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Identification of *Mutator* insertional mutants of starch-branching enzyme 1 (*sbe1*) in *Zea mays* L.

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Abstract

Starch-branching enzymes (SBE) alter starch structure by breaking an α-1,4 linkage and attaching the reducing end of the new chain to a glucan chain by an α1,6 bond. In maize, three isoforms of SBE have been identified. In order to examine the function of the SBEI isoform, a reverse-genetics PCR-based screen was used to identify a mutant line segregating for a *Mutator* transposon within *Sbe1*. Compared to wild-type controls, *Sbe1* transcripts accumulate at extremely low levels in leaves of the homozygous mutant. Antibodies failed to detect SBEI in leaf tissue of mutants or wild-type controls. In contrast, the level of SBEI in endosperm is undetectable in homozygous mutants while easily detected in wild-type controls. Starches extracted from mutant leaves and endosperm have structures indistinguishable from starches of wild-type controls as determined by size-exclusion chromatography (SEC) of intact starch and high-performance SEC of debranched starch. To investigate the possibility of compensation for the lack of SBEI by expression of the homologous sequence reported by Kim *et al.* (1998), a genomic fragment (*Sbe1b*) of this sequence was cloned. Northern hybridizations of mutant leaf, root, tassel, endosperm and embryo tissues with non-specific *Sbe1b* probes failed to reveal expression of the homologous sequence. These results suggest that the homologous sequence is not compensating for a lack of SBEI in *sbe1::Mu* mutants. Further study of this *sbe1* mutation in the presence of other genetic mutations may help to understand the role of SBEI in determining starch structure in leaves and endosperm.

Abbreviations: DAE, days after emergence; DAP, days after pollination; SBE, starch-branching enzyme

Introduction

Starch biosynthesis occurs through the action of four enzymes: ADP-glucose pyrophosphorylase (glucose 1-phosphate adenylyltransferase, EC 2.7.7.27), starch synthase (EC 2.4.1.21), starch-branching enzyme (1,4-α-glucan branching enzyme, EC 2.4.1.18) and debranching enzyme (EC 2.4.1.41) (reviewed in Smith *et al.*, 1997). Multiple isoforms of each of these enzymes have been identified which differ in their expression pattern and *in vitro* properties. These enzymes create two forms of starch: amylose, an essentially linear glucan chain consisting of α-1,4 linkages, and amylopectin, a highly branched glucan chain with multiple branch-points formed by α-1,6 linkages.

Starch-branching enzymes (SBEs) catalyze the formation of branch-points within glucan chains by hydrolyzing an α-1,4 linkage and reattaching the chain to a glucan chain by an α-1,6 bond. Introduction of branches into the glucan chain increases the number of nonreducing ends, thereby facilitating starch synthesis. Three isoforms of starch-branching enzymes, SBEI, SBEIIa and SBEIIb, have been identified in maize (Boyer and Preiss, 1978; Dang and Boyer, 1988). The *in vitro* properties of SBEI differ from those of SBEIIa and SBEIIb (Guan and Preiss, 1993;

Takeda *et al.*, 1993). SBEI has a lower K_m for amylose and tends to produce shorter constituent chains than SBEIIa or SBEIIb when reacted with amylose *in vitro*.

The genomic DNA and/or cDNA sequences encoding each of the three isoforms have been cloned and sequenced (Baba *et al.*, 1991; Fisher *et al.*, 1993, 1995; Gao *et al.*, 1997, Kim *et al.*, 1998, 1999; Blauth *et al.*, 2001). The expression pattern of the three isoforms differs considerably (Gao *et al.*, 1996). *Sbe1* is expressed moderately during middle stages of kernel development (12–20 DAP), strongly during the later stages of kernel development (22–43 DAP) and is moderately expressed in vegetative tissues as determined by northern hybridization with the *Sbe1* cDNA. In contrast, *Sbe2a* is expressed moderately in endosperm tissues and more strongly in vegetative tissues, while the expression of *Sbe2b* is restricted to the endosperm embryo and tassels. Kim *et al.* (1988) discovered a genomic sequence homologous to *Sbe1* through Southern blot analysis using the *Sbe1* cDNA as a probe. This homologous sequence maps to a locus separate from that of *Sbe1* (chromosome 6 vs. chromosome 10). This sequence has not been isolated nor has it been shown whether this sequence is expressed to form an active protein *in vivo* or *in vitro*. This sequence will be referred to as *Sbe1b* to differentiate it from *Sbe1*.

Mutations for the three SBE isoforms resulting in the loss of enzyme activity have been identified for only SBEIIa and SBEIIb (Vineyard and Bear, 1952; Fisher *et al.*, 1993; Stinard *et al.*, 1993; Blauth *et al.*, 2001). Compared to wild-type controls, the *Sbe2a* mutant displays an early senescence phenotype and accumulates leaf starch with a reduced amount of branching and longer branches (Blauth *et al.*, 2001). In subsequent backcross generations however, the early senescence phenotype was eliminated, indicating that it was due to a tightly linked locus and not the *sbe2a::Mu* insertion (personal communication, Yuan Yao). Compared to wild-type controls, the *Sbe2b* mutant, *ae*, accumulates 20% less starch within the endosperm, which has a reduced amount of branching and longer branches (Garwood *et al.*, 1976). The discovery of a mutant lacking SBEI activity would greatly aid our exploration of the role of each of these isoforms in determining the final starch structure.

To discover such a mutant we utilized a reverse-genetics strategy using the transposon family *Mutator*. This transposon family has been useful in mutagenesis studies in maize since it shows exceptionally high rates of forward mutation (reviewed in Chandler and Hardeman, 1992). Reverse-genetics strategies using *Mutator* transposons have identified mutants of previously characterized genes, *An1* (Benson *et al.*, 1995), *Zag1* (Mena *et al.*, 1996), and *Sbe2a* (Blauth *et al.*, 2001). In the investigation reported here, we identify and characterize *Mutator* insertional mutants for *Sbe1* in order to gain an understanding of the role of SBEI in starch synthesis.

Materials and methods

Identification of putative sbe1::Mu *mutants*

Plant populations segregating for multiple *Mutator* (*Mu*) insertions were screened for the presense of a *Mutator* transposon within *Sbe1* using a *Mu*-TIR-specific primer (*Mu*TIR9242: 5′-AGAGAAGCCAACGCCA(AT)CGCCTC(CT)ATTTCGTC-3′) and several *Sbe1*-specific primers (1A1: 5′-CTGGACCCCAAGCTGGAGATATTCAAG-3′, 1A3: 5′-CTCCACGTATCTATGAAGCCCATGTAGGT-3′, 1A4: 5′-TGGGATGCGATTTGCCTGGGAAATACAG-3′, 1A5: 5′-CTCTGGAAGCTTTGACGTCGATGCTC-3′). Amplification was conducted by personnel at Pioneer Hi-bred International in a 50 μl reaction containing 10% sucrose, 10 mM $MgCl_2$, 10% DMSO, 0.25 mM dNTP, 1 μM MuTIR primer, 1 μM target primer, 1× HotTub Buffer (Amersham), and 50 ng DNA. Reactions were denatured at 94 °C for 2 min and then run for 35 cycles at 94 °C for 2 min, 62 °C for 1 min, 72 °C for 2 min, with a final extension of 2 min at 72 °C. Of the total 50 μl amplification products, 20 μl were separated and visualized on a 2% agarose gel. Out of ca. 40 000 plants screened, 17 were identified as potentially containing *Mu* insertions within *Sbe1*. F_2 seed from these 17 families were analyzed further to identify *Mu* insertional mutants for *Sbe1* (*sbe1::Mu*).

Plant material

Plants were field-grown in the summer at the Pennsylvania State University Horticultural Research Farm (Rock Springs, PA). Plants were grown in the fall and spring under standard greenhouse conditions with supplemental lighting on a 14/10 h day/night cycle. Plants identified as containing *Mu* insertions within *Sbe1* were backcrossed to W64A in order to develop mutants with a known genetic background (Figure 1).

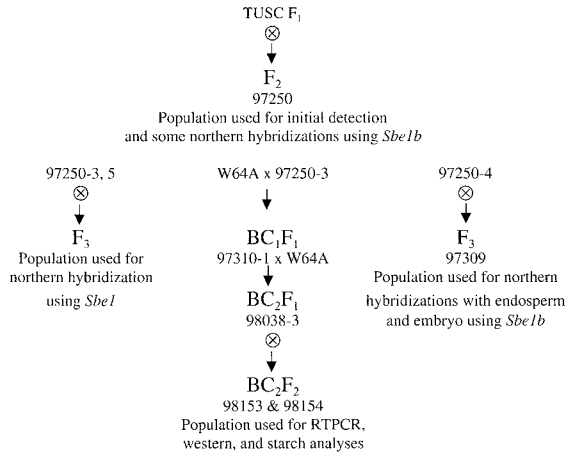

Figure 1. Pedigree of populations used in this study. Independent F_1 plants were identified by Pioneer personnel (TUSC F_1). Self-progeny of these plants were self pollinated and also crossed to inbred line W64A to create a BC_2F_2 population for analysis.

Genotyping

Genomic DNA was extracted from leaf tissue at about 10 days after emergence (DAE) following the microprep procedure described by Dellaporta (1994). The resulting pellet was dissolved in 200 μl TE, of which 1 μl was used for amplification via PCR. Each 25 μl reaction contained 2.5 U *Taq* DNA polymerase (Promega), 1× Promega buffer, 2.5 mM $MgCl_2$, 0.4 μM each primer, and ca. 50 ng target DNA. To detect *sbe1::Mu* alleles, the *Mu*-TIR primer was used in combination with one of two *Sbe1*-specific primers (1A4, 1A5). To detect *Sbe1* alleles lacking a *Mutator* transposon at a specific area, two *Sbe1*-specific primers flanking the area in question were used in combination (e.g. 1A4 and 1A5). After heating the reactions to 80 °C, 1 mM total dNTP was added to each reaction before resuming the following conditions in a thermal cycler (Ericomp, San Diego, CA): one cycle of 5 min at 94 °C, 40 cycles of 45 s at 94 °C, 1 min at 60 °C, 2 min at 72 °C, with a final extension of 5 min at 72 °C. Amplification products were separated on a 2% agarose gel using standard procedures (Sambrook *et al.*, 1989).

Amplification products were probed with *Sbe1* and sequenced to verify the initial identification of *sbe1::Mu* mutants. After gel electrophoresis, amplification products were transferred onto nylon membrane by capillary transfer following manufacturer's instructions. These filters were probed with the full-length *Sbe1* cDNA clone (Fisher *et al.*, 1995) labeled by random hexamer labeling (Boehringer Mannheim, Indianapolis, IN) under the following hybridization conditions. Filters were prehybridized at 65 °C for 1 h in 0.5 M Na_2HPO_4 pH 7.2, 7% SDS, and 40 μg/ml denatured salmon sperm DNA (Church and Gilbert, 1984). Labelled probe was added to the prehybridization solution and incubated at 65 °C for 3 h. Filters were washed once in 5% SEN (5% SDS, 1 mM EDTA, 0.04 M Na_2HPO_4 pH 7.2) and twice in 1% SDS, 1 mM EDTA, 0.04 M Na_2HPO_4 pH 7.2) for 15 min at 65 °C and were exposed for ca. 2 h. Amplification products to be sequenced were excised from the agarose gel and purified using a Millipore filter unit (Millipore) before being sequenced by the DNA Sequencing Facility at Pennsylvania State University.

Genomic Southern hybridizations

Genomic DNA was prepared from 30 DAE leaf tissue following the miniprep method described by Dellaporta (1994). Genomic DNA (10 μg) was digested with restriction enzyme *Bam*HI, separated on a 0.8% agarose gel, and transferred onto Hybond-N+ as described above. Hybridization conditions were as described above with prehybridization incubation extending to 4 h, hybridization to 24 h, and exposure to ca. 2 days.

RNA expression analysis

Total RNA was isolated from 30 DAE leaf tissue and 20 days after pollination (DAP) endosperm as described by de Vries *et al.* (1988). Total RNA was fractionated, transferred to Hybond-N+ as described above, and probed following the same methods as in genomic Southern hybridizations with RNase-free solutions. RT-PCR was performed using a Superscript preamplification system (Gibco-BRL) followed by PCR amplification as described by the manufacturer using gene-specific primers to amplify *Sbe2a* transcripts in the region upstream of the *Mu* insertion (1A3 and 1A8: CTATTTCACCCATCGACCACTCAGAGTC) and at the point of insertion (1A4 and 1A5). Amplification products were separated on 2% agarose gels, excised, purified and sequenced as described above.

Protein and starch granule extractions

Total protein and starch were extracted from a 4 g wet weight sample of 30 DAE leaves. Tissue was harvested at the end of the 14 h light cycle, flash-frozen with liquid nitrogen and stored at −70 °C. Tissue was then

ground in liquid nitrogen with a mortar and pestle, and 1 ml/g tissue of protein extraction buffer containing 100 mM sodium citrate pH 7.0, 5 mM dithioerythritol (Dang and Boyer, 1988) and protease inhibitors (Complete, Boehringer Mannheim) was added. The homogenate was centrifuged at 4 °C for 10 min at 6000 × *g*. The pellet was resuspended in 10 ml of extraction buffer and homogenized (Polytron PT10/35, Kinematica, Switzerland). The homogenate was centrifuged as above. The supernatant served as a crude extract of total proteins. Starch granules contained in the pellet were resuspended in cold water, filtered successively through 400 μm and 200 μm mesh and centrifuged as described above. The resulting pellet was washed four times with acetone, and dried overnight at 40 °C.

For total proteins extracted from 20 DAP endosperm tissue, 0.5 g wet weight of endosperm dissected from the top half of developing ears were flash frozen with liquid nitrogen and ground in 500 μl of protein extraction buffer as above. This homogenate was centrifuged at 4 °C for 10 min at 6000 × *g*, the supernatant serving as a crude extract of total proteins. Starch granules from 40 DAP endosperm were isolated as described by Boyer *et al.* (1976) from the bottom half of the same ears used for protein extraction.

Western analysis

Total protein concentrations from extracts were estimated using the Bradford method (Stoscheck, 1990). From these crude extracts of total proteins, 10 μg were fractionated on a 7.5% Tris-HCl polyacrylamide gel at 90 V for 4 h. Proteins were then blotted onto Immunobulin PVDF membrane (Millipore) using a HEP-1 semi-dry electroblotting device (Owl Scientific) and Towbin's buffer (Towbin *et al.*, 1979). Immunodetection of SBEI, SBEIIa and SBEIIb was performed using an ECL western blotting detection kit (Amersham). Antibodies were generated against peptides from unique regions of SBEI (amino acids 502–520: DYLKNKDDSEWSMGEIAHT), SBEIIa (amino acids 77–91: EASSGVEAEERPELS) and SBEIIb (amino acids 143–161: RRIRSDIDEHEGGLTAFSR) by injection of multiple antigen peptides in Freund's adjuvant into New Zealand white rabbits (Research Genetics, Huntsville, AL). The antibody to SBEIIb is isoform-specific, detecting a protein of 80 kDa. However the antibody to SBEIIa binds to SBEIIa (90 kDa) and as yet unidentified proteins; the antibody to SBEI binds to SBEI, SBEIIa and SBEIIb (84 kDa, 90 kDa and 80 kDa, respectively) and other as yet unidentified proteins (Martha James, personal communication).

Starch analysis

The starch granules were solubilized in 90% DMSO by heating in a boiling water bath for 3 h with periodic vortexing. Insoluble material consisting mainly of small particles of cellular debris were pelleted by microcentrifugation for 5 min at full speed. The starch was precipitated from the supernatent by adding ethanol to 80%, and pelleted by centrifuging for 5 min at 2500 × *g*. The starch was washed once with 80% ethanol and three times with acetone and dried overnight at 50 °C (Klucinec and Thompson, 1998).

Size-exclusion chromatography of dispersed starch was performed using Sepharose CL-2B (Supelco) with 0.01 M NaOH pH 12 as the mobile phase, following the method of Klucinec and Thompson (1998). A total number of 100 fractions of 5 ml each were collected. The total carbohydrate content of the fractions was determined as described by DuBois et al. (1956).

High-performance size-exclusion chromatography (HPSEC) of debranched starches was conducted using the method of Klucinec and Thompson (1998). Briefly, between 2.5 mg and 5 mg of starch was mixed with a sufficient quantity of 90% DMSO to result in a 5% starch concentration. The mixtures were heated in a boiling water bath for 10 min to disperse the starch. Samples were diluted 1:8 in 50 °C 0.05 N sodium acetate buffer pH 3.75. After cooling to 37 °C, 0.5 units of isoamylase (Megazyme USA, Bozeman, MT) were added; these samples were held at 37 °C with constant agitation for 24 h. The digested samples were diluted 1:10 with DMSO, heated in a boiling water bath for 10 min, and centrifuged at 6000 × *g* for 10 min. A 50 μl sample was injected into the chromatograph.

Construction and screening of a sub-genomic library for Sbe1b *genomic DNA*

Genomic DNA (30 μg) from maize inbred W64A was digested with *Bam*H1 and separated on a 0.8% agarose gel. DNA fragments approximately 3.2 kb in size were isolated from the gel and ligated into *Bam*HI/CIAP-treated ZAP Express vector (Stratagene, La Jolla, CA). Packaging and amplification of the library was performed as described in the manufacturer's instructions. About 6×10^4 pfu were screened with ^{32}P-labeled 0.6 kb *Sbe1* genomic DNA as described in Kim

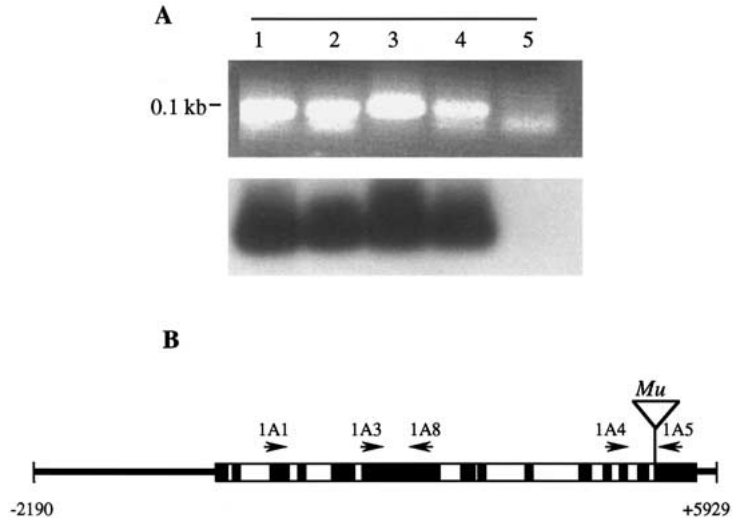

Figure 2. Detection of the presence and location of a *Mutator* transposon within *Sbe1*. A. Detection of the *sbe1::Mu* mutant allele segregating in 5 individuals from line 97250 via PCR with primers 1A5 and *MuTIR* results in a 0.1 kb fragment. Gel electrophoresis (top) and subsequent hybridization to the full-length cDNA *Sbe1* clone (bottom). B. Relative location of primer binding sites A1A, 1A3, 1A8, 1A4, 1A5 and *Mutator* insertions in *Sbe1*. The thick line represents 5′ and 3′ UTR, black boxes represent exons and open boxes indicate introns. The position of the *Mutator* insertions (*Mu*) is indicated for lines 98250 (position +5201/2) and 98154 (position +5210/1) as determined via PCR using *MuTIR* and *Sbe1* primers and subsequent sequencing of amplification products.

et al. (1998). Positive plaques hybridizing strongly to the probe were selected and purified through three rounds of screening. Phagemids containing the inserts were excised *in vivo* from the positive plaques with ExAssist helper phage with XLOLR strain following the manufacturer's instructions (Stratagene). DNA sequences were determined with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and were analyzed with software from DNASTAR (Madison, WI).

Results

Identification of the sbe1::Mu *mutant*

Seventeen unrelated plants were identified by Pioneer Hi-bred International as potentially containing a *Mutator* insertion within *Sbe1* (*sbe1::Mu*). F_2 progeny from one of these 17 plants (line 97250) were found to segregate for the presence of *sbe1::Mu* as determined by amplification with *Sbe1*- and *MuTIR*-specific primers and subsequent hybridization to the full-length *Sbe1* cDNA clone (Figure 2). Comparison of DNA sequence data from these amplification products to that of the *Sbe1* genomic sequence indicates the presence of a *Mutator* (*Mu*) transposon within the 14th exon in the 761st codon (Figure 2B).

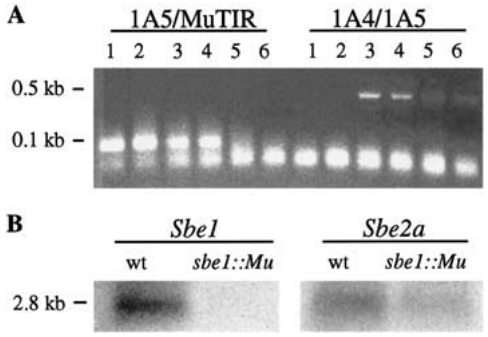

Figure 3. Selection of homozygous *sbe1::Mu* mutants and wild-type controls and subsequent analysis of *Sbe1* expression. A. Detection of *sbe1::Mu* allele via PCR using primers 1A5/*MuTIR* results in a 0.1 kb fragment, and detection of wild-type *Sbe1* allele via PCR using primers 1A4/1A5 results in a 0.5 kb fragment. Plants analyzed include homozygous mutant (lanes 1, 2), heterozygous (lanes 3, 4), and homozygous wild-type (lanes 5, 6) plants from line 97250. Homozygous mutant and wild-type individuals were self-pollinated; the resulting progeny were used for northern hybridizations below. B. Expression of *Sbe1* in seedlings of homozygous mutant (*sbe1::Mu*) and wild-type control (wt) plants. Northern blots were probed with the full-length cDNA *Sbe1* clone; the cDNA *Sbe2a* clone was used as a control.

To verify the identification of a *sbe1::Mu* mutant and the successful disruption of *Sbe1* stable expression resulting from the *Mu* insertion, we identified homozygous mutants and wild-type controls from line 97250 (Figure 3A) and investigated the expression of *Sbe1*

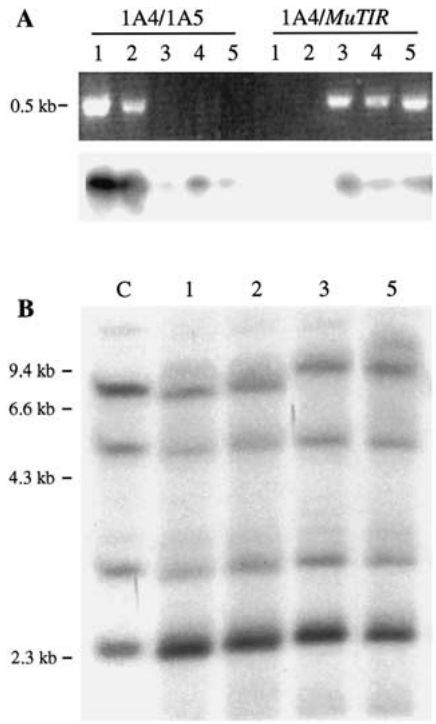

Figure 4. Selection of homozygous *sbe1::Mu* mutants (lanes 3, 4, 5) and wild-type controls (lanes 1, 2) in a BC_2F_2 population. A. PCR amplification using primers 1A4 and 1A5 was used to detect the wild-type allele, while amplification with primers 1A4 and *MuTIR* were used to detect the mutant allele in lines 98153 and 98154. Amplification products were separated using gel electrophoresis (top) and were subsequently hybridized to the *Sbe1* cDNA clone (bottom). B. Southern hybridization of genomic DNA with the *Sbe1* cDNA clone, showing insertions in homozygous mutants. Inbred line W64 is shown as a control (C). Molecular weight standards are indicated to the left of figure.

in their self progeny's seedling tissue using northern hybridization with the full-length *Sbe1* cDNA clone as a probe (Figure 3B). *Sbe1*, or sequences highly homologous to *Sbe1*, are expressed at a moderate level in full-sibling control plants containing wild-type *Sbe1* alleles, whereas their expression is undetectable in homozygous *sbe1::Mu* mutants.

Characterization of the sbe1::Mu *mutant*

In order to determine the effects of a lack of SBEI activity on the structure of leaf and endosperm starch, homozygous mutant plants along with homozygous wild-type controls were selected from a BC_2F_2 population (lines 98153 and 98154). This population was produced from the cross between the originally identified mutant described above, and the inbred line W64A (Figure 1). Three mutant plants and two wild-type controls were selected by PCR with *Sbe1*- and *Mu*TIR-specific primers (Figure 4A). The resulting bands were isolated and sequenced to verify the position of the *Mu* insertion. These results indicate that the *Mu* transposon was in the 14th exon at a position 9 nucleotides further into the exon than identified in line 97250 (Figure 2B), suggesting transposon movement with a lack of a footprint at the original insertion site. Although germinal excisions of *Mu* elements are rare, the lack of a footprint at the excision site is not uncommon (Chandler and Hardemon, 1992).

To check for multiple *Mu* insertions within *Sbe1* or its homologous sequence, DNA was isolated from 30 DAE leaves, blotted, and probed with the *Sbe1* cDNA clone which has been shown by Kim *et al.* (1998) to bind to both sequences (Figure 4B). Results indicate that two homozygous mutants (lanes 3 and 5) have insertions in the 8 kb bands, whereas wild-type plants lack these insertions. The third homozygous mutant (lane 4) was not included due to degradation of the DNA sample. This 8 kb fragment has been previously shown to contain the 3′ end of the gene including the 14th exon (Kim *et al.*, 1998) and so likely represents the *Mu* insertion detected via PCR. No other insertions are evident within *Sbe1* or the bands corresponding to *Sbe1b* as identified by Kim *et al.* (1998).

To investigate the expression of *Sbe1* in these mutants, RNA was extracted from 30 DAE leaves and analyzed using RT-PCR (Figure 5A). Reactions using *Sbe1* primers upstream from the point of insertion yield amplification products both with *sbe1::Mu* mutants and their wild-type controls. However, when these reactions are repeated using *Sbe1* primers that flank the *Mu* insertion, transcripts are easily detected in wild-type controls, but difficult to detect in *sbe1::Mu* mutants, although products from both reactions result in bands of the same size. This low level of *Sbe1* transcripts detected in the *sbe1::Mu* mutants may be the result of transcription of the mutant allele followed by splicing of the insertion or due to rare excision events restoring the *Sbe1* DNA sequence. These results suggest that the *Sbe1* transcript in *sbe1::Mu* mutants is produced at a very low level, or that the transcripts are unstable.

Western analysis was conducted to ensure the absence of SBEI in tissues of *sbe1::Mu* mutants from which the starch is to be characterized (Figure 5B). Extractions from 30 DAE leaves failed to show detectable levels of SBEI in *sbe1::Mu* mutants or wild-type controls. Western analysis using extractions from 20 DAP endosperm show that SBEI is not detectable in

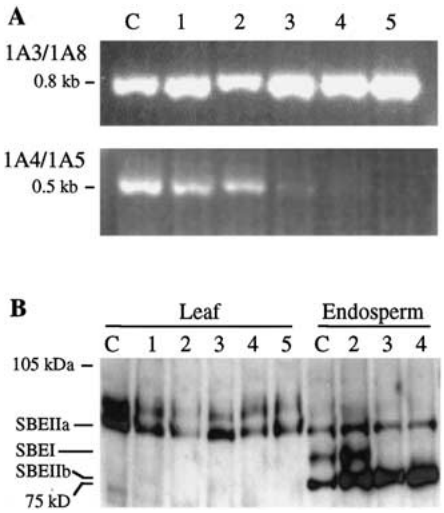

Figure 5. Expression of *Sbe1* in *sbe1::Mu* mutants (lanes 3, 4, 5), full-sibling wild-type controls (lanes 1, 2) in a $BC2F_2$ population and the inbred line W64 (C). A. RT-PCR of the *Sbe1* locus with 30 DAE leaf tissue from lines 98153 and 98154. Sequence upstream of the *Mutator* insertion was amplified using primers 1A3 and 1A8 (top); sequence at the insertion site was amplified using primers 1A4 and 1A5 (bottom). B. Western analysis of with 30 DAE leaf tissue (Leaf) and 20 DAP endosperm tissue (Endosperm) from lines 98153 and 98154. The SBEI antibody used is not isoform-specific, but isoform-specific antibodies were used to verify the identity of SBEI, SBEIIa and SBEIIb.

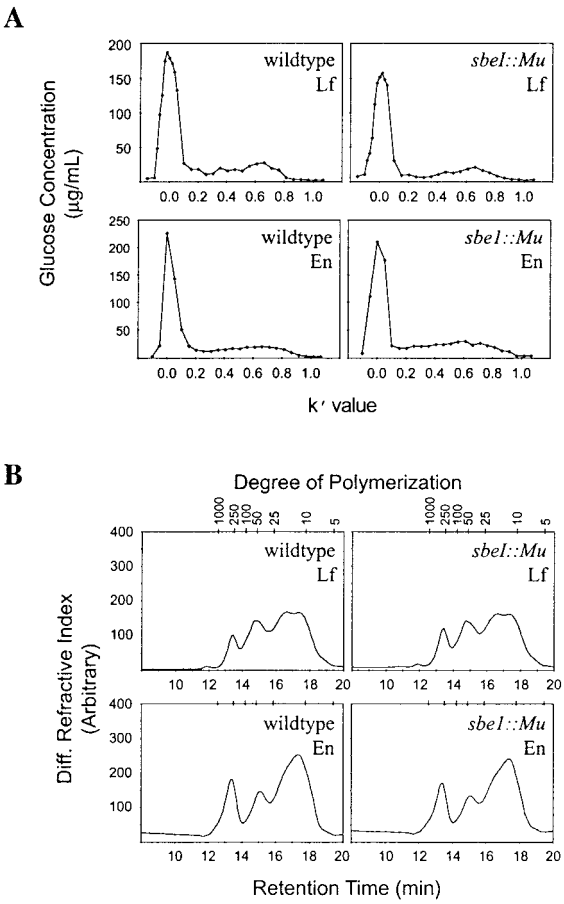

Figure 6. Analysis of starch samples from *sbe1::Mu* 30 DAE leaves and 20 DAP endosperm from a BC_2F_2 population. A. Size-exclusion chromatography (SEC) of whole starches from leaves (top) and endosperm (bottom) from lines 98153 and 98154. Starches from homozygous *sbe1::Mu* mutants are shown to the right, while starches from their full-sibling wild-type controls are shown to the left. B. High-performance SEC (HPSEC) of whole starches from leaves (top) and endosperm (bottom) from lines 98153 and 98154. Starches from homozygous *sbe1::Mu* mutants are shown to the right, while starches from their full-sibling wild-type controls are shown to the left.

sbe1::Mu mutants, while SBEI is present at high levels in wild-type controls. Levels of SBEIIa and SBEIIb in leaves and endosperm of *sbe1::Mu* mutants are similar to those found in wild-type controls. Visible leaf and kernel phenotypes of the mutants do not differ from those of wild-type controls.

The structure of the starch from leaves and endosperm of *sbe1::Mu* mutants and wild-type controls was determined by size-exclusion chromatography (SEC) of intact starch and high-performance SEC (HPSEC) of debranched starch (Figure 6). SEC of both leaf starch and endosperm starch produced similar chromatograms for *sbe1::Mu* mutants and wild-type controls (Figure 6A). Similarly, HPSEC of both leaf starch and endosperm starch produced indistinguishable chromatograms for *sbe1::Mu* mutants and wild-type controls (Figure 6B).

Isolation of a genomic fragment homologous to Sbe1

One possible explanation for the failure to detect differences in starch structure caused by the *sbe1::Mu* mutation is compensation for the lack of SBEI by activity of a protein produced from the homologous sequence reported by Kim *et al.* (1988). To investigate this possibility, a genomic fragment of this sequence (*Sbe1b*) was cloned. According to genomic Southern blot analysis, part of *Sbe1b* genomic DNA runs at approximately 3.2 kb in size on an agarose gel when digested with the *Bam*HI restriction enzyme (Kim *et al.*, 1998). Based on this information, we created a sub-genomic library of ca. 3.2 kb *Bam*HI DNA fragments. Screening of the library with the ^{32}P-labeled 0.6 kb *Sbe1* genomic DNA (Kim *et al.*, 1998) identified seven positive plaques which strongly hybridized to the probe. DNA sequence analysis after *in vivo* excision of the plaques indicated that they are all identical

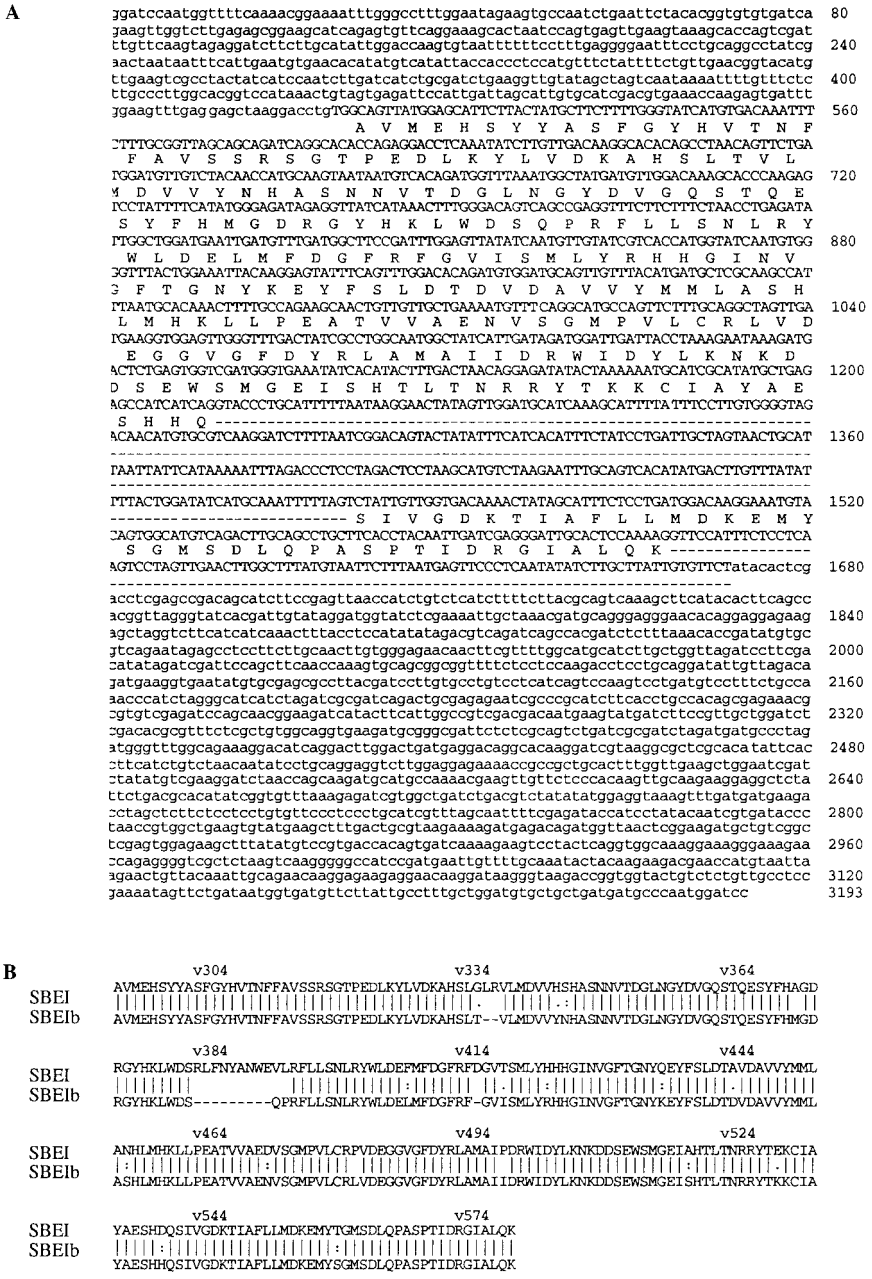

Figure 7. Sequence of the *Sbe1b* genomic DNA fragment and SBEIB deduced amino acid sequence. A. Nucleotide sequence of the *Sbe1b* genomic DNA *Bam*HI fragment. The region exhibiting 87.4% sequence similarity with the *Sbe1* gene (Kim *et al.*, 1999) is shown in capital letters, while flanking regions are presented in lower-case letters. Nucleotides are numbered at the right. The deduced amino acid sequences are shown below the string of exon sequences. The dashes indicate putative intron sequences. B. Alignment of deduced amino acid sequences between maize *Sbe1* and *Sbe1b* conserved regions (exons 6 and 7). Identical amino acids are indicated with vertical bars, similar amino acids with colons and related amino acids with dots. Dashes indicate gaps to maximize alignment and numbers represent the positions of amino acids from the translation initiation site of the *Sbe1* cDNA (Fisher *et al.*, 1995).

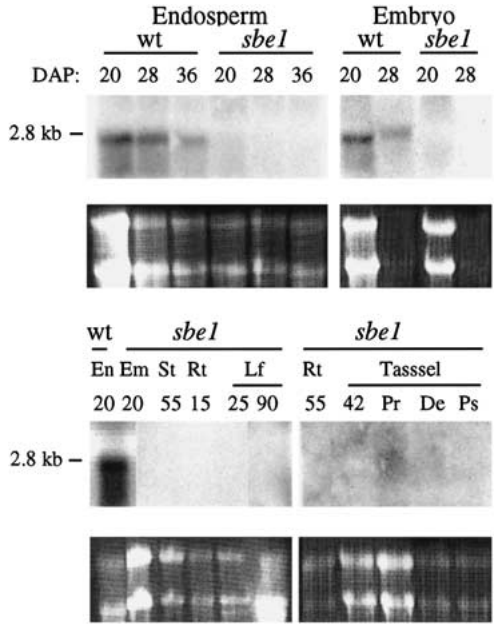

Figure 8. Northern hybridization of *sbe1::Mu* mutants (*sbe1*) and wild-type controls (wt) using the genomic fragment of the sequence homologous to *Sbe1* as a probe. RNA was sampled from various tissues: endosperm (En, 20, 28 and 36 DAP from line 97309), embryo (Em; 20 and 28 DAP from line 97309), Stem (St; 55 DAE from 97250-3), Root (Rt; 15 and 55 DAE from 97250-3), Leaf (Lf; 25 and 90 DAE from 97250-3), and Tassel (Ts; 42 DAE; Pr, pre-dehiscence; De, dehiscence; Ps, post-dehiscence; from 97250-3). For each autoradiogram, gel electrophoresis results are shown below, indicating levels of total RNA. Each of two hybridization events (top and bottom) included a 20 DAP wild-type endosperm sample as a positive control.

clones which have a 3193 bp insert containing *Sbe1*-like DNA sequence (Figure 7). Sequence alignment of the *Sbe1b* genomic DNA with the maize *Sbe1* genomic clone revealed a conserved region in the middle of the *Sbe1b* clone, which is 1220 bp long and shares 87.4% identity. However, DNA sequences surrounding the conserved region differ significantly from each other. The conserved region spans from inside exon 6 to intron 7 of the *Sbe1* genomic clone. Consistent with the DNA sequence comparison, the amino acid sequence deduced from the conserved exon regions (Exons 6 and 7) of the *Sbe1b* genomic clone shares 88.2% overall similarity with that of the *Sbe1* gene (Figure 7B).

Northern analysis for Sbe1b *expression*

To investigate the possibility of a *Sbe1b* protein compensating for the loss of SBEI in *sbe1::Mu* mutants, studies were conducted to detect transcripts of the homologous sequence in *sbe1::Mu* mutants (lines 97250 and 97309). RNA blots were probed with the genomic *Sbe1b* fragment which will also hybridize to *Sbe1* (Figure 8). Homozygous *sbe1::Mu* mutants do not express detectable amounts of transcripts from either sequence in endosperm, embryo, stem, root, leaf or tassel tissues.

Discussion

In this study and in an earlier study of *sbe2a::Mu* mutants (Blauth *et al.*, 2001), SBEI was not present in wild-type leaves at levels detectable via western hybridization. However, Dang and Boyer (1988) attributed starch-branching activity in leaves to both SBEIIa and SBEI based upon their elution profile as compared to that from endosperm tissue. RNA transcripts that hybridize to the full-length *Sbe1* cDNA were detected via northern hybridization in wild-type leaves by Gao *et al.* (1996), and in wild-type seedlings in this study. We also detected transcripts in leaves that were amplified via RT-PCR using primers designed to bind to *Sbe1*. These results suggest that the SBEI-like activity detected by Dang and Boyer (1988) was due to an as yet unidentified starch-branching enzyme whose transcripts have high homology with those of *Sbe1*. This suggestion would explain our inability to detect differences in structure between leaf starch from the *sbe1::Mu* mutant and leaf starch from wild-type controls. Alternatively, since the SBEI antibody used in this study and by Blauth *et al.* (2001) was generated against a polypeptide sequence from a portion of SBEI, it is possible that the SBEI-like activity detected by Dang and Boyer (1988) was due to a trunkated form of SBEI undetectable by the antibody used. Detailed studies using isoform-specific antibodies and measuring SBE activity of SBE mutants are needed to clarify this apparent conflict.

In endosperm tissue, SBEI is undetectable in the *sbe1::Mu* mutant and easily detected in wild-type controls, but endosperm starch does not appear to be altered. Although this lack of a small observed difference might be due to the SEC methodology used, it is clear that any effect of the lack of SBEI on starch in endosperm is far smaller than the effect of the lack of SBEIIa or SBEIIb observed on starch in the leaf and endosperm, respectively. Similarly, potato tubers with a reduction of SBEI lack a major change in starch structure, including change in chain length distribu-

tion, as compared to wild-type controls (Flipse *et al.*, 1996).

From expression analysis, we conclude that the lack of altered phenotype in *sbe1::Mu* mutant endosperm is not due to compensation by activity of an enzyme encoded by the homologous sequence of *Sbe1* identified by Kim *et al.* (1998). Since no evidence was found for the expression of this sequence, it is possible that *Sbe1b* is a pseudogene. However, further analysis is needed to rule out low-level expression or expression limited to a specific tissue or developmental stage not observed in this study. It is possible that the lack of SBEI activity was compensated by other enzymes. Studies with isogenic, multiple mutants of *sbe1::Mu*, *sbe2a::Mu* and *ae* needed to explore these relationships further.

It is also possible that differences do exist in endosperm starch structure in *sbe1::Mu* mutants that were not detectable with the procedures used. Changes in the starch structure that do not alter the chain length distribution, such as the relationship among branch points along the glucan chain, would not be detected by the measures used here. More detailed analyses of starch fine structure with isogenic lines will be needed to explore this possibility.

Alternatively, perhaps SBEI does not have a significant role in determining starch quantity or quality in leaves or endosperm at the stages analyzed. If true, it is puzzling why this gene is so highly conserved in the plant kingdom. Regardless, the lack of mutant phenotype in leaves and phenotype in *sbe1::Mu* mutants is an important observation. The identification of this mutant will allow new strategies towards an understanding of the role of SBEI in starch synthesis.

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