R (the recessive, reference allele) and btl-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 btl-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 ~10⁶ dpm/μg. The dSpm probe fragment was obtained by Alul digestion of DNA from a clone of bz-m13 (see Raboy et al., 1989). Acc125 and Acc550 are fragments of the btl-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 NaPO₄, 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 NaPO₄, 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 NaPO₄, 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 btl-m allele was digested with Sall 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 Xhol-digested, alkaline phosphatase-treated XZAP DNA (Short et al., 1988). Ligated DNA was packaged using Gigapack Gold (Stratagene, La Jolla, CA). A total of 1 × 10⁶ 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 Sall fragment in opposite orientations within the pBluescript SK− vector were used to generate a series of exonuclease III deletion clones (ExolIII/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, 32P-GATP, 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
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