Plant Physiol. (1997) 114: 69–78

Independent Genetic Control of Maize Starch-Branching Enzymes IIa and IIb[1]

Isolation and Characterization of a *Sbe2a* cDNA

Ming Gao[2], Dane K. Fisher[3], Kyung-Nam Kim, Jack C. Shannon, and Mark J. Guiltinan*

Department of Horticulture, Intercollege Graduate Program in Plant Physiology, and The Biotechnology Institute, The Pennsylvania State University, University Park, Pennsylvania 16802

In maize (*Zea mays* L.) three isoforms of starch-branching enzyme (SBEI, SBEIIa, and SBEIIb) are involved in the synthesis of amylopectin, the branched component of starch. To isolate a cDNA encoding SBEIIa, degenerate oligonucleotides based on domains highly conserved in *Sbe2* family members were used to amplify *Sbe2*-family cDNA from tissues lacking SBEIIb activity. The predicted amino acid sequence of a *Sbe2a* cDNA matches the N-terminal sequence of SBEIIa protein purified from maize endosperm. The size of the mature protein deduced from the cDNA also matches that of SBEIIa. Features of the predicted protein are most similar to members of the SBEII family; however, it differs from maize SBEIIb in having a 49-amino acid N-terminal extension and a region of substantial sequence divergence. *Sbe2a* mRNA levels are 10-fold higher in embryonic than in endosperm tissue, and are much lower than *Sbe2b* in both tissues. Unlike *Sbe2b*, *Sbe2a*-hybridizing mRNA accumulates in leaf and other vegetative tissues, consistent with the known distribution of SBEIIa and SBEIIb activities.

SBE catalyzes the formation of α(1–6) branches in amylopectin, the highly branched component of starch. It cleaves α(1–4) bonds on linear glucosyl chains and reattaches the released glucan segments to the same or another glucosyl chain by α(1–6) linkages. The reaction creates not only branches, but also new, nonreducing ends for further α(1–4) glucan elongation. Multiple forms of SBE have been characterized in many species, including rice (Mizuno et al., 1992; Nakamura et al., 1992; Yamanouchi and Nakamura, 1992), spinach, pea, potato, and maize (*Zea mays* L.) (for review, see Preiss, 1991). In maize three SBE isoforms, SBEI, SBEIIa, and SBEIIb, were resolved by DEAE-cellulose chromatography (Boyer and Preiss, 1978a, 1978b). Maize SBEI is distinct from SBEIIa and SBEIIb in amino acid composition, peptide maps, substrate specificity, optimal reaction conditions, enzyme kinetic properties, and immunological reactivities, whereas SBEIIa and SBEIIb are very similar in these respects (Preiss, 1991; Guan and Preiss, 1993; Takeda et al., 1993). cDNAs encoding SBEI and SBEIIb have been isolated (Baba et al., 1991; Fisher et al., 1993, 1995; Stinard et al., 1993) and their expression patterns characterized (Gao et al., 1996).

Preiss and co-workers (Singh and Preiss, 1985; Preiss, 1991) concluded that SBEIIa and SBEIIb may be the products of a single gene because of their similarities (for review, see Preiss, 1991). This one-gene hypothesis predicts that the differences between SBEIIa and SBEIIb could result from posttranscriptional modification, e.g. alternative splicing of the same pretranscript, or from posttranslational modification of a common precursor such as phosphorylation or glycosylation.

An alternative hypothesis is that SBEIIa and SBEIIb in maize endosperm are the products of two separate genes. Results from studies of the *amylose-extender* (*ae*) locus, the structural gene encoding SBEIIb, and its expression pattern support this hypothesis (Dang and Boyer, 1988, 1989; Stinard et al., 1993; Fisher et al., 1996a; Gao et al., 1996). Mutant endosperm from a number of independently derived *ae* alleles are deficient in SBEIIb activity but contain approximately normal levels of SBEIIa (Boyer and Preiss, 1978b; Baba et al., 1982; Hedman and Boyer, 1983). In homozygous endosperm of one of these *ae* alleles (*ae-B1*), the *Sbe2b* transcript was reduced to below the level of detection by northern analysis, yet SBEIIa activity was not affected (Fisher et al., 1996a). This strongly supports the hypothesis that endosperm SBEIIa and SBEIIb are encoded by separate genes. Likewise, no *Sbe2b* transcripts were detected in maize leaves (Stinard et al., 1993; Gao et al., 1996), which contain only SBEIIa and SBEI activities (Dang and Boyer, 1988, 1989).

To be consistent with the reported biochemical and immunological similarities of the SBEIIa and SBEIIb isoforms,

[1] This work was supported by the Pennsylvania Agricultural Experiment Station (CRIS 3303) and by a grant no. DE-FG02-96ER20234 from the U.S. Department of Energy, Energy Bioscience Program, to M.J.G., J.C.S., and Donald Thompson.

[2] Present address: Department of Biochemistry and Biophysics, 2154 Molecular Biology Building, Iowa State University, Ames, IA 50011.

[3] Present address: Monsanto Corporation, 700 Chesterfield Parkway North, Chesterfield, MO 63198.

* Corresponding author; e-mail mjg@psupen.psu.edu; fax 1–814–863–1357.

Abbreviations: DAE, days after emergence; DAP, days after pollination; pfu, plaque-forming units; RPA, RNase protection assay; RT-PCR, reverse transcriptase-coupled PCR; SBE, starch-branching enzyme; ssDNA, single-stranded DNA.

the two-gene hypothesis predicts that they share very high amino acid sequence similarity. However, the nucleotide sequence of the *Sbe2a* cDNA could be substantially divergent from that of the *Sbe2b* cDNA because of potentially synonymous codon usage. It is possible that separate, tissue-specific genes encode *Sbe2a* in leaf and endosperm. Considering this possibility, the two-gene hypothesis also predicts that the *Sbe2a* gene and/or a closely related duplicate locus, but not the *Sbe2b* gene, should be expressed in leaves and *ae-B1* endosperm (devoid of SBEIIb activity). Moreover, a low steady-state level of the *Sbe2a* message in these tissues, together with the potentially substantial sequence difference between *Sbe2a* and *Sbe2b*, could explain why no *Sbe2b*-hybridizing transcripts were detected in leaves or in *ae-B1* endosperm by northern analysis, even under low-stringency conditions (Fisher et al., 1996a; Gao et al., 1996).

To test the two-gene hypothesis, we sought a maize *Sbe2a* cDNA. We report here the isolation and characterization of a novel, nearly full-length *Sbe2*-family cDNA. As predicted, mRNA hybridizing to this cDNA is detected at very low levels in leaves and in *ae-B1* endosperm. Its deduced amino acid sequence shares high sequence similarity with the deduced SBEIIb sequence, but its DNA sequence is more divergent. A 49-amino acid N-terminal extension of the deduced SBEIIa protein is consistent with its slightly larger molecular weight relative to SBEIIb, as estimated by SDS-PAGE. The 14 N-terminal amino acids of mature SBEIIa protein purified from *ae-B1* endosperm perfectly match a sequence deduced from the putative *Sbe2a* cDNA and fit the consensus chloroplast transit peptide cleavage site. Overlapping partial cDNAs isolated from embryo, roots, and *ae-B1* endosperm tissue perfectly match the sequence determined from the leaf cDNA. These results suggest that this cDNA, distinct from *Sbe2b*, encodes SBEIIa in maize.

MATERIALS AND METHODS

Nomenclature

Sbe1, *Sbe2a*, and *Sbe2b* refer to the structural genes for maize SBEI, SBEIIa, and SBEIIb enzymes, respectively.

RT-PCR

Primers for PCR amplification of *Sbe2*-family cDNAs were derived from sequences almost perfectly conserved in maize (*Zea mays* L.) *Sbe2b*, rice *Rbe3*, and pea *Sbe1*: Sbe2/forward, 5′ TGG ATG TGG GAT TCT CGC CTW(A or T); Sbe2/reverse, 5′ ACA TAT CCT TGT CCA TCA ACC. All RT-PCR amplifications were performed using the SuperSCRIPT system (GIBCO-BRL) and *Taq* polymerase (Promega). The Sbe2/reverse primer was used for first-strand cDNA syntheses with 1 to 5 μg of total RNA. The RT-PCR products were either blunt-ended with T4 DNA polymerase and cloned into *Sma*I-cut pBluescript SK$^-$ (Stratagene) (Simon et al., 1994), or directly cloned into pCR (version 2.0, Invitrogen, San Diego, CA) following the manufacturer's instructions.

Library Screening, DNA Sequencing, and Sequence Analysis

A maize leaf (5 weeks old, B73-inbred) cDNA library (Uni-Zap XR, Stratagene, catalog no. 937005) was screened by hybridization to radioactively labeled DNA fragments following the manufacturer's instructions. All DNA probes were labeled with [^{32}P]dCTP by the random-primed labeling method. All phage lift membranes were hybridized and washed under high-stringency conditions as described previously (Church and Gilbert, 1984). DNA sequencing was performed using Sequenase (United States Biochemical). Both strands of the cDNA clone (pLf-8-1Aa) were sequenced by a combination of subcloning and synthetic primers. All sequence analysis was performed using Lasergene software (DNASTAR, Inc., Madison, WI). DNA and protein similarity scores were determined by algorithms of Martinez-Needleman-Wunch (gap penalty, 1.10; gap-length penalty, 0.33; and minimum match, 9) and Lipman-Pearson (gap penalty, 4; gap-length penalty, 12; and Ktuple, 2), respectively.

RNA Extraction, Northern Analysis, and RNase Protection Assay

Preparation of plant materials, RNA extractions, and northern analysis were performed as described previously (Fisher et al., 1996a; Gao et al., 1996). The template for synthesizing the *Sbe2a*-specific probe was the 393-bp 5′ end fragment of pLf-8-1A (1–380 bp of the cDNA, plus a 13-bp adapter sequence). The fragment was derived from *Eco*RI digestion of a PCR fragment amplified with a T3 vector primer and a gene-specific primer P2-20 (corresponding to the sequence from 380 to 360 bp). The fragment was filled in with Klenow and ligated to the *Eco*RV site of pBluescript SK$^-$ (Stratagene). Sense and antisense RNA were transcribed in vitro with T7 RNA polymerase (BrightStar BIOTINscript, Ambion, Inc., Austin, TX) from two *Eco*RI-linearized plasmids containing the 5′ end fragment in sense or antisense orientation relative to the T7 promoter. The antisense RNA (455 bp), containing a 393-bp cDNA and a 62-bp vector sequence, was maximally labeled with biotin-14-CTP and used as a probe in the RPA.

The *Sbe2b*-specific probe used in the RPA was the 214-bp 5′ end *Eco*RI to *Xho*I fragment of the full-length *Sbe2b* cDNA. The fragment was subcloned into *Eco*RI- and *Xho*I-digested pBluescript SK$^-$ and pBluescript KS$^-$. The sense RNA (270 bp, including a 66-bp vector sequence) was transcribed with T7 RNA polymerase from a *Xho*I-linearized pBluescript KS$^-$ plasmid containing the *Sbe2b* fragment. The *Sbe2b* antisense RNA probe (244 bp, including a 30-bp vector sequence) was similarly transcribed from the pBluescript SK$^-$ plasmid. The *Sbe2b* antisense probe was labeled with either one-half or one-fourth of the maximal concentration of biotin-14-CTP to bring the signal strength for the *Sbe2b* mRNA within the same range as that of the *Sbe2a* mRNA. This allowed quantification of signals for both transcripts in the linear range of the x-ray film with a single exposure. The *Sbe2a* and *Sbe2b* sense RNA (455 and 270 bp, respectively) used as standards were not

labeled with biotin. RNA markers were in vitro-transcribed from a marker template (Century, Ambion) with maximal biotin-14-CTP concentration. All in vitro-transcribed RNA species were gel-purified and quantified spectrophotometrically.

RPA was carried out using a kit (RPAII, Ambion) according to the manufacturer's instructions. Total RNAs from different tissues were co-precipitated with the *Sbe2a* and *Sbe2b* antisense probes (1 ng of probe/20 μg of total RNA), hybridized for 18 h, and digested with a mixture of RNase A and T1. The RNase-digested products were then precipitated, resolved on a 5% denaturing polyacrylamide gel, and blotted to nylon membrane (Hybond-N^+, Amersham) with a semidry blotting apparatus (Fisher Scientific). The protected, biotinylated fragments were detected with streptavidin-alkaline phosphatase conjugates catalyzing a chemiluminescent reaction detected by x-ray film (BrightStar BioDetect system, Ambion). The films were scanned with a densitometer and quantified using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). All measurements were made only on bands with optical densities in the linear range of the x-ray film.

Two controls containing 2 ng of both *Sbe2a*- and *Sbe2b*-labeled probes mixed with 20 or 40 μg of yeast total RNA corresponding to the highest amount of sample total RNA analyzed were included in all RPA experiments. One was digested with RNases (negative control); the other was not digested after hybridization (positive probe control). One-tenth of the positive probe control was analyzed on the gel. Five RNA standards containing known amounts of the in vitro-synthesized *Sbe2a* and *Sbe2b* sense RNAs mixed with 20 μg of yeast total RNA were also included. Quantities of *Sbe2b*/*Sbe2a* RNA standard included in the RPA were 225.5/14.4, 340.8/19.2, 113.6/4.8, 71.0/2.4, 51.1/1.4, and 454.4/9.6 (pg), respectively. To construct a standard curve for calculations of the transcript levels in samples, the signal strengths of the probe fragments protected by the RNA standards were plotted against the amount of RNA standards in moles.

Isolation and N-Terminal Sequencing of SBEIIa from *ae-B1* Endosperm

SBEIIa from endosperm of 22 DAP *ae-B1* kernels was purified using a procedure modified from that of Guan and Preiss (1993). Twenty-eight grams of frozen endosperm was extracted, ammonium sulfate-fractionated, and dialyzed according to Guan and Preiss (1993). The dialyzed SBE fraction was applied to a preequilibrated DEAE-Sepharose Fast-flow column (Pharmacia; 1.6 × 70 cm, 120-mL resin bed volume; about 10 mg protein mL^{-1} bed volume). The column was washed with 2 resin bed volumes of buffer A (50 mM Tris-acetate buffer [pH 7.5] containing 10 mM EDTA, 2.5 mM DTT, and 5% glycerol). SBEIIa was eluted with a linear gradient of 12 resin bed volumes of 0 to 0.4 M KCl in buffer A, following the method of Guan and Preiss (1993). Fractions were assayed for branching enzyme activity as described previously (Fisher et al., 1996a). To confirm the presence of a single species of SBE in all SBEIIa fractions, aliquots were fractionated on an 8% polyacrylamide gel, blotted, and detected with an antibody recognizing both SBEIIa and SBEIIb (Fisher et al., 1996a; Mu-Forster et al., 1996).

Fractions containing SBEIIa activity were pooled, precipitated with 55% ammonium sulfate, and redissolved in a minimal volume of buffer A. The redissolved SBEIIa fraction was then loaded on an ω-aminooctyl-agarose column (Sigma; 1.6 × 20 cm, 30-mL resin bed volume, and 0.1 mg mL^{-1} resin bed volume) equilibrated with buffer A. The column was washed with 4 resin bed volumes of buffer A, and SBEIIa was eluted with a linear gradient of 10 resin bed volumes of 0 to 1 M KCl in buffer A. Fractions with SBEIIa activity were pooled and concentrated with filters (Centriplus-30, Amicon Inc., Beverly, MA).

The concentrated SBEIIa fraction was next applied to a Sephacryl S-300-HR column (Sigma; 1.6 × 40 cm, 60-mL resin bed volume) and eluted with buffer A at a flow rate of 0.2 mL/min. Aliquots from 1-mL column fractions containing SBEIIa activity were checked for purity with SDS-PAGE (8% gel) and silver staining. Peak fractions essentially free of protein contaminants were pooled and concentrated with filters (Centriplus-30, Amicon). The SBEIIa protein of near homogeneity was further resolved by preparative SDS-PAGE, blotted onto a PVDF membrane (Trans-Blot, Bio-Rad), and visualized with black stain (0.1% amido black 10B [Sigma] in 10% acetic acid). N-terminal sequencing of the blotted SBEIIa from *ae-B1* endosperm was performed by the Wistar Protein Microsequencing Facility (Philadelphia, PA).

Genomic Southern Analysis

Maize genomic DNA was extracted from 16 DAP W64A kernels. Ten grams of frozen kernels was ground to a fine powder in liquid nitrogen, added to 35 mL of extraction buffer (100 mM Tris-HCl, pH 8.8, 100 mM NaCl, 20 mM EDTA, and 1% Sarkosyl), and extracted by sitting at room temperature for 10 min. An equal volume of Tris-HCl-equilibrated phenol (pH 8.0, 35 mL) was added, and the mixture was vortexed for 3 min and centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was extracted twice with an equal volume of chloroform:isoamyl alcohol (50:1), and the genomic DNA was precipitated by adding one-tenth volume of NaOAc (3 M, pH 5.2) and 1 volume of isopropanol. The genomic DNA was wound on a glass rod, redissolved in 5 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and digested with RNase A (50 μg/mL) for 1 h and then with proteinase K (50 μg/mL) for 1 h at 37°C. The DNA preparation was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), precipitated as before, wound on a glass rod, and washed with 70% ethanol. DNA was dissolved in 1 mL of TE buffer. Ten micrograms of DNA was digested with each restriction enzyme, fractionated on a 0.8% agarose gel, and alkaline-blotted to a membrane (Hybond-N^+) following the manufacturer's instructions. The blot was hybridized sequentially with ssDNA probes specific to *Sbe2b*, or *Sbe2a* cDNAs generated as described by Konat et al. (1994). The template used for synthesizing the *Sbe2a*-specific ssDNA probe was the PCR-amplified 5′ end fragment (1–381 bp). The primer

P2-20 was also used for PCR amplification of the *Sbe2a* ssDNA probe. The template specific to *Sbe2b* was the 5′ end *Eco*RI and *Kpn*I fragment (1–353 bp). The primer used for the amplification of the *Sbe2b* ssDNA probe was P2-6R3, corresponding to the sequence from 345 to 338 bp of the full-length *Sbe2b* cDNA. The blot was hybridized at 65°C for 16 h, and washed under high-stringency conditions as described by Church and Gilbert (1984).

RESULTS

Isolation of a Novel *Sbe2*-Family cDNA

Our approach was to first isolate a partial *Sbe2a* cDNA from tissues lacking *Sbe2b* transcript by RT-PCR using conserved *Sbe2* primers. Sequence comparison between the maize *Sbe2b* cDNA and its counterparts in pea (*Sbe1*) and rice (*Rbe3*) revealed two almost completely conserved sequences of 25 bp each, which are not conserved in cDNAs encoding maize SBEI or other plant SBEI family members. We reasoned that since maize SBEIIa is very similar in many biochemical and immunological properties to SBEIIb (Fisher and Boyer, 1983; Preiss, 1991; Guan and Preiss, 1993; Takeda et al., 1993), these two stretches of sequences might also be conserved in the hypothetical cDNA encoding maize SBEIIa. Therefore, two degenerate primers derived from these two sequences were used for RT-PCR amplification of cDNA from maize leaf total RNA. Leaf tissue was chosen because it contains SBEII activity with chromatographic characteristics similar to those of maize endosperm SBEIIa, but is devoid of detectable SBEIIb activity and *Sbe2b* transcripts.

A single 538-bp cDNA fragment was amplified from 25-d-old seedling leaf RNA. The fragment was cloned and seven positive isolates were sequenced at both ends. One clone was a nonspecific amplification product from 18S rRNA, and the other six were identical. The complete sequence of the RT-PCR-amplified cDNA is shown in Figure 1 as part of a subsequently isolated, nearly full-length putative *Sbe2a* cDNA. This RT-PCR-amplified cDNA fragment showed 78 and 40% sequence similarity to the corresponding region of the maize *Sbe2b* and *Sbe1* cDNAs, respectively. Thus, the cDNA fragment, although distinct from *Sbe2b* and *Sbe1*, was likely amplified from an mRNA encoding a leaf *Sbe2*-family protein. cDNA fragments with identical sequences were also amplified, cloned, and sequenced from total RNA from seedling roots, embryo, and *ae-B1* endosperm (not shown).

To isolate a full-length cDNA, the putative *Sbe2a* cDNA fragment was used as a probe to screen 5.4×10^5 pfu of a maize leaf cDNA library (Stratagene). Of 11 independently isolated clones, the largest (designated pL-5-2A) contained a 2.3-kb insert. As summarized in Table I, another 15 independent clones were isolated by screening 2.5×10^6 pfu. Among them, two clones (pGl-7-1A and pLf-8-1A) of 2795 bp were further analyzed. Their size is very close to that of a hybridizing mRNA (2.8–2.9 kb) estimated from northern analysis of total RNA from various tissues (see below). The cDNA sequence and the deduced amino acid sequences from the longest open reading frame of the two clones are shown in Figure 1. Several cDNAs with shortened 3′ untranslated regions were found, indicating either imprecise or alternative polyadenylation addition sites.

The Putative *Sbe2a* cDNA Encodes a Protein Very Similar to SBEIIb

The sequence of the near-full-length putative *Sbe2a* cDNA showed about 67% similarity overall to that of the *Sbe2b* cDNA (Fig. 2). The sequence between 380 and 2443 bp is very similar to the corresponding *Sbe2b* cDNA sequence (78% similarity). However, a short 5′ end fragment (0–380) and the 3′ untranslated region (2443–2763) of this putative *Sbe2a* cDNA strongly diverge from their counterparts in *Sbe2b*, with only 29 and 38% sequence similarity, respectively (Fig. 2). The pattern of similarity between the putative *Sbe2a* and *Sbe2b* cDNA in maize is very similar to that of the two genes encoding two SBEII enzymes in *Arabidopsis thaliana*, i.e. a conserved central region and diverged termini (Fisher et al., 1996b). These and other data (see below) indicate that although this cDNA is most similar to the *Sbe2* family, it is clearly distinct from *Sbe2b*.

Consistent with the DNA sequence comparison, the amino acid sequence deduced from the longest open reading frame of the putative *Sbe2a* cDNA shares 77% overall similarity with that of the *Sbe2b* cDNA (Fig. 3). The amino acid sequence corresponding to the highly conserved region (amino acids 124–810) is 89% similar to that of SBEIIb. Thus, this leaf putative *Sbe2a* cDNA encodes a protein very similar to, but not identical to, endosperm SBEIIb. Both amino acid and codon usage differences between the two cDNAs contribute to the overall nucleotide sequence difference of the two cDNAs.

As in all other SBE isoforms in the SBEII family (Burton et al., 1995; Fisher et al., 1996b), the central conserved region of the amino acid sequence deduced from the putative *Sbe2a* cDNA starts with a stretch of three consecutive Pros (SBEIIa positions, amino acids 124–126). Residues predicted to fold into the $(\beta\text{-}\alpha)_8$ barrel domain conserved in plant-branching enzymes (Burton et al., 1995) are also highly conserved (Fig. 3). Residues participating in the active sites of glucanases, which are conserved in all other branching enzymes (Burton et al., 1995), are also completely conserved in the amino acid sequence deduced from the putative *Sbe2a* cDNA (Fig. 3).

The N-Terminal Sequence of Purified SBEIIa Matches the Deduced Sequence of the Putative *Sbe2a* cDNA

The N-terminal sequence of purified SBEIIa protein was determined, and the data provide conclusive evidence that the putative *Sbe2a* cDNA encodes the SBEIIa isoform defined biochemically. SBEIIa protein was purified from *ae-B1* endosperm by a procedure modified from that of Guan and Preiss (1993). Endosperm of this mutant is devoid of the potentially contaminating SBEIIb isoform. Enriched SBEIIa fractions from a DEAE-Sepharose column were purified further through an amino ω-octo agarose column. A sharp, symmetrical peak of SBE activity was reproducibly eluted in fractions containing approximately

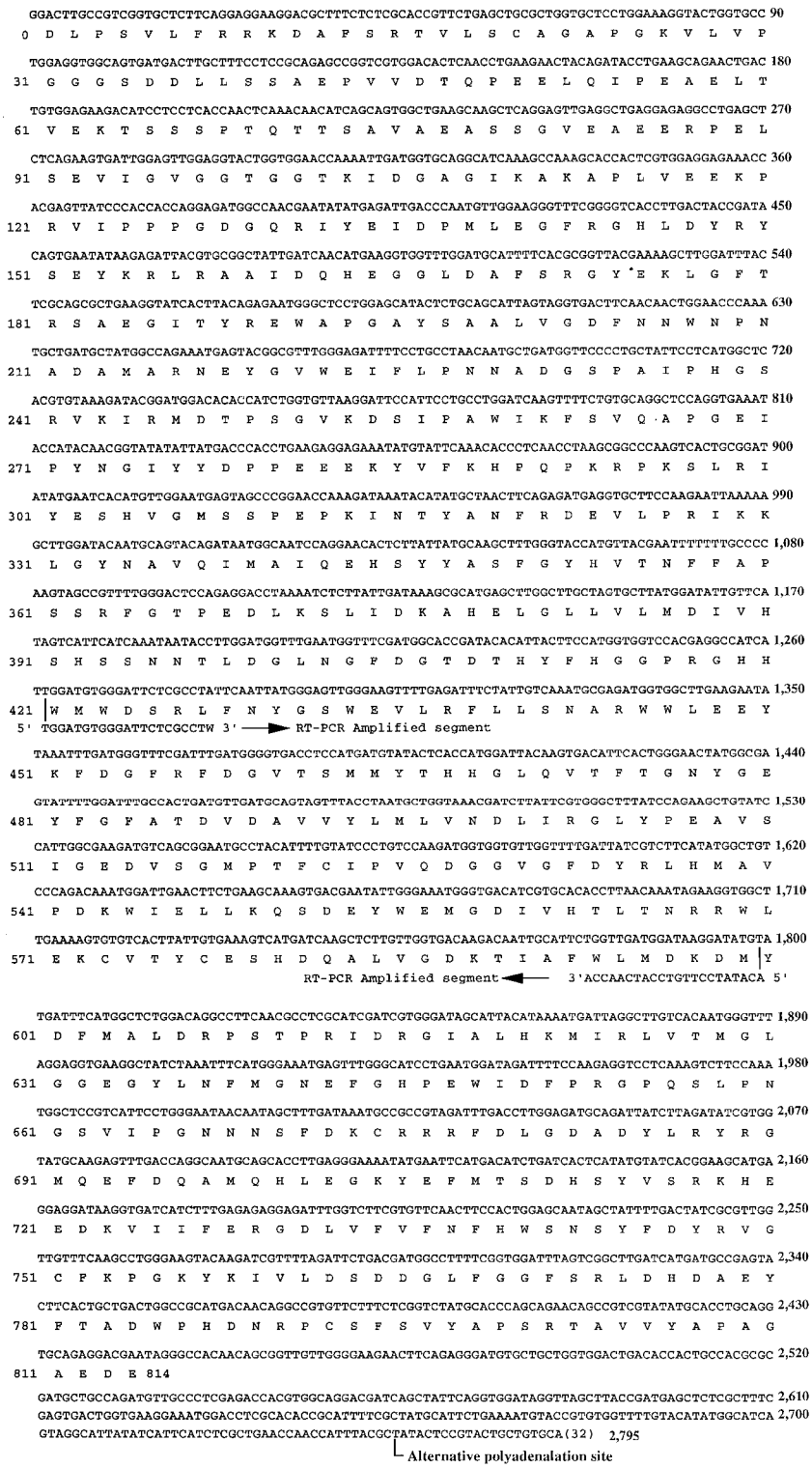

Figure 1. Nucleotide sequence and conceptual translation of a nearly full-length *Sbe2a* cDNA. Nucleotides and amino acids are numbered at the right and the left, respectively. The cDNA segment amplified by RT-PCR from leaf and *ae-B1* endosperm total RNA and the primers used are indicated by the arrows. Positions of synthetic oligonucleotides used for RT-PCR are indicated. The alternative polyadenylation site indicates where the poly(A) tails in the different cDNAs begin.

0.5 to 0.75 M KCl in buffer A. The identity of the enzyme was confirmed by western analysis of the fractions with an anti-SBEII antibody that recognizes both the 85-kD SBEIIb and the larger, 89-kD SBEIIa (Fisher et al., 1996a; Mu-Forster et al., 1996).

The SBEIIa protein was purified further through a Sephacryl S-300-HR column. An aliquot of pooled peak SBEIIa fractions essentially free of other proteins (as indicated by SDS-PAGE analysis) was compared by western analysis with SBEIIa and SBEIIb from the wild-type W64A

Table I. *Summary of maize leaf cDNA library screens*

Screen	Probe	pfu Screened	No. of Clones Isolated	Designation of Longest Isolates
1	538-bp cDNA fragment from RT-PCR amplification of leaf RNA	5.4×10^5	11	pL-5-2A, 2.3 kb
2	0.6-kb 5′ *EcoRI*/*KpnI* fragment from pL-5-2A	4.8×10^5	5	pFL-8 1A, 2.65 kb
3	5′ 500-bp fragment from pFL-8-1A	1.0×10^6	5	pML-16-1A and pML-6-1A, 2.77 kb
4	5′ 360-bp fragment from pML-6-1A	1.0×10^6	5	pLf-8-1A and pGl-7-1A, 2.79 kb

endosperm in fractions from a DEAE-Sepharose Fast-flow column (Fig. 4). The purified SBEIIa protein co-migrated with the larger, 89-kD band in the control, SBEIIa-enriched fraction from W64A endosperm on the western blot. The 85-kD band detected in the control, SBEIIa-enriched fraction from W64A endosperm is contaminating SBEIIb (Fisher et al., 1996a), which is not detected in SBEIIa fractions from *ae-B1* endosperm. The molecular mass of the mature SBEIIa calculated from the deduced amino acid sequence (amino acids 11–814; Fig. 3) is 89,583 D, matching very closely that of SBEIIa from endosperm determined by SDS-PAGE (89 kD) (Fisher et al., 1996a).

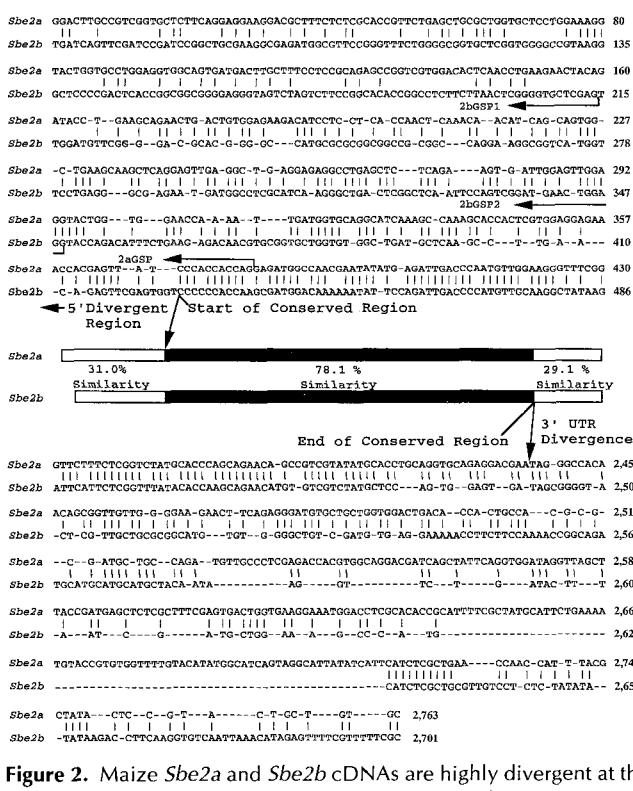

Figure 2. Maize *Sbe2a* and *Sbe2b* cDNAs are highly divergent at the 5′ and 3′ ends. Sequences that are identical in both cDNAs are indicated by vertical bars. Sequence positions where the 5′ region of low similarity ends and the 3′ region of low similarity begins are indicated by arrows. Sequences of the central portions of the two cDNAs that are highly conserved are not shown. The sequences of *Sbe2a* and *Sbe2b* gene-specific probes for genomic Southern, northern, and RPA analysis are indicated by arrows and labeled as 2aGSP, 2bGSP2, and 2bGSP1, respectively. DNA sequence comparison by the Martinez-Needleman-Wunch algorithm (gap penalty, 1.10; gap-length penalty, 0.33; and minimum match, 9) was implemented with Lasergene software (DNASTAR, Inc.).

A portion of the *ae-B1* pooled SBEIIa peak fractions from a Sephacryl S-300-HR column was fractionated by preparative SDS-PAGE, blotted onto a PVDF membrane, and subjected to N-terminal peptide sequencing. The N-terminal sequence of SBEIIa from *ae-B1* endosperm was 'AGA'PGKVLVPGGGS (single quotation marks indicate

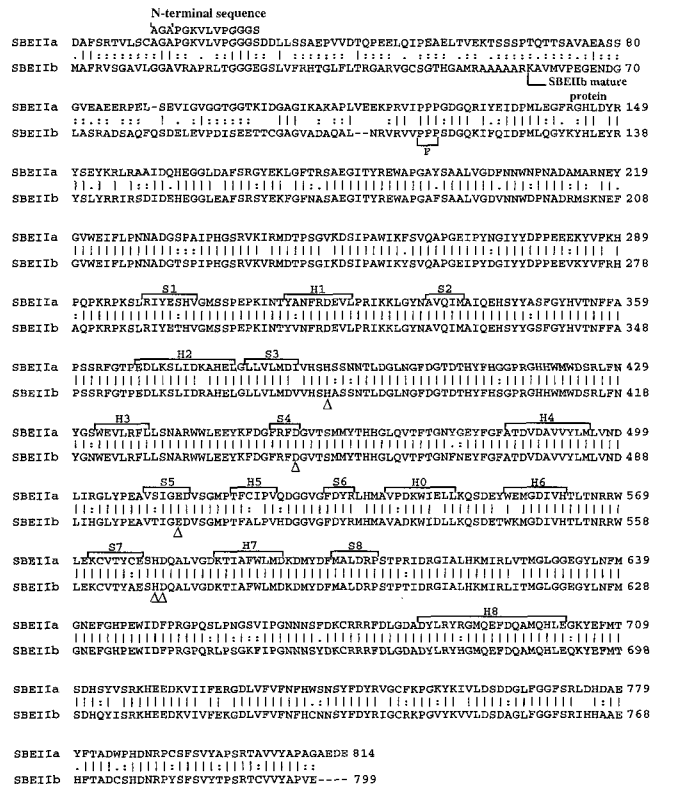

Figure 3. Maize SBEIIa and SBEIIb share very high sequence and structural similarities. Identical amino acids are indicated with vertical bars, similar amino acids with colons, and related amino acids with dots. The amino acid sequences in regions of predicted β-strands (S1–S8) and α-helices (H1–H8 and H0) are indicated with brackets. The residues participating in the active site of glucanases are denoted with Δ. The three Pros at the beginning of the conserved region are indicated by brackets and labeled P. The N-terminal sequence of purified SBEIIa protein from *ae-B1* endosperm is presented on top of the corresponding deduced amino acid sequences. The first three amino acids, labeled with single quotation marks, are the most prevalent of several alternatives in the first three cycles of sequencing. The start of the SBEIIb mature protein as determined by N-terminal sequencing is also indicated (Fisher et al., 1993). Protein sequence comparison by the Lipman-Pearson algorithm (gap penalty, 4; gap-length penalty, 12; and Ktuple, 2) was implemented by Lasergene software (DNASTAR, Inc.).

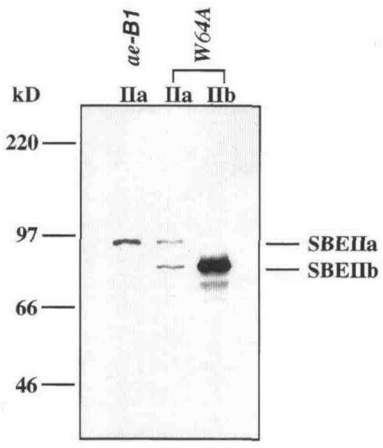

Figure 4. Immunological comparison of SBEIIa purified from *ae-B1* endosperm with SBEIIa- and SBEIIb-enriched fractions from W64A endosperm. One microgram of *ae-B1* endosperm SBEIIa purified through a Sephacryl S-300-HR column to near homogeneity and 10 μg of total protein from W64A endosperm SBEIIa and SBEIIb fractions purified through a DEAE-Sepharose Fast-flow column were separated by SDS-PAGE, blotted, and detected with an anti-SBEII antibody (Fisher et al., 1996a; Mu-Forster et al., 1996). The SBEIIa fraction of W64A endosperm contains some contaminating SBEIIb, as described previously (Fisher et al., 1996a). Positions of molecular mass markers (not shown) are indicated on the left.

the most prevalent amino acid of several alternatives in the first three cycles). The 14 N-terminal amino acids of the purified SBEIIa protein perfectly match the amino acid sequence deduced from the putative *Sbe2a* cDNA (Fig. 3). The sequence at the predicted cleavage site (RTVLSC ↓ A) is similar to the proposed consensus ($R^{I}/_{V}X^{A}/_{C}\downarrow A$) of chloroplast transit peptides (Gavel and von Heijne, 1990), suggesting that the mature SBEIIa protein starts with Ala. The precise match of 14 N-terminal amino acids of the *ae-B1* endosperm SBEIIa to that of the deduced SBEIIa protein strongly supports the conclusion that the newly isolated *Sbe2* cDNA encodes maize SBEIIa.

The *Sbe2a* Gene Is Expressed at a Very Low Level in Most Tissues

The expression of the putative *Sbe2a* gene in various tissues and organs of normal W64A and *ae-B1* plants was investigated by northern analysis using a *Sbe2a*-specific probe (2aGSP). This 2aGSP probe was derived from the highly divergent, 380-bp 5′ end of the *Sbe2a* cDNA (Fig. 2). This probe does not hybridize to the *Sbe2b* cDNA under high-stringency conditions (see below). As shown in Figure 5, a transcript of about 2.8 to 2.9 kb was detected at a low level in endosperm of both normal W64A and *ae-B1* plants. Embryos of both normal W64A and *ae-B1* showed the highest level of *Sbe2a* transcript among all of the tissues examined. The *Sbe2a* gene was also expressed in young stem and tassel tissues. A very weak *Sbe2a* transcript level was detected in RNA extracted from roots of 9-d-old seedlings. The presence of the *Sbe2a* transcript in seedling roots was also confirmed by RT-PCR (data not shown).

To unequivocally detect the two very similar *Sbe2a* and *Sbe2b* transcripts and evaluate their expression levels, RNA samples were further analyzed by RPA, which has the advantage of higher sensitivity and specificity than northern analysis. More importantly, it can accommodate multiple probes in a single hybridization experiment, eliminating potential artifacts of serial membrane hybridizations. The antisense probes specific to *Sbe2a* and *Sbe2b* were derived from the 5′ 455- and 244-bp divergent regions of the two genes, including short stretches of transcribed vector sequences. Transcripts of the two genes in total RNA were expected to specifically protect the complementary 380- and 214-bp sequences of the antisense probes against RNase digestion, as illustrated in Figure 6A. Known amounts of sense RNA fragments in vitro-transcribed from subcloned *Sbe2a* and *Sbe2b* cDNAs (see "Materials and Methods") were used as standards.

The transcript levels of the *Sbe2a* and *Sbe2b* genes in various tissues measured by RPA are summarized in Table II. Consistent with northern analysis (Fig. 4), a low level of *Sbe2a* transcript was detected in W64A endosperm. On a total RNA basis, it was approximately 10 times lower than in the embryo, and 35 times lower than the *Sbe2b* level in normal endosperm. Note that in order to have the signal strength of both protected fragments in the linear range of the x-ray film for a single exposure, the *Sbe2b* antisense probe used in analysis of total RNA from endosperm and embryo was labeled with one-fourth of the maximal biotin-14-CTP concentration used for labeling of *Sbe2a*. The *Sbe2a* transcript in *ae-B1* endosperm was approximately 3-fold higher than in normal endosperm. This is similar to the increase in transcript level of several starch synthetic genes in nonallelic maize endosperm mutants reported by Giroux et al. (1994), possibly indicating a metabolite feedback control mechanism.

As in the northern analysis, embryos of both normal plants and *ae-B1* mutants have the highest levels of the *Sbe2a* transcript on a total RNA basis (Table II). However, in total RNA from normal embryos, the *Sbe2b* transcript level is approximately 3-fold higher than that of *Sbe2a*. In contrast, the *Sbe2b* transcript was not detected by RPA in embryos from *ae-B1* mutant kernels, which is consistent with previous northern analysis results (Fisher et al., 1996a). Young roots from 2 DAE seedlings showed a *Sbe2a* transcript level similar to that of embryos. This is in con-

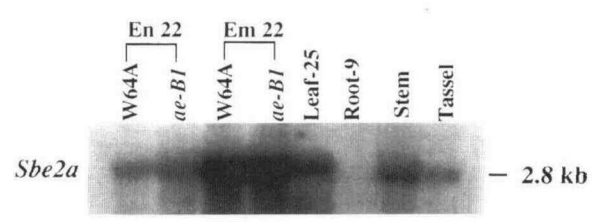

Figure 5. Expression of *Sbe2a* in various tissues of normal W64A and *ae-B1* maize plants. Thirty micrograms of RNA from 22 DAP endosperm (En 22) and 22 DAP embryo (Em 22) of W64A or *ae-B1* kernels, 25 DAE leaves (Leaf-25), 9 DAE root (Root-9), young stem, and tassels were fractionated on a 1.2% formaldehyde gel, blotted, and probed with a *Sbe2a*-specific probe (2aGSP, 380-bp 5′ end fragment, Fig. 2). Autoradiography was for 4 d.

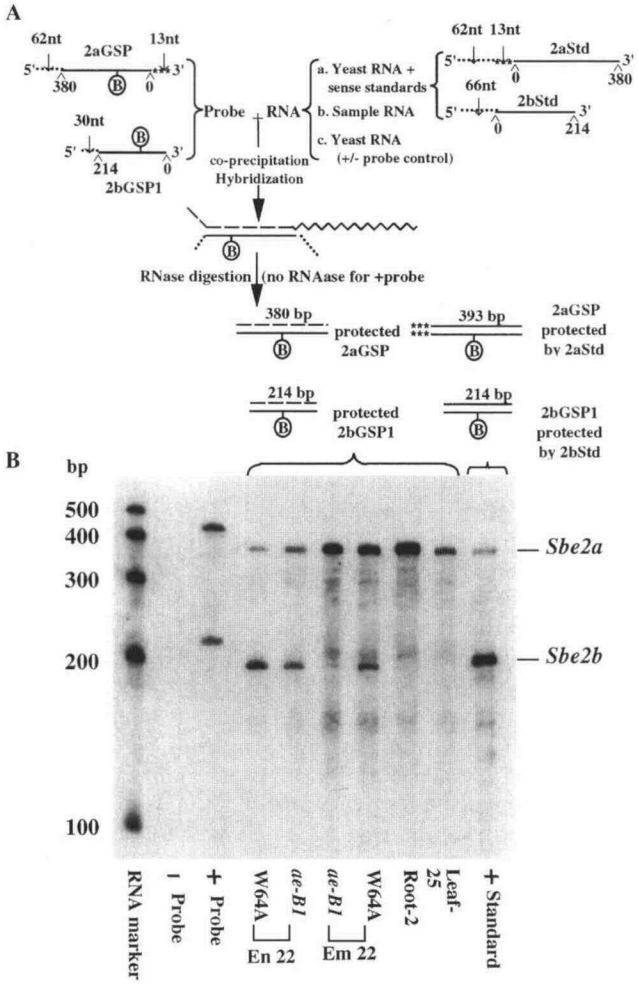

Figure 6. RPA of *Sbe2a* and *Sbe2b* mRNA in various tissues of *ae-B1* and normal W64A plants. Antisense probes specific to *Sbe2a* (2aGSP) and *Sbe2b* (2bGSP1) were used in RPA. Twenty micrograms of total RNA from various tissues except 2 DAE root (40 μg) were used. Two nanograms of each of the two antisense RNA probes mixed with 20 μg of yeast RNA were used for controls. RNase A and T1 were omitted from the digestion buffer for the positive probe control (+Probe), and added to the RNase digestion control (−Probe). The in vitro-transcribed sense RNA of *Sbe2b* (2bStd) and *Sbe2a* (2aStd) were used as standards for quantification. An example with a *Sbe2b* to *Sbe2a* molar ratio of 26 (225.5–14.4 pg) is shown (+ Standard). Note that the *Sbe2b* probes for RPA analysis of endosperm and embryo total RNA and for the positive control were labeled with one-fourth of the maximal biotin-14-CTP concentration, and with one-half of the maximal for RPA analysis of root and leaf total RNA. The *Sbe2a* probe was maximally biotinylated. A, Schematic illustration of the RPA analysis. B, Gel analysis of protected probe fragments. The protected, biotinylated probe fragments were resolved on a 5% denaturing polyacrylamide gel, blotted, and detected with streptavidin-alkaline phosphatase conjugates. The light emitted from the chemiluminescent reaction catalyzed by alkaline phosphatase was detected by exposure to x-ray film for 2.5 min.

trast to the very low level of the *Sbe2a* transcripts detected by northern analysis and RT-PCR in roots of 9 DAE seedlings. If SBEIIa is higher in the relatively starch-rich root tips, then the reduction in *Sbe2a* transcript levels in older seedling roots may result from a dilution of the root-tip mRNA with mRNA from the rest of the root. Whether the decrease in the *Sbe2a* transcript in developing roots has any physiological significance awaits further analysis. An intermediate level of the *Sbe2a* transcript was observed in maize leaves.

There are several reasons we believe that the residual *Sbe2b* transcripts in *ae-B1* endosperm detected by RPA were most likely truncated and nonfunctional. First, no SBEIIb protein or activity was detected in *ae-B1* endosperm (Fisher et al., 1996a). Second, the *Sbe2b* transcript of normal size was not detectable in the *ae-B1* endosperm by northern analysis, although a weak smear of possibly truncated messages was detected (Fisher et al., 1996a). Finally, the RPA procedure can detect even severely truncated transcripts because the *Sbe2b* antisense probe is short and derived from the extreme 5′ end of the *Sbe2b* cDNA.

As observed with northern analysis (Fisher et al., 1996a), no residual *Sbe2b* transcripts were detected by RPA in the embryo of *ae-B1* mutant kernels, even with the *Sbe2b* probe labeled with one-half of the maximal biotin-14-CTP content. No *Sbe2b* transcripts were detectable by RPA in young roots and leaf tissues, which is also consistent with biochemical and northern analysis (Dang and Boyer, 1988, 1989; Gao et al., 1996).

Sbe2a and *Sbe2b* Genes Reside at Different Genomic Restriction Fragments

To investigate the genomic structure of the *Sbe2a* and *Sbe2b* loci, Southern genomic analysis was performed with gene-specific probes. Genomic DNA extracted from W64A kernels was digested with several restriction enzymes, blotted, and analyzed by hybridization with gene-specific *Sbe2a* (2aGSP) and *Sbe2b* (2bGSP2) probes. The gene-specific probes were derived from the highly divergent 5′ end regions of the two cDNAs (Fig. 2). As shown in Figure 7, the two gene-specific probes hybridized specifically to their own cDNA controls. Only one strongly hybridizing band was detected by the *Sbe2a*-specific probe in genomic DNA separately cut with four restriction enzymes (Fig. 7A). The *Sbe2b*-specific probe detected one highly hybridizing band in

Table II. *The transcript level of Sbe2a and Sbe2b in 20 μg of total RNA from various maize tissues*

The data are the average of two or three measurements with SE values no more than 10%. All quantitative measurements were made within the linear response range of the film.

RNA Sample	*Sbe2b* mRNA	*Sbe2a* mRNA	*Sbe2b/Sbe2a* Ratio
	fmol		
Endosperm (22 DAP)			
Normal	1.59	0.045	35.3
ae-B1	NA[a]	0.150	
Embryo (22 DAP)			
Normal	1.3	0.470	2.8
ae-B1	–[b]	0.476	
Leaves (25 DAE)	–	0.249	
Roots (2 DAE)	–	0.440	

[a] NA, Not applicable. [b] Below background.

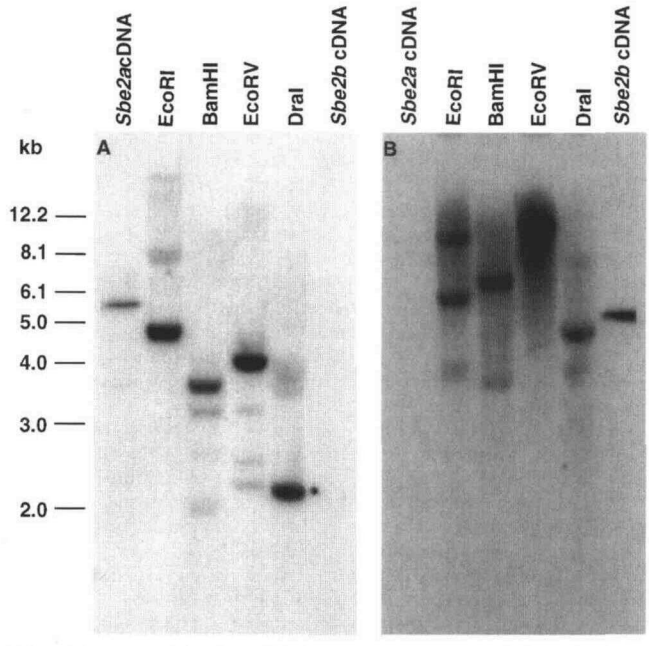

Figure 7. Maize *Sbe2a* and *Sbe2b* genes are encoded at different genomic restriction fragments. Maize genomic DNA was digested with various restriction enzymes, fractionated on a 0.8% agarose gel, alkaline-blotted, and hybridized sequentially with *Sbe2a*-specific (2aGSP) and *Sbe2b*-specific (2bGSP1) probes. Linearized plasmids containing *Sbe2a* or *Sbe2b* cDNAs were run as controls. Exposure was for 3 d. A, Hybridization to the *Sbe2a*-specific probe. B, Hybridization to the *Sbe2b*-specific probe.

genomic DNA cut with *Bam*HI, *Eco*RV, and *Dra*I, and two bands in the genomic DNA cut by *Eco*RI (Fig. 7B). None of the bands in corresponding lanes are of the same molecular mass. This evidence supports the conclusion that SBEIIa and SBEIIb are encoded by different genes. The high-molecular-mass band (about 11 kb) detected by the *Sbe2b*-specific probe in the *Eco*RI-cut genomic DNA could have resulted from incomplete digestion. Alternatively, the two bands may have resulted from an *Eco*RI cut in an intron, because no *Eco*RI sites are found in the cDNA probe.

DISCUSSION

Based on the data presented above and on our previous molecular genetic analysis of alleles of *ae* mutants (Fisher et al., 1996a), we conclude that maize SBEIIa and SBEIIb are the products of two unique genes. First, although the sequences of the putative *Sbe2a* and *Sbe2b* cDNAs and their deduced amino acid sequences share high similarity over an extended region, they also have regions of substantial divergence (Figs. 2 and 3). The amino acid sequence deduced from the putative *Sbe2a* cDNA also shares common structural features with the amino acid sequences of all plant SBEII isoforms (Fig. 3). Thus, we can conclude that the putative *Sbe2a* cDNA encodes an SBE belonging to the SBEII family, but distinct from SBEIIb.

Second, the 14 N-terminal amino acids of SBEIIa protein purified from *ae-B1* endosperm perfectly match the sequence deduced from the *Sbe2a* cDNA. In addition, the molecular mass (89,583 D) calculated from the deduced SBEIIa mature protein sequences closely matches that of endosperm SBEIIa experimentally determined by SDS-PAGE (Fig. 4). SBEIIa extracted from *ae-B1* and normal W64A endosperm showed the same behavior on a DEAE-Sepharose Fast-flow column, had identical mobility on SDS-PAGE, and was recognized by the same antibody (Fig. 4). These results support the conclusion that the SBE protein purified from *ae-B1* endosperm is SBEIIa, with characteristics identical to those originally used when naming the IIa and IIb isoforms (Boyer and Preiss 1978b; Preiss 1991; Fisher et al., 1996a).

Third, the expression pattern of the putative *Sbe2a* gene is consistent with the known distribution of SBEIIa activity. Very low levels of *Sbe2a* transcript were detected in leaves, endosperm, and embryos of normal and *ae-B1* mutant plants by a combination of northern analysis, RPA, and RT-PCR methods (Figs. 5 and 6). SBEIIa enzyme activity was also reported in these tissues (Dang and Boyer, 1988, 1989). Moreover, a cDNA fragment identical to the leaf *Sbe2a* cDNA was amplified from total RNA of *ae-B1* endosperm, indicating that the same gene encodes SBEIIa in both leaves and endosperm. In contrast, no *Sbe2b* message could be detected in maize leaves by northern analysis, RPA, or RT-PCR methods, consistent with the previous conclusion that SBEIIa is the only SBEII-type isoform in maize leaves (Dang and Boyer, 1988, 1989). Finally, maize genomic DNA fragments detected by *Sbe2a*- and *Sbe2b*-specific probes are completely different, indicating that the two cDNAs are located on different restriction fragments in the genome. Taken together, these data support the conclusion that the putative *Sbe2a* cDNA indeed encodes maize leaf and endosperm SBEIIa.

SBEIIa and SBEIIb may perform different functions in the synthesis of maize starch, because it is clear from the phenotype of *ae* mutant kernels that the loss of SBEIIb function cannot be compensated for by SBEIIa and SBEI. The loss of SBEIIb in *ae* endosperm results in the synthesis of a novel type of amylopectin with longer average chain lengths and a reduction in total starch content (Boyer et al., 1976; Inouchi et al., 1983; Shannon and Garwood, 1984). Unlike SBEIIa, SBEIIb is not expressed in leaf tissues, where transitory starch is produced. As expected, leaf transitory starch does not contain the novel amylopectin characteristic of *ae* endosperm starch. The specific functions of SBEIIa and SBEIIb in starch synthesis await further investigation.

The SBEIIa and SBEIIb mature proteins share 89% sequence similarity over 685 amino acids, accounting for approximately 85% of the total length of the SBEII isozymes (Fig. 3). The predicted $(\beta\text{-}\alpha)_8$ barrel structure and amino acids participating in the active sites of plant SBEs are also highly conserved in maize SBEIIa and SBEIIb. The loop size between β-strand 8 and α-helix 8, which was suggested to have an impact on the size of branched glucosyl chains (Burton et al., 1995), are the same in SBEIIa and SBEIIb (Fig. 3). In addition, the amino acids in the loop are highly conserved in the two isozymes. Both isozymes indeed seem to preferentially transfer glucosyl chains shorter than SBEI (Takeda et al., 1993). Other in vitro catalytic properties of SBEIIa and SBEIIb are also extremely similar (Preiss, 1991; Guan and Preiss, 1993; Takeda et al., 1993).

The only major structural difference between SBEIIa and SBEIIb mature proteins is a stretch of highly divergent amino acid sequences from their N termini to the three highly conserved Pros. The N-terminal divergent region of SBEIIa mature protein has a 49-amino acid extension compared with the SBEIIb mature protein. In addition, the 54 amino acids in the N-terminal divergent region of SBEIIb mature protein share a very limited similarity with their counterparts in SBEIIa mature protein (Fig. 3). A short, highly divergent region at the N termini was also observed in the two members of the SBEII family in *A. thaliana* (Fisher et al., 1996b). These short stretches of divergent amino acids at the N termini of SBEIIa and SBEIIb may define differences in their noncatalytic properties.

An intriguing question arises from the comparison between the transcript levels of the *Sbe2a* and *Sbe2b* genes and their encoded protein levels. The *Sbe2b* transcript level is 35-fold higher than that of *Sbe2a* in normal endosperm (Table II). However, the SBEIIa protein level is approximately one-fourth to one-half of the SBEIIb protein level, as estimated from the protein content of SBEIIa and SBEIIb fractions from the DEAE-Sepharose Fast-flow column when correcting for contaminating SBEIIb in the SBEIIa fractions. This large discrepancy may indicate that the *Sbe2a* message may be more efficiently translated or that the SBEIIa protein is more stable than the SBEIIb protein. The cloning of the *Sbe2a* cDNA clears up the long-standing controversy regarding the genetic control of SBEIIa and SBEIIb and provides a new tool for studying the biochemical properties, genetic regulation, and roles of SBEs in starch synthesis.

ACKNOWLEDGMENTS

We would like to thank Drs. Richard Cyr, Donald Thompson, and Teh-Hui Kao for critical review of the manuscript and Chris Dervenis for technical assistance.

Received November 20, 1996; accepted January 21, 1997.

LITERATURE CITED

Baba T, Arai Y, Ono T, Munakata A, Yamaguchi H, Itoh T (1982) Branching enzyme from amylomaize endosperms. Carbohydr Res **107:** 215–230

Baba T, Kimura K, Mizuno K, Etoh H, Ishida Y, Shida O, Arai Y (1991) Sequence conservation of the catalytic regions of amylolytic enzymes in maize branching enzyme-I. Biochem Biophys Res Commun **181:** 87–94

Boyer CD, Garwood DL, Shannon JC (1976) The interaction of the *amylose-extender* and *waxy* mutants of maize (*Zea mays* L.): fine structure of *amylose-extender waxy* starch. Starch/Stärke **28:** 405–409

Boyer CD, Preiss J (1978a) Multiple forms of (1,4)-alpha-D-glucan-(1,4)-alpha-D-glucan-6-glycosyl transferase from developing *Zea mays* L. kernels. Carbohydr Res **61:** 321–334

Boyer CD, Preiss J (1978b) Multiple forms of starch branching enzyme of maize: evidence for independent genetic control. Biochem Biophys Res Commun **80:** 169–175

Burton RA, Bewley J, Smith AM, Bhattacharyya MK, Tatge H, Ring S, Bull V, Hamilton WDO, Martin C (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. Plant J **7:** 3–15

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

Dang PL, Boyer CD (1988) Maize leaf and kernel starch synthases and branching enzymes. Phytochemistry **27:** 1255–1259

Dang PL, Boyer CD (1989) Comparison of soluble starch synthases and branching enzymes from leaves and kernels of normal and *amylose extender* maize. Biochem Genet **27:** 521–532

Fisher DK, Boyer CD, Hannah LC (1993) Starch branching enzyme II from maize endosperm. Plant Physiol **102:** 1045–1046

Fisher DK, Gao M, Kim K-N, Boyer CD, Guiltinan MJ (1996a) Allelic analysis of the maize (*Zea mays* L.) *amylose-extender* locus suggests that independent genes encode starch branching enzymes IIa and IIb. Plant Physiol **110:** 611–619

Fisher DK, Gao M, Kim K-N, Boyer CD, Guiltinan MJ (1996b) Two closely related cDNAs encoding starch branching enzyme from *Arabidopsis thaliana*. Plant Mol Biol **30:** 97–108

Fisher DK, Kim KN, Gao M, Boyer CD, Guiltinan MJ (1995) A cDNA encoding starch branching enzyme I from maize endosperm. Plant Physiol **108:** 1313–1314

Fisher MB, Boyer CD (1983) Immunological characterization of maize starch branching enzymes. Plant Physiol **72:** 813–816

Gao M, Fisher DK, Kim K-N, Shannon JC, Guiltinan MJ (1996) Evolutionary conservation and expression patterns of maize starch branching enzyme I and IIb genes suggests isoform specialization. Plant Mol Biol **30:** 1223–1232

Gavel Y, von Heijne G (1990) A conserved cleavage-site motif in chloroplast transit peptides. FEBS Lett **261:** 455–458

Giroux MJ, Boyer G, Feix G, Hannah LC (1994) Coordinated transcriptional regulation of storage product genes in the maize endosperm. Plant Physiol **106:** 713–722

Guan HP, Preiss J (1993) Differentiation of the properties of the branching isozymes from maize (*Zea mays*). Plant Physiol **102:** 1269–1273

Hedman KD, Boyer CD (1983) Allelic studies of the *amylose-extender* locus of *Zea mays* L.: levels of the starch branching enzymes. Biochem Genet **21:** 1217–1222

Inouchi N, Glover DV, Takaya T, Fuwa H (1983) Development changes in fine structure of starches of several endosperm mutants of maize. Starch/Stärke **35:** 371–376

Konat GW, Laszkiewicz I, Grubinska B, Wiggins R (1994) Generation of labeled DNA probe by PCR. *In* HGG Griffin, M Annette, ed, PCR Technology, Current Innovations. CRC Press, Boca Raton, FL, pp 37–42

Mizuno K, Kimura K, Arai Y, Kawasaki T, Shimada H, Baba T (1992) Starch branching enzymes from immature rice seeds. J Biochem **112:** 643–651

Mu-Forster C, Huang R, Powers JR, Harriman RW, Knight M, Singletary GW, Keeling PL, Wasserman BP (1996) Physical association of starch biosynthetic enzymes with starch granules of maize endosperm. Granule-associated forms of starch synthase I and starch branching enzyme II. Plant Physiol **111:** 821–829

Nakamura Y, Takeichi T, Kawaguchi K, Yamanouchi H (1992) Purification of two forms of starch branching enzyme (Q-enzyme) from developing rice endosperm. Physiol Plant **84:** 329–335

Preiss J (1991) Biology and molecular biology of starch synthesis and its regulation. Oxf Surv Plant Mol Cell Biol **7:** 59–114

Simon MM, Palmetshofer A, Schwarz T (1994) From RNA to sequenced clones within three days: a complete protocol. BioTechniques **16:** 633–637

Singh BK, Preiss J (1985) Starch branching enzymes from maize. Immunological characterization using polyclonal and monoclonal antibodies. Plant Physiol **79:** 34–40

Stinard PS, Robertson DS, Schnable PS (1993) Genetic isolation, cloning, and analysis of a mutator-induced, dominant antimorph of the maize amylose extender 1 locus. Plant Cell **5:** 1555–1566

Takeda Y, Guan HP, Preiss J (1993) Branching of amylose by the branching isoenzymes of maize endosperm. Carbohydr Res **240:** 253–263

Yamanouchi H, Nakamura Y (1992) Organ specificity of isoforms of starch branching enzyme (Q-enzyme) in rice. Plant Cell Physiol **33:** 985–991