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MOLECULAR CHARACTERIZATION OF STARCH BRANCHING ENZYME GENES, *Sbe1*, *Sbe2b* AND *Sbe2a* IN MAIZE (*Zea mays* L.)

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ABSTRACT

Starch branching enzymes (SBE) play a central role in amylopectin synthesis. This research was focused on the genetic regulation of the three maize (*Zea mays* L.) SBE isozymes, SBEI, SBEIIa and SBEIIb, and on their individual roles in amylopectin synthesis.

In order to gain insight into individual roles of maize SBE isozymes in starch synthesis, expression of the maize starch branching enzyme (SBE) genes *Sbe1* and *Sbe2b* were characterized during kernel development and in vegetative tissues. The onset of *Sbe1* and *Sbe2b* expression during endosperm development was similar to that of other genes involved in starch biosynthesis (*Wx*, *Sh2* and *Bt2*). However, the expression of *Sbe2b* peaked earlier than that of *Sbe1* in developing endosperm and embryos resulting in a shift in the ratio of *Sbe1* to *Sbe2b* relative message levels during kernel and embryo development. Transcripts hybridizing to the *Sbe2b* probe were not detectable in leaves or roots which nonetheless have SBEII enzymatic activity, suggesting that there may be another divergent starch branching enzyme II-like gene(s) in maize. A similar expression pattern is shared between the maize genes and related genes in pea, which together with their evolutionary conservation, suggests that the SBE isoforms may play unique roles in starch biosynthesis during plant development.

In order to provide conclusive evidence for independent genetic control of SBEIIa and SBEIIb, a near-full-length *Sbe2a* cDNA was isolated and characterized. A 538 bp cDNA fragment was first cloned from leaf total RNA by RT-PCR using primers derived from sequences almost completely conserved in pea *Sbe1* (homologous to maize *Sbe2b*), rice *rbe3* and maize *Sbe2b*. The cDNA fragment showed 78% sequence similarity to *Sbe2b* cDNA, but only 40% similarity to maize *Sbe1* cDNA, suggesting that it was

amplified from an mRNA encoding a *Sbe2* family protein. A near-full-length *Sbe2* family cDNA of 2.795 kb was then isolated by screening a maize leaf cDNA library. This leaf *Sbe2* family cDNA, compared to the endosperm *Sbe2b* cDNA counterparts, is highly conserved in the central region (380 to 2445 bp, 78 % similarity), but quite divergent at the 5' end (0 to 380 bp, 29% similarity) and the 3' UTR region (2445-2763, 38% similarity). The deduced amino acid sequence from the longest open reading frame of the *Sbe2* family cDNA shares 77% similarity to the deduced SBEIIb sequence. The amino acid sequences corresponding to the central conserved regions of the *Sbe2* family cDNA and the *Sbe2b* cDNA share 89% sequence similarity. The predicted $(\beta\text{-}\alpha)_8$ structures and amino acids participating in the active sites of plant SBEs are also highly conserved in the amino acid sequence deduced from the *Sbe2* family cDNA. These results suggest that the *Sbe2* family cDNA encodes leaf SBEIIa.

The N-terminal 14 amino acids of mature SBEIIa protein purified from *ae-B1* endosperm perfectly match the amino acid sequence deduced from the putative *Sbe2a* cDNA. The molecular weight (89.583 kD) calculated from the deduced SBEIIa mature protein sequence closely matches that of endosperm SBEIIa experimentally determined by SDS PAGE (89 kD). Moreover, a portion of the putative *Sbe2a* cDNA, a 538 bp cDNA fragment as amplified from the leaf total RNA, was also cloned from *ae-B1* endosperm total RNA by RT-PCR. Northern and RNase protection assay (RPA) analysis with the probe specific to the putative *Sbe2a* cDNA detected a very low level of transcript in endosperm and embryo of both normal W64A and *ae-B1* kernels, leaves, roots, young stems and tassels of normal W64A plants. Southern genomic analysis with gene specific probes showed that the putative *Sbe2a* cDNA is encoded at a locus distinct from the *Sbe2b* locus in the maize genome. Taken together, these results and the data from molecular genetic analysis of *ae* mutants lead to the conclusion that this putative *Sbe2a* cDNA indeed

encodes maize SBEIIa.W64A and B73 inbred, together with results from molecular genetic analysis of maize mutants deficient

The expression pattern of three SBE genes in various tissues of both in starch synthetic enzymes, suggests that the three SBE isoforms may differentially contribute to the synthesis of different amylopectin branch structures, controlling the quantity of synthesis of different types of amylopectin. Functional specialization may be achieved by differences in both the catalytic (SBEI vs. SBEIIa or IIb) and non-catalytic (SBEIIa vs. IIb) properties as suggested by their structural and *in vitro* catalytic properties.

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CHAPTER 1

INTRODUCTION

Starch is the most significant carbohydrate reserve in higher plants. It can be found as cold-water-insoluble granules in all organs of most higher plants (reviewed in [19]). The storage organs of higher plants, including seeds, fruits, tubers, bulbs and storage roots, are the major sites of starch accumulation. Starch accumulates in specialized plastids, the amyloplast in storage organs such as seeds, chloroplasts in leaf tissues and chromoplasts such as those in carrot roots, which are all differentiated from proplastids during plant development. Starch granules in these plastids are different in morphology and structure, suggesting that different sets of starch synthetic enzymes are expressed in these plastids differentiated from the same proplastids. Starch granules in these plastids of higher plants are usually classified as transitory [19]. Leaf chloroplast starch, an example of transitory starch, functions as a temporary storage of photoassimilates during the day and is mobilized to simple sugars for translocation to other plant parts at night or whenever assimilate demand by "sink tissues" exceed current assimilate production. Reserve starch granules in storage organs of higher plants are formed during one phase of the plant's life cycle, e.g. seed formation, and utilized during another phase, e. g. seed germination. The reserve starch in storage organs of many higher plants is not only a primary food source for humans and other animals but also is a very important raw material for various industrial applications.

Starch is mainly composed of two glucan polymers, amylose and amylopectin, which are arranged into a three-dimensional, semicrystalline granule structure [16].

Amylose consists of predominantly linear chains of α(1-4) linked glucose residues, each about 1,000 residues long. A small number of α(1-6) branches about one per 1,000 glucosyl units may occur in amylose [18, 19]. Amylose usually makes up 11 to 35% of the reserve starch granule, depending on the species, cultivars and the developmental stages of the source tissues (reviewed in [19]). Amylopectin consists of highly branched glucan chains with α(1-6) linkage at the branch points. About 65 to 75% of the reserve starch is amylopectin [16, 18].

The two component polysaccharides of starch have distinctive properties because of their different chemical structures. The highly branched amylopectin is quite stable in aqueous solution, while the linear amylose spontaneously crystallizes (retrogradation) out of aqueous solution depending on concentration, degree of polymerization and temperature [18]. Amylose in solution exists as an α-helix and complexes with fatty acids, low molecular weight alcohols and iodine. Thus, the two component polysaccharides contribute to different aspects of the structural and biochemical properties of starch granules. Amylose molecules are thought to be packed in the form of single helix structures in amorphous regions of starch granules, while the amylopectin molecules determine the crystal structure of starch granules (reviewed in [16]).

Starch biosynthesis in higher plants involves the activity of three enzymes, ADPG pyrophosphorylase (AGPase), starch synthase and starch branching enzyme (SBE) (reviewed in [16-18]). Multiple isoforms of starch synthase and SBE have been reported (reviewed in [16, 18]). Multiple isozymes of AGPase have been reported in barley endosperm [22] and proposed to be present in maize endosperm [10]. Starch branching enzymes catalyze the formation of α-1,6 linkages at branching points in amylopectin. SBE

plays a central role in the synthesis of amylopectin, although recent cloning of the maize *sugary* 1 gene revealed that debranching enzymes are also involved in the formation of amylopectin [14]. The mechanisms and regulation of starch synthesis in plant tissues such as maize endosperm are, however, not completely understood. Relatively detailed knowledge about the mechanisms and regulation of starch biosynthesis are vital for potential modification of starch for special dietary and industrial applications by recombinant DNA techniques.

Maize endosperm have been reported to contain three forms of SBE (SBEI, SBEIIa and SBEIIb) [2, 3]. SBEI and SBEIIa are also found in maize leaves [5]. The biochemical properties of these SBE isozymes have been extensively studied [9, 18, 21]. However, two important questions about SBEs in maize remain to be addressed. First, what are the individual roles of each SBE isozyme in the synthesis of amylopectin in maize leaves and endosperm? Secondly, how are the three SBE isoforms genetically regulated? Answers to these two questions are of interest for understanding fundamental aspects of amylopectin synthesis and formation of starch granules, and also are critical for potential molecular manipulation of amylopectin synthesis for the production of special purpose starches for industry. This thesis was centered on increasing our understanding of these two important questions from the perspectives of gene expression and regulation.

One hypothesis about the individual role of the three SBE isozymes is that all of them are needed in different aspects of the synthesis of normal amylopectin in endosperm, and that SBEI and SBEIIa are essential for the synthesis of normal amylopectin in leaves. Branches in an amylopectin molecule are arranged in a cluster structure which has a great impact on the crystallinity of starch granules [15, 16]. These SBE isoforms may be specialized in the formation of different branches to form an orderly cluster structure. Loss of either of the isoforms may result in defects or elimination of the cluster structure.

A novel type of amylopectin containing branches of longer average chain lengths was indeed found in endosperm of *amylose-extender* (*ae*), a maize endosperm mutant deficient in SBEIIb activity [1, 13].

An alternative hypothesis is that any one of the SBEs is enough for the formation of amylopectin branches. Multiple forms of SBE in maize may be employed as a genetic mechanism to control the quantity of amylopectin synthesis in various tissues or at different developmental stages of the same tissue via their different expression levels or different interactions with starch synthases and debranching enzymes. This hypothesis predicts that the three isoforms should be expressed differentially in different tissues or at different developmental times in the same tissue.

Some or all of the three isozymes may be employed both for functional specialization in the formation of branch structures of amylopectin and for genetic control over the quantity of the synthesis of amylopectin with special branch structures. In addition to the formation of a novel amylopectin, loss of SBEIIb in *ae* endosperm resulted in reduction of total starch content and amylopectin up to 20% and 70%, respectively [16]. Thus, SBEIIb in maize endosperm may be specialized in the production of larger quantities of a particular type of amylopectin. Examination of the expression of genes encoding the three SBE isozymes in various developmental stages and tissues could shed light on the roles of a particular SBE isozyme. This was greatly facilitated by the cloning of cDNAs encoding maize SBE I and SBEIIb in our lab [7, 8]. Expression of both genes in various maize tissues and organs at different developmental stages was examined by northern analysis, and reported in this thesis (Chapter 2 and Appendix 1).

Comparison of SBEIIa and SBEIIb in amino acid composition, peptide mapping, and immunological properties showed these two isoforms to be indistinguishable, which earlier led to the conclusion that they were encoded by a single gene [18, 20]. However,

the genetic data indicates that the two isozymes are products of two genes [4-6, 11, 12]. To distinguish whether SBEIIa and SBEIIb are products of one gene or two genes is very important in understanding the regulation of these two isozymes, and is thus crucial for potential genetic engineering of starch. The one-gene hypothesis would suggest a posttranscriptional or posttranlational regulation, while the two-gene hypothesis would indicate independent genetic control. Research reported in this thesis was designed to test the two gene hypothesis. A novel cDNA encoding leaf SBEIIa was isolated from a maize leaf cDNA library, and this gene was also shown to encode endosperm SBEIIa as reported in this thesis (Chapter 3).

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CHAPTER 2

EVOLUTIONARY CONSERVATION AND EXPRESSION PATTERNS OF MAIZE STARCH BRANCHING ENZYME I AND IIB SUGGESTS ISOFORM SPECIALIZATION

2.1. Introduction

Starch biosynthesis in plants involves the activity of three groups of enzymes, ADPG pyrophosphorylase, starch synthase and starch branching enzyme (SBE) (reviewed by [28, 31, 36]). Recent molecular cloning of the *sugary1* locus in maize has also revealed the importance of starch debranching enzyme in the determination of starch composition in endosperm [18]. Starch is composed of two structurally different glucans, amylose and amylopectin. Amylose is an essentially linear polymer of α-1, 4 linked glucosyl residues while amylopectin is a branched polymer of α-1,4 linked glucosyl residues with α-1,6 linkages at branch points. Starch branching enzyme hydrolyzes α-1,4 bonds and reattaches the released α-1,4 glucan segments to the same or another glucosyl chain by an α-1-6 linkage. The reaction creates not only branch points but also new non-reducing ends for further elongation of the α-1,4 glucan branches.

Multiple forms of SBE have been characterized in many species, including spinach leaf, pea embryo, potato tuber, maize endosperm and leaf [9, 30, 31] , and recently in rice seed and other vegetative tissues [25, 26, 41]. The SBE isoforms in maize [8], rice [27] and pea [35] have been classified into two groups, SBEI and II, based on their chromatographic properties. In maize, two isoforms of SBEII (IIa and IIb) have been further resolved [7]. The biochemical differences of the SBE isoforms in maize have been

well characterized [31]. In maize, SBEI is quite different from SBEIIa and IIb in molecular size, chromatographic and enzyme kinetic properties and immunological reactivity, whereas SBEIIa and IIb are quite similar, but not identical, in most biochemical properties (reviewed by [31]). However, the particular role of each SBE isoform in starch biosynthesis remains unknown.

The recent isolation of genes encoding various SBE isoforms in several plant species has greatly facilitated the investigation of the role of SBE isoforms in starch biosynthesis. The first SBE cDNA was isolated for the gene encoding pea SBEI at the *rugosus (r)* locus ([5], EMBL acc. # 80009), used by Mendel in his study on the law of inheritance [5, 23]. cDNAs encoding SBE have been isolated from potato tubers [21, 29], rice seed [19, 24, 25, 27] and cassava [32]. The developmental expression patterns of several cloned SBE genes have been well characterized in potato [21, 32, 39], rice [19, 24, 25] and in pea embryo [4, 9]. In maize, a partial cDNA encoding SBEI was first isolated by Baba et al. [2]; a full length cDNA encoding maize SBEI was later isolated by Fisher et al., [16]. Our laboratory first reported the isolation of a full length cDNA (*Sbe2b*) encoding SBEIIb using the pea *Sbe1* cDNA as a heterologous hybridization probe [13]. The identity of this cDNA as the product of the *amylose-extender* gene in maize was subsequently verified by transposon tagging [37].

Sequence comparisons of the various SBE genes and their encoded proteins have revealed that two distinct classes of genes encoding SBE may be clearly distinguished [9, 36]. As listed in Table 2.1, these classes are referred by the prototypic family member names maize SBEI and SBEII as recommended by the Commission on Plant Gene Nomenclature [10]. In order to gain insight into the role of each maize SBE isoform in starch synthesis, the expression of the cloned *Sbe1* and *Sbe2b* genes at different developmental stages were examined. This chapter presents evidence that the *Sbe1* and

Sbe2b genes are expressed differentially during maize kernel development. The onset of *Sbe1* and *Sbe2b* mRNA accumulation in developing endosperm is similar to that of other genes involved in starch biosynthesis (*Wx*, *Sh2* and *Bt2)*. However, the timing of their maximal expression levels differ, resulting in a shift of the ratio of *Sbe1* to *Sbe2b* mRNAs during development, similar to that described for corresponding family members during pea embryo development [9]. Furthermore, while SBEI and II enzymatic activity can be detected in maize leaves and roots, we show that only the *Sbe1* cDNA hybridizes to mRNA from these tissues, indicating that there could be another divergent gene encoding vegetative SBEII activity in maize. The evolutionary conservation of the genes encoding SBEI and II in pea and maize and their distinct expression patterns suggest specific roles of the SBE isoforms in starch biosynthesis.

2.2. Materials and Methods

2.2.1. Plant Materials

Maize (*Zea mays* L.) inbred W64A was field grown, and inbred B73 was grown in 4-liter pots outside from late in May to late Oct. of 1993. Plants were self or sib-pollinated and developing kernels were harvested at various days after pollination (DAP). Kernels at later developmental stages were hand dissected into endosperm and embryo fractions. Leaves and primary roots from W64A seedlings were sampled at various days after emergence (DAE). The seedlings were germinated and grown in perlite in a growth chamber at 30^{o} C under a 14 h light and 10 h dark photoperiod regime. Leaf blades and sheaths at different positions, stem segments of the top most 5 cm and the next 5 cm and a tassel just before anthesis were harvested from one maize B73 plant grown in a 4 liter-pot outside. All of the samples were immediately frozen in liquid nitrogen and stored at -70^{o}C.

Table 2.1. Nomenclature system for plant starch branching enzyme genes based on enzymological precedence of the maize endosperm isoforms as suggested by the Commission on Plant Gene Nomenclature [10], and categorization of individual SBE family members from various species. In this manuscript, protein designations are in all caps and Roman numerals, all genes, cDNAs and mRNAs are designated in italics with first letter caps only and Arabic numerals. A previously published nomenclature system is also listed [9]. cDNAs from maize have been categorized based on encoded sequence identity to N-terminal amino acid sequence data of purified SBEI and SBEII protein. All other cDNAs can be categorized into either the SBEI family or the SBEII family based on sequence relatedness to the maize cDNAs as reported [10, 15], although their published designations may not correspond numerically.

Enzymological Precedence	SBEI Family EC 2.2.4.1.18:1	SBEII Family EC 2.2.4.1.18:2
Commission on Plant Gene Nomenclature [10]	Individual Family Members maize *Sbe1* pea *Sbe2* rice *BE-1* potato *Sbe* cassava *Sbe*	Individual Family Members maize *Sbe2b* pea *Sbe1* rice *Rbe3* arabidopsis *Sbe2.1, Sbe2.2*
Previously Proposed Nomenclature System [9]	Family B	Family A

2.2.2. cDNA cloning

Our laboratory reported the cloning of a *Sbe2b* cDNA by screening maize λgt10 cDNA libraries constructed from endosperm poly(A)+ RNA (14, 22, 29 DAP) with the pea *Sbe1* cDNA [5] as a probe [13] . A full length cDNA clone of the maize *Sbe1* gene was isolated by screening the same maize endosperm cDNA libraries with a PCR-amplified 426 bp fragment of the 5' coding region of *Sbe1* from maize genomic DNA [16]. It should be noted that the *Sbe1* and *Sbe2b* genes exist as multigene families in the maize genome with two to three members each (M. Guiltinan, unpublished data). While the cDNA probes for each isoform class used in this study do not hybridize to each other, they do cross-hybridize to each of the related genes in their respective families and thus are expected to hybridize with mRNA from each. Thus, the transcript levels presented here represent mRNA from one or multiple genes in a given family, representing the total message for a given isoform class.

2.2.3. Northern blotting and RNA Quantification

Total RNAs from all samples were extracted as described [22] and fractionated on a 2.2 M formaldehyde, 1.2% (w/v) agarose gel. All experiments were repeated on RNA extracted from independent ears. Total RNA was dried in a SpeedVac (Savant), and denatured at 65° C for 15 min. in 30 µl of loading buffer containing 50% (v/v) formamide, 2.2 M formaldehyde, 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), 4% (v/v) glycerol, 0.8% (w/v) bromophenol blue and 1 µg ethidium bromide. Denatured RNA samples were chilled on ice for 3-5 min., loaded and allowed to rest in wells for 5-10 min. before running. They were run at 2-3 v cm^{-1} for 4-5 h until

bromophenol blue neared the bottom of the gel. Fractionated RNA was blotted on nylon membranes (Hybond N, Amersham) by the manufacturer's instruction, and fixed by a 30-second exposure under a UV lamp. Membranes were prehybridized and hybridized at 65° C in 10 ml of the buffer containing 6x SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 100 μg mL^{-1} salmon sperm DNA, and 5x Denhardt's (0.1% (w/v) Ficoll, 0.1% (w/v) PVP, 0.1% (w/v) BSA). The probes were labeled with [^{32}P] dCTP using a random primed DNA labeling kit (Boehringer Mannheim Biochemica, Cat. No. 1004760), and purified with Sephadex G-50 spun columns [33]. Membranes were washed with 2x SSC for 5 min., 2x SSC and 0.1% (w/v) SDS twice for 10 min. each at room temperature, and 0.1x SSC and 0.1% (w/v) SDS at 65° C for 15 min. Membranes were exposed to x-ray film with one intensifying screen at -70° C. Radioactivity was directly quantified with a ß-scope (Betagen, Mountain View, CA.). Membranes were stripped of the radioactive probes in a boiling 0.1% (w/v) SDS solution and allowed to cool to room temperature. Membranes were then sequentially hybridized with other probes. The actual amount of the total RNA loaded was based on the total RNA concentration calculated from spectrophotometric readings (absorbance at 260 nm). Minor RNA loading differences between samples from the same tissue or organ were calibrated using a tomato cDNA probe hybridizing to the 26 S rRNA. The relative mRNA level (R.L.) of each band on a membrane was then calculated and expressed as the percentage of the peak expression level in the same tissue at a different developmental stage or the control as specified in figure legends, based on ß-scope data.

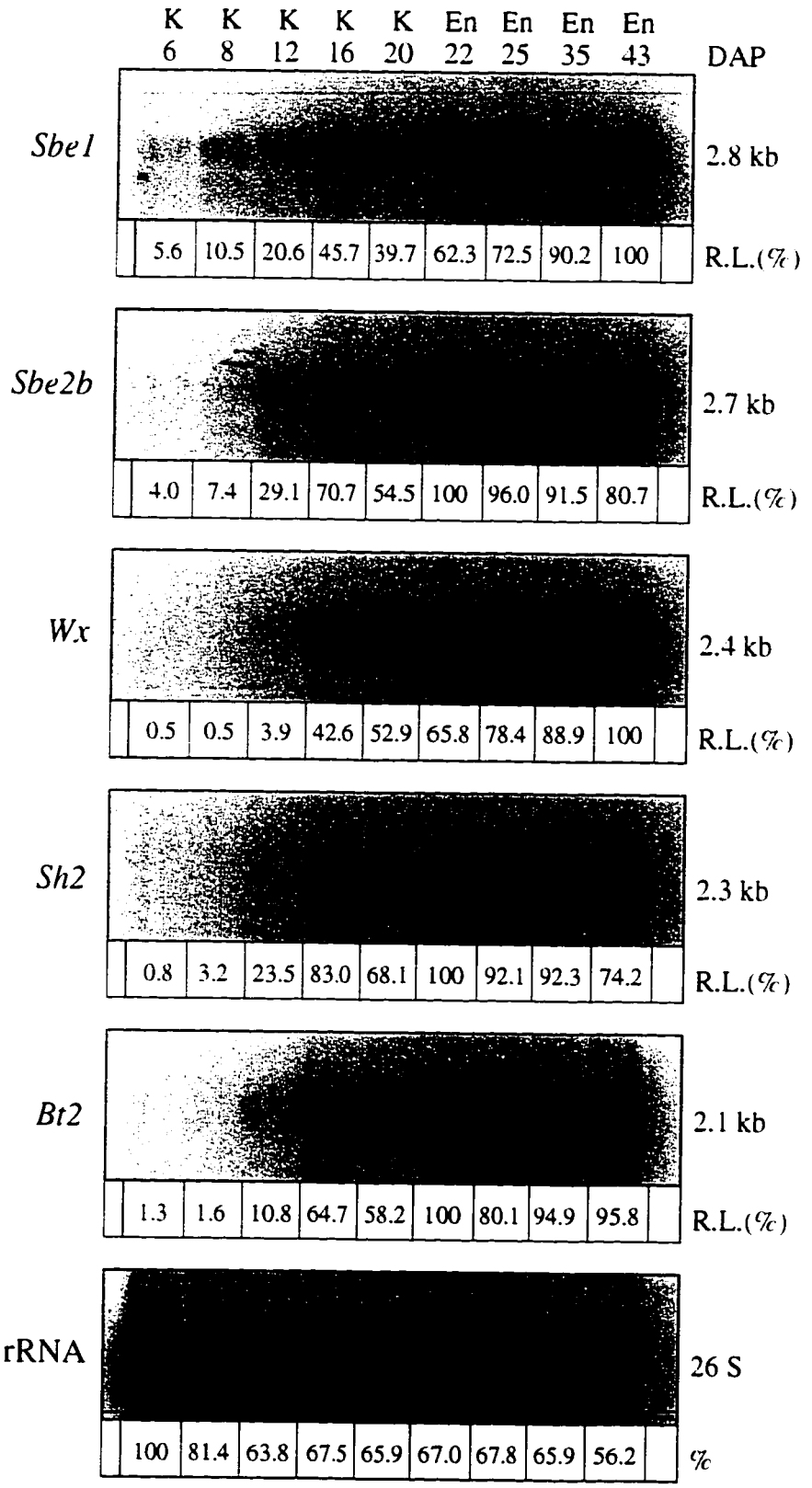
2.3. Results

2.3.1. Expression of *Sbe1* and *Sbe2b* genes during maize kernel development

To investigate the roles of the *Sbe1* and *Sbe2b* genes in starch biosynthesis, we characterized their expression patterns during maize kernel development and compared them with those of several other starch biosynthetic genes, including *Wx*, encoding the granule bound starch synthase [34], and *Sh2* [6] and *Bt2* [3], encoding the large and small subunits of ADPG pyrophosphorylase, respectively. Total RNA was extracted from whole kernels, endosperm and embryos of kernels at various developmental stages and analyzed by northern blotting. Total RNA from endosperm of kernels at mid or later stages were used in the blots shown in Fig. 2.1. Because of the difficulty of separating the endosperm and embryo of very young kernels, total RNA was extracted from whole kernels at the early stages of development. The endosperm tissue takes up much of the cytoplasmic content of kernels at 12-20 DAP [20], and thus in a practical sense, the transcript levels in total RNA from kernels at these early stages is quantitatively representative of the endosperm of the same age (data not shown).

The *Sbe1* gene was expressed at a very low level in W64A kernels at 6 and 8 DAP and began to accumulate to high levels after 12 DAP (Fig. 2.1.). Similarly, the *Sbe2b* transcript level was first detectable at 6 DAP, and continued to rise thereafter. The expression of *Sbe1* peaks in endosperm at a developmental stage at or later than 43 DAP. The transcript level of the *Wx* gene likewise increased throughout kernel development. Thus, the developmental timing of the *Sbe1* gene expression parallels that of the *Wx* gene except that *Wx* mRNA was not significantly detectable in kernels before 12 DAP. Very similar expression patterns for the two genes were also observed in B73 kernels during development (see appendix). The *Sbe2b* expression pattern was very similar to those of the

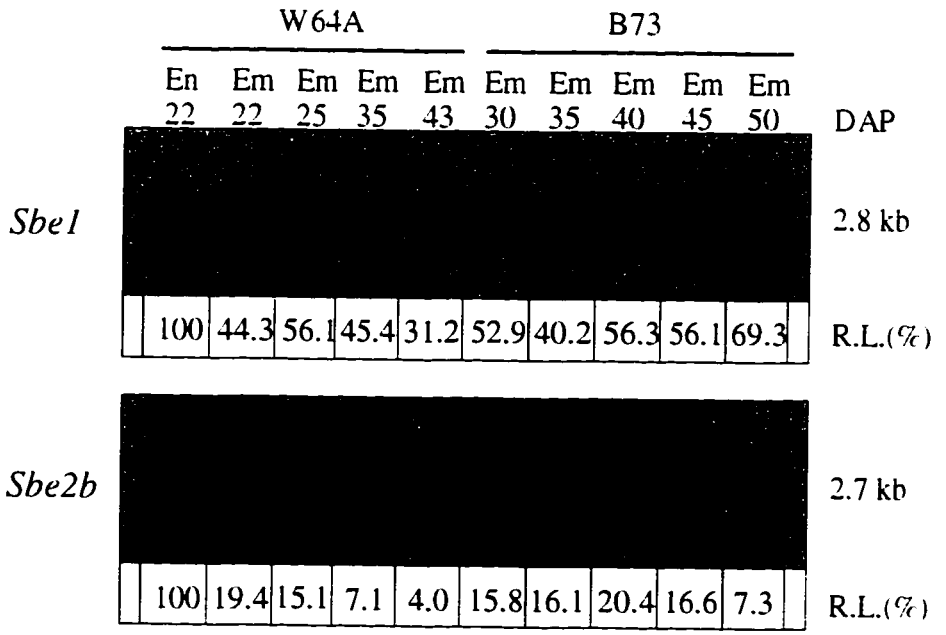
Fig. 2.1. Northern blot analysis of the expression of starch biosynthetic genes during development of W64A maize kernels. 7.5 μg of total RNA extracted from kernels (K) and endosperm (En) at various days after pollination (DAP) was fractionated, blotted and probed consecutively with radioactively labeled *Sbe1* [16], *Sbe2b* [13], *Sh2* [6], *Bt2* [3], and *Wx* [34] cDNAs. The relative mRNA level (R.L.) of each gene in a sample was expressed as the percentage of its maximal transcript level in a particular sample (e.g., 22 DAP endosperm for *Sbe2b*), calibrated for the 26S rRNA levels in the two samples. R.L. for the *Sbe1*, *Sbe2b* and *Sh2* genes represent the average of three or four repeated blots with the absolute value of standard error less than 9%. The data shown below *Bt2* and *Wx* are the average from two repeated blots with the absolute value of standard error less than 15.3 %. The data below the 26S rRNA bands are the percentage of the maximal 26S rRNA level on the particular blot. All repeat experiments were performed on RNA extracted from independent ears.


K 6
K 8
K 12
K 16
K 20
En 22
En 25
En 35
En 43
DAP
Sbe1
2.8 kb
5.6 10.5 20.6 45.7 39.7 62.3 72.5 90.2 100
R.L.(%)
Sbe2b
2.7 kb
4.0 7.4 29.1 70.7 54.5 100 96.0 91.5 80.7
R.L.(%)
Wx
2.4 kb
0.5 0.5 3.9 42.6 52.9 65.8 78.4 88.9 100
R.L.(%)
Sh2
2.3 kb
0.8 3.2 23.5 83.0 68.1 100 92.1 92.3 74.2
R.L.(%)
Bt2
2.1 kb
1.3 1.6 10.8 64.7 58.2 100 80.1 94.9 95.8
R.L.(%)
rRNA
26 S
100 81.4 63.8 67.5 65.9 67.0 67.8 65.9 56.2
%

Sh2 and *Bt2* genes. As shown in Fig. 2.1., although barely detectable at earlier stages, the message levels of all three genes first accumulated to significant levels in kernels at 12 DAP, then gradually increased until peaking at 22 DAP. They dropped slightly in endosperm older than 22 DAP, but were maintained at a high level as the endosperm matured. Very similar developmental expression patterns for all three genes were also observed in B73 kernels except that the expression peaked later at about 30 DAP (see appendix). This difference between the inbreds examined is likely due to differences in the environmental conditions during the growth of the plants. It should be noted that quantitative levels of transcripts encoded by different genes can not be compared since the specific activity, length and the hybridization times were slightly different for each probe. However, qualitatively, it is valid to compare the developmental timing of the peak expression of the five genes, based on these data. Significantly, the peak expression of *Sbe1* and *Wx* genes occurred later than those of *Sbe2b*, *Sh2* and *Bt2* in developmental timing.

To see if the *Sbe1* and *Sbe2b* genes also exhibit differential expression patterns in developing embryos, total RNA from embryos of both inbreds were analyzed by northern blot. As in endosperm RNA, the *Sbe1* message level peaked later than that of *Sbe2b* in developing embryos of both inbreds (Fig. 2.2.). As indicated by the relative transcript level, the expression of *Sbe2b* peaks at or before 22 DAP in W64A embryos and at about 40 DAP in B73 embryos, whereas *Sbe1* was expressed maximally in W64A embryos at 25 DAP and B73 embryos at 45-50 DAP. Although we cannot make a quantitative comparison of the *Sbe1* and *Sbe2b* message level, we can compare the relative expression levels of each class between embryos and endosperm. The highest level of the *Sbe1* transcript in embryos was about 50 to 70% of the *Sbe1* transcript level in 22 DAP W64A endosperm on a per unit RNA basis. The *Sbe2b* gene was expressed in embryos at about 20% of the *Sbe2b* transcript level in 22 DAP W64A endosperm on a per unit RNA basis.

Fig. 2.2. Northern analysis of the expression of *Sbe1* and *Sbe2b* in developing embryos of W64A and B73 maize inbreds. 15 μg of total RNA extracted from embryos (Em) at various days after pollination was fractionated, blotted and probed consecutively with the *Sbe1* and *Sbe2b* cDNAs. 7.5 μg of total RNA from 22 DAP endosperm of W64A plants was used as a positive control. After calibration of minor loading differences between embryo RNA samples with a rDNA clone, the relative message level (R.L.) of each gene in embryos at various developmental ages was calculated and expressed as a percentage of that in the control.


W64A
B73
En 22
Em 22
Em 25
Em 35
Em 43
Em 30
Em 35
Em 40
Em 45
Em 50
DAP
Sbe1
2.8 kb
100
44.3
56.1
45.4
31.2
52.9
40.2
56.3
56.1
69.3
R.L.(%)
Sbe2b
2.7 kb
100
19.4
15.1
7.1
4.0
15.8
16.1
20.4
16.6
7.3
R.L.(%)

A very similar expression pattern of corresponding family members in developing pea embryos has also been observed [9]. The significance of the differential timing of expression levels of the *Sbe1* and *Sbe2b* genes in starch biosynthesis in endosperm and embryo remains to be investigated.

2.3.2. Expression of *Sbe1* and *Sbe2b* in other maize organs

To investigate the tissue specificity of expression of the *Sbe1* and *Sbe2b* genes and the possible involvement of the products of both genes in starch biosynthesis in organs other than kernels, we characterized their expression in other maize tissues. The *Sbe1* gene was expressed in seedling leaves and young roots of W64A plants at a low and constant level of about 10% of that in 22 DAP endosperm (Fig. 2.3.). No essential difference in the level of *Sbe1* transcript was observed among adult leaf blades from upper, middle and lower parts of a B73 plant and in the leaf sheath (Fig. 2.4.). By contrast, the *Sbe2b* transcript was not detectable in seedling leaves and young roots of W64A plants (Fig. 2.3.), even under low stringency hybridization and with increased loading of the leaf total RNA up to 50 μg (data not shown). Similarly, the *Sbe2b* transcript was not detectable in adult leaf blades and sheath of a B73 plant (Fig. 2.4.). This result does not rule out the possibility that the *Sbe2b* transcript may be expressed at very low levels in leaf, undetectable by this method, or that a separate, divergent, SBEII-like gene is expressed in these tissues. RT-PCR of leaf mRNA using PCR primers designed from highly conserved regions of SBEII family members did not result in the amplification of *Sbe2b* sequences to our detection limits, although large numbers of such clones were obtained from similar reactions using endosperm RNA. This is consistent with previous biochemical evidence of

Fig. 2.3. Northern blot analysis of the expression of *Sbe1* and *Sbe2b* in primary roots and seedling leaves of W64A plants. 15 μg of total RNA from primary roots and seedling leaves collected at various days after emergence were fractionated, blotted and probed consecutively with *Sbe1* and *Sbe2b* cDNAs. 15 μg of total RNA from 22 DAP endosperm of W64A plants was loaded in the first lane as a control. The minor loading differences between leaf RNA samples, and between root RNA samples, respectively, were calibrated with a rDNA clone. The relative message level (R.L.) of each gene in leaves and roots of various developmental age was expressed as the percentage of that in the control.

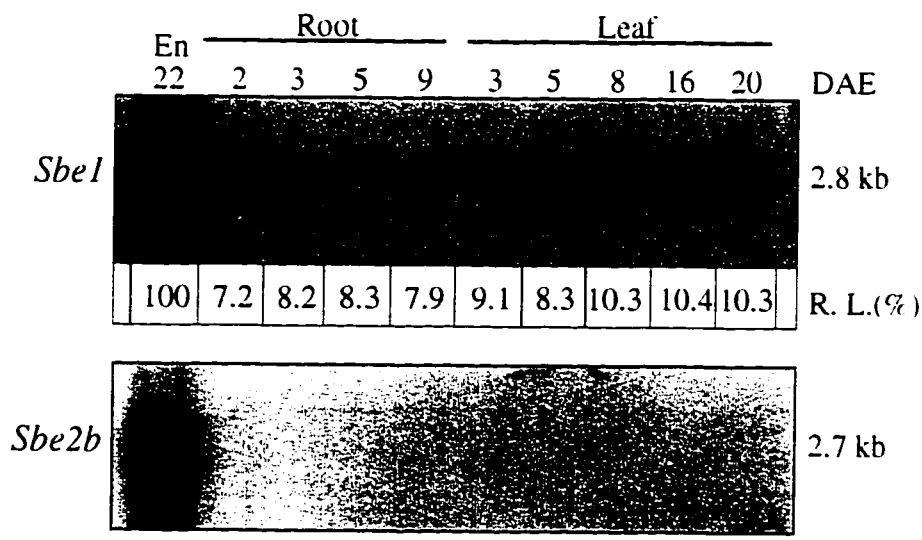
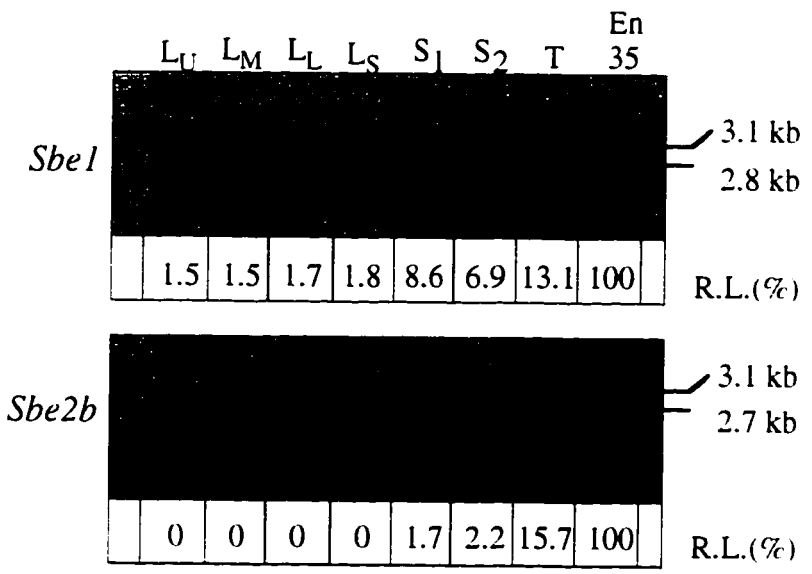

En
Root
Leaf
22 2 3 5 9 3 5 8 16 20
DAE
Sbe1
2.8 kb
100 7.2 8.2 8.3 7.9 9.1 8.3 10.3 10.4 10.3
R. L.(%)
Sbe2b
2.7 kb

Fig. 2.4. Northern blot analysis of the expression of *Sbe1* and *Sbe2b* in the leaf, the young stem and the tassel of a B73 plant just prior to anthesis. 30 μg each of total RNA from leaf blades at upper (L_U), middle (L_M) and lower (L_L) parts, leaf sheath (L_S), stem segments of top most 5 cm (S_1) and the next 5 cm (S_2), and a tassel before anthesis of a B73 plant were fractionated, blotted and probed consecutively with *Sbe1* and *Sbe2b* cDNA clones. 7.5 μg of total RNA from 35 DAP endosperm of B73 plants was used as a control. The minor loading differences among the RNA samples of the leaf blades and sheath were calibrated with a rDNA clone. The relative message level (R.L.) is expressed as the percentage of that in the control.


L_U L_M L_L L_S S_1 S_2 T En 35
Sbe1
3.1 kb
2.8 kb
1.5 1.5 1.7 1.8 8.6 6.9 13.1 100
R.L.(%)
Sbe2b
3.1 kb
2.7 kb
0 0 0 0 1.7 2.2 15.7 100
R.L.(%)

a lack of SBEIIb activity in maize leaves [11, 12] and supports the conclusion that the *Sbe2b* gene is not expressed to significant levels in leaf (data not shown).

The *Sbe1* gene was expressed in the topmost 10 cm of the stem at about 6-8% of that in 35 DAP endosperm. The *Sbe2b* gene was also expressed at an extremely low level (1-2% of that in 35 DAP endosperm) in the upper stem segments. Both *Sbe1* and *Sbe2b* genes were expressed in the tassel just before anthesis (Fig. 2.4.). The *Sbe1* transcript level in the tassel corresponded to about 13% of that in 35 DAP endosperm of B73 plants on a per unit RNA basis. The *Sbe2b* gene was expressed in the tassel at about 16% of that in 35 DAP endosperm. A faint *Sbe1* hybridizing band at about 3.1 kb was reproducibly detected in the tassel and the young stem just above the 2.8 kb *Sbe1* band. A similarly sized 3.1 kb transcript in the tassel total RNA was also detected with the *Sbe2b* cDNA although *Sbe1* and *Sbe2b* cDNAs do not cross hybridize significantly (data not shown). This 3.1 kb transcript(s) were not detected with either the *Sbe1* or the *Sbe2b* probe in maize kernels, young roots and leaves, and its identity remains to be further investigated.

2.4. Discussion

The maize *Sbe1* gene was expressed in all maize tissues or organs examined, including endosperm, embryos, leaf blade and sheath, young stem, young roots and tassel. Moreover, the expression remained at the same low level in young leaves and roots from their emergence. These results suggest that the *Sbe1* gene is constitutively expressed in vegetative tissues. However since our probe cross hybridizes to two or three *Sbe1* genes in the genome, we cannot rule out the possibility that different genes are expressed with tissue specific and complementary distributions.

Unlike in other organs, the *Sbe1* transcript level steadily increased in kernels from an early stage (12 DAP for W64A) until at least 43 DAP (the oldest kernel sampled). Thus, the *Sbe1* gene is strongly induced in developing kernels (mostly in endosperm). The *Sbe1* and *Wx* genes shared a very similar expression pattern in kernels older than 16 DAP, suggesting that the induced expression of *Sbe1* and the expression of *Wx* may be coordinately regulated. The rice *rbe1* cDNA is most similar to the maize *Sbe1* cDNA [19]. Consistent with our results, it was reported that the *rbe1* gene is also constitutively expressed at low levels in most tissues, and is strongly induced during seed development [19]. Interestingly, the timing of its expression is also similar to that of the rice *Wx* gene. The SBE from potato tuber has a high degree of immunological similarity with maize SBEI, in both native and denatured forms [40], and cDNA sequence is most similar to the maize *Sbe1* and the rice *rbe1* cDNAs. Similar to maize *Sbe1* and rice *rbe1*, the potato *Sbe* gene is expressed in a fashion similar to that of the two genes encoding a granule bound starch synthase and the small subunit of ADPG pyrophosphorylase in potato tubers [39]. It is also expressed at an extremely low level in other potato organs, and was strongly induced in leaves by metabolizable carbohydrates such as sucrose [21]. These evidences may suggest some type of interaction between SBEs and other starch synthetic enzymes.

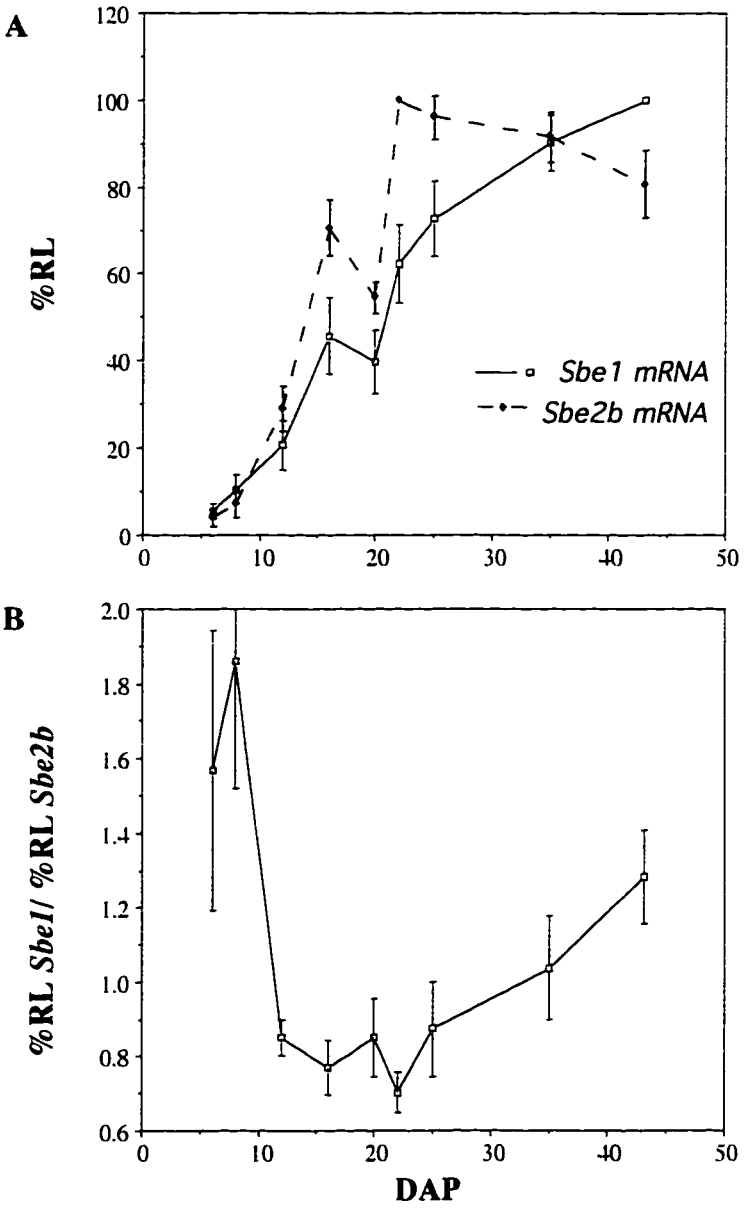
The expression pattern of the *Sbe1* gene during development of the W64A inbred kernel differs from that reported for the Oh 43 inbred kernels [2]. It was reported that the expression of the *Sbe1* gene peaked early in kernel development (10-14 DAP) and dropped quickly thereafter. We also examined the expression pattern of the *Sbe1*, *Sbe2b*, *Sh2*, *Bt2* and *Wx* genes in the maize B73 inbred. The expression pattern of all five genes in B73 was similar to that observed in W64A. The *Sbe1* gene was maximally expressed at a later stage, and the expression of *Sbe2b* peaked at an earlier stage during development of endosperm (see appendix) and embryo (Fig. 2.2.) of the B73 inbred kernels. It is possible that the differences in timing of expression observed in the two inbred genotypes reflects

the effects of different growing conditions on endosperm development (see results and discussion in appendix). In addition, we never detected a 1.5 kb transcript of the *Sbe1* gene previously reported [2].

The maize *Sbe1* and *Sbe2b* genes are differentially expressed in endosperm and embryos during kernel development. As summarized in Fig. 2.5., the expression of the *Sbe1* gene peaked later than that of the *Sbe2b* gene in both developing endosperm and embryos (Fig. 2.1. and 2.2.). This is similar to what has been reported for the genes encoding corresponding family members in pea embryo development [9]. The maize SBEI and IIb isoforms show differences in the length of chains synthesized by recombinantly expressed proteins (11-14 chain length glucans and 8-9 chain length glucans respectively) [38]. If the SBE enzymatic activities are reflected by the *Sbe* mRNA levels during endosperm development, the differential expression of the *Sbe1* and *Sbe2b* genes may result in starch with on average, longer chain lengths later in development. Although evidence for such a shift in chain length over development has been reported for pea embryos [9], similar data have not been reported in maize endosperm.

DNA sequence comparison of plant SBE cDNAs show that they fall into two classes corresponding to cDNAs encoding SBEI and II maize isoforms (Table 2.1. and [9]). Although the N- and C-terminal domains of these proteins have diverged substantially, the internal sequences (from amino acids 1-595 of the mature SBEI protein) show high conservation within the groups. The SBEI class of genes includes maize *Sbe1*, rice *rbe1*, pea *Sbe2b* and potato *Sbe*, which share 76 to 92% amino acid similarity in the conserved central region (determined using Clustal analysis with a PAM250 table). The SBEII family includes maize *Sbe2b*, rice *rbe3*, pea *Sbe1* and arabidopsis *Sbe2b* genes [15] which share 81 to 92% amino acid similarity. The percentage similarity in this central region is much lower between the two classes of genes (55-60%). This suggests that while

Fig. 2.5. Relative transcript levels for *Sbe1* and 2 during kernel development. A. Mean relative transcript levels (R.L.) for *Sbe1* and 2 were measured in four replicate experiments as described in Fig. 2.1. Error bars represent 1 standard error of the mean. B. Mean ratios of *Sbe1* R.L. to *Sbe2b* R.L. during kernel development. Standard errors of the mean ratios were determined from the same data in Fig. 2.1. and 5A from ratios determined independently from each of the replicate experiments.


A
120
100
80
60
40
20
0
%RL
0
10
20
30
40
50
Sbe1 mRNA
Sbe2b mRNA
B
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
%RL Sbe1/ %RL Sbe2b
0
10
20
30
40
50
DAP

the *Sbe1* and II genes are related, they had evolved their distinctive features prior to the monocot-dicot divergence. The evolutionary conservation of these sequences along with the similarities in expression patterns noted above, strongly suggests that these enzymes play unique and essential roles in plant development.

The following characteristics may apply to all genes encoding the SBEI isoforms: (1) they are constitutively expressed in most vegetative tissues, but strongly induced during the development of storage tissues and embryos; (2) the timing of their expression is similar to the gene encoding granule bound starch synthase during the development of storage tissue; and (3) for species with multiple *Sbe* genes, maximal expression of genes encoding the SBEI isoforms occurs later in development of storage and embryo tissues than those encoding SBEII.

As discussed above, a very similar expression pattern can also be observed among the genes encoding SBEII family members. The maize *Sbe2b* gene is maximally expressed earlier in both developing endosperm and embryos than the maize *Sbe1* gene (Fig. 2.5.). The pea *Sbe1* gene also reaches its maximal expression level earlier in developing pea embryos than the pea *Sbe2* gene [4, 9]. The maize *Sbe2b* and pea *Sbe1* are thus very similar not only in sequence but also in their expression pattern. The expression of the maize *Sbe2b* gene in endosperm is very similar in developmental timing with the *Sh2* gene encoding the large subunits of ADPG pyrophosphorylase (Fig. 2.1.). It would be interesting to see if the expression of other *Sbe2* class genes are also similar to those of genes encoding ADPG pyrophosphorylase, the rate-limiting enzyme for starch biosynthesis, in the corresponding species.

In addition to endosperm and embryos, the maize *Sbe2b* gene is also highly expressed in young tassels (Fig. 2.4.). An extremely low level of the *Sbe2b* transcripts was also detected in young stem tissues below the tassel (Fig. 2.4.). No *Sbe2b* transcripts

were detectable in young leaves and roots (Fig. 2.3.), even under low stringency condition. The same *Sbe2b* gene was also cloned by transposon tagging from the maize *amylose-extender1* locus and no transcript was detectable in seedling leaves [37]. Consistent with our results, the rice *Sbe3* gene (similar in cDNA sequence to the maize *Sbe2b* gene) was demonstrated to be seed specific [19]. Thus it appears that the *Sbe2b* gene, and perhaps other similar genes, are not expressed at detectable levels in leaves or other vegetative tissues. However, the major starch branching enzyme in maize leaves was shown to be similar to the endosperm SBEIIa enzyme, and no SBEIIb activity was detected in maize leaves [11, 12]. Thus SBEIIa and IIb may be the products of different genes [11, 12, 17]. Our results indicating the lack of detectable expression of the *Sbe2b* gene in leaves and roots which have significant SBEIIa activity, implicate another, yet-to-be identified gene encoding SBEII in maize. We have recently extended these observations by molecular analysis of 16 allelic amylose extender isogenic lines which also indicates the presence of a separate gene encoding SBEIIa [14].

2.5. References-Chapter 2

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CHAPTER 3

MOLECULAR CLONING AND CHARACTERIZATION OF MAIZE *Sbe2a* GENE

3.1. Introduction

Starch branching enzyme (SBE) catalyzes 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 non-reducing ends for further α(1-4) glucan elongation. Multiple forms of SBE have been characterized in many species, including rice [23, 24, 32], spinach, pea, potato and maize (reviewed by [25]). In maize, three SBE isoforms, SBEI, SBEIIa and SBEIIb were resolved by DEAE-cellulose chromatography [4]. 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, while SBEIIa and SBEIIb are very similar in these respects [18, 25, 30]. cDNAs encoding SBEI and SBEIIb have been isolated [2, 10, 13, 29], and their expression patterns characterized [15]. However, it remains controversial whether SBEIIa and SBEIIb are encoded by a single gene or by separate genes [8, 9, 25, 28].

Preiss and co-workers [25, 28] concluded that SBEIIa and SBEIIb were the products of a single gene because of their similarities (reviewed in [25]). 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 [8, 9, 11, 15, 29]. Mutant endosperm from a number of independently derived *ae* alleles are deficient in SBEIIb activity, but contain approximately normal level of SBEIIa [1, 5, 20]. 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 [11]. Likewise, no *Sbe2b* transcripts were detected in maize leaves [15, 29], which contain only SBEIIa and SBEI activity [8, 9].

To be consistent with the known data, the two-gene hypothesis must predict that the *Sbe2a* gene would encode amino acid sequences very similar to that of SBEIIb, explaining the biochemical and immunological similarities of the two isoforms. However, the nucleotide sequence of the *Sbe2a* cDNA could be substantially divergent from that of the *Sbe2b* cDNA because of potential synonymous codon usage, depending on when they were diverged. The two-gene hypothesis also predicts that the *Sbe2a* gene, but not the *Sbe2b* gene, should be expressed in leaves and *ae-B1* endosperm. 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 *ae-B1* endosperm by northern analysis even under low stringency hybridization condition [11, 15].

To test the two-gene hypothesis, we sought to isolate a *Sbe2a* cDNA. We report here the isolation and characterization of a novel near-full-length *Sbe2* family cDNA. As

predicted, it is expressed at a very low level in leaves and *ae-B1* endosperm. Its deduced amino acid sequences share a very high sequence similarity to the deduced SBEIIb sequences, however, its DNA sequence is more divergent. A 49 aa N-terminal extension of SBEIIa protein is consistent with its slightly larger molecular weight of SBEIIa relative to SBEIIb as estimated by SDS-PAGE. The 14 N-terminal amino acids of mature SBEIIa protein purified from *ae-B1* endosperm match perfectly the sequences deduced from the putative *Sbe2a* cDNA. These results suggest that this cDNA, distinct from *Sbe2b*, encodes SBEIIa in maize.

3.2. Results

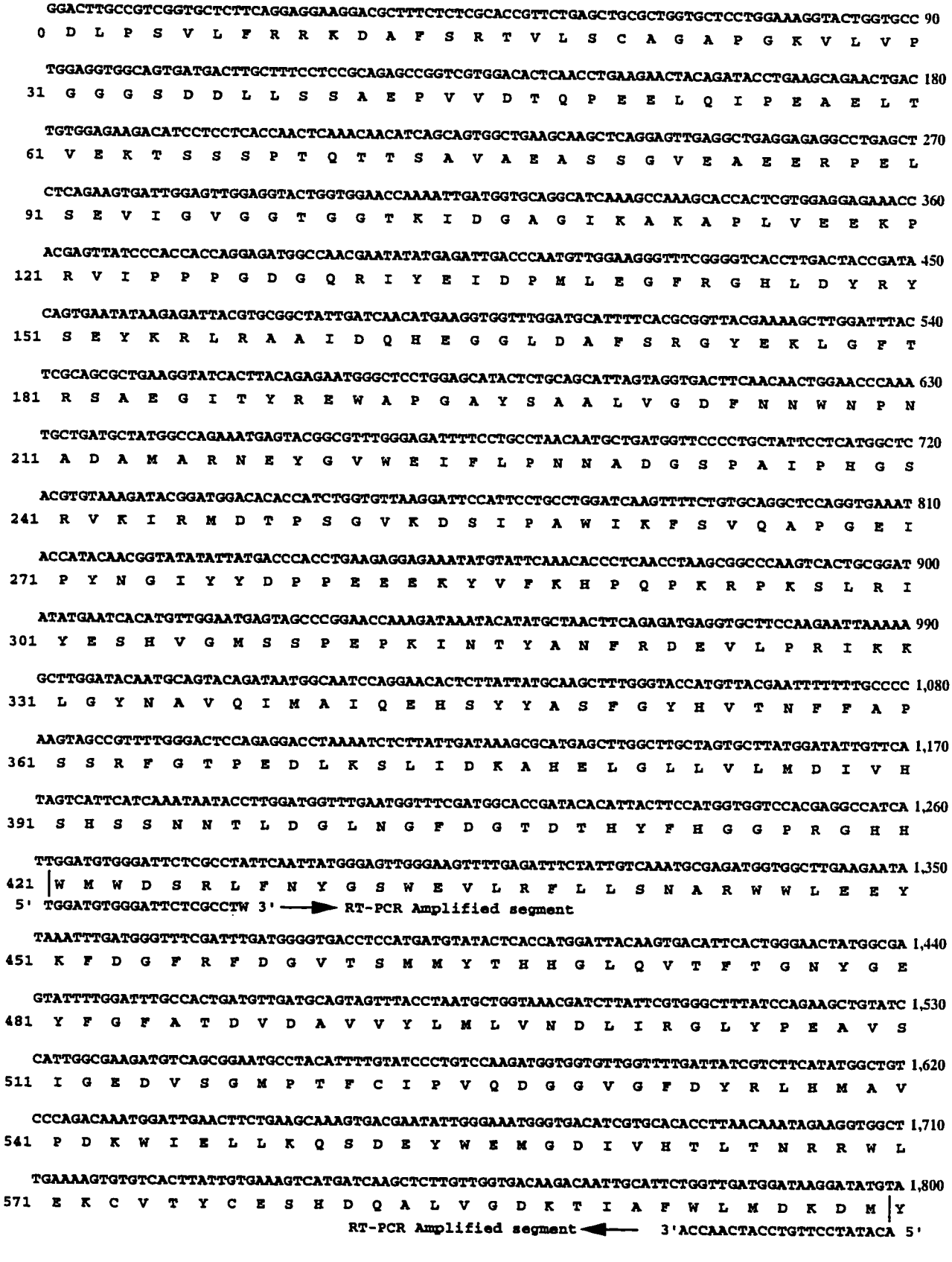
3.2.1. Isolation of a Novel *Sbe2* Family cDNA

The approach was first to isolate a partial *Sbe2a* cDNA from tissues lacking *Sbe2b* transcript by RT-PCR using *Sbe2* primers. Sequence comparison between the maize *Sbe2b* cDNA and its counterparts in pea (*sbe1*) and rice (*rbe3*), revealed a highly conserved region near the middle of their coding regions. All three cDNAs contain two almost identical 25 base pair (bp) sequences, 538 bp apart. Moreover, these sequences are not conserved in cDNAs encoding other SBE isoforms. We reasoned that since maize SBEIIa is very similar to SBEIIb in many biochemical and immunological properties [14, 18, 25, 30], these stretches of sequences might also be conserved in the putative gene encoding maize SBEIIa. Therefore, two degenerate primers were used for RT-PCR amplification of an *Sbe2* family cDNA from maize leaf total RNA. Leaf tissue was chosen because it contains SBEIIa activity, but no detectable SBEIIb activity or potentially contaminating *Sbe2b* transcripts.

A single 538 bp cDNA fragment was amplified from 25 days old seedling leaf RNA. The fragment was cloned and seven positive isolates were sequenced at both ends. One clone was a non-specific amplification product from 18S ribosomal RNA, and the other six were identical. The complete sequence of the RT-PCR amplified cDNAs is shown in Fig. 3.1. as a part of a later-isolated near-full-length putative *Sbe2a* cDNA. This cDNA fragment showed 78% sequence similarity to the corresponding region of the maize *Sbe2b* cDNA, and 40% similarity to *Sbe1*. Thus, as predicted the cDNA fragment, while distinct from *Sbe2b* and *Sbe1*, was most likely amplified from an mRNA encoding a leaf *Sbe2* family protein. The same cDNA fragment was also amplified, cloned and sequenced from total RNA from seedling roots and *ae-B1* endosperm (not shown).

Fig. 3.1. Nucleotide Sequence and Conceptual Translation of a Near 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 different cDNAs begin.


GGACTTGCCGTCGGTGCTCTTCAGGAGGAAGGACGCTTTCTCTCGCACCGTTCTGAGCTGCGCTGGTGCTCCTGGAAAGGTACTGGTGCC 90
0 D L P S V L F R R K D A F S R T V L S C A G A P G K V L V P
TGGAGGTGGCAGTGATGACTTGCTTTCCTCCGCAGAGCCGGTCGTGGACACTCAACCTGAAGAACTACAGATACCTGAAGCAGAACTGAC 180
31 G G G S D D L L S S A E P V V D T Q P E E L Q I P E A E L T
TGTGGAGAAGACATCCTCCTCACCAACTCAAACAACATCAGCAGTGGCTGAAGCAAGCTCAGGAGTTGAGGCTGAGGAGAGGCCTGAGCT 270
61 V E K T S S S P T Q T T S A V A E A S S G V E A E E R P E L
CTCAGAAGTGATTGGAGTTGGAGGTACTGGTGGAACCAAAATTGATGGTGCAGGCATCAAAGCCAAAGCACCACTCGTGGAGGAGAAACC 360
91 S E V I G V G G T G G T K I D G A G I K A K A P L V E E K P
ACGAGTTATCCCACCACCAGGAGATGGCCAACGAATATATGAGATTGACCCAATGTTGGAAGGGTTTCGGGGTCACCTTGACTACCGATA 450
121 R V I P P P G D G Q R I Y E I D P M L E G F R G H L D Y R Y
CAGTGAATATAAGAGATTACGTGCGGCTATTGATCAACATGAAGGTGGTTTGGATGCATTTTCACGCGGTTACGAAAAGCTTGGATTTAC 540
151 S E Y K R L R A A I D Q H E G G L D A F S R G Y E K L G F T
TCGCAGCGCTGAAGGTATCACTTACAGAGAATGGGCTCCTGGAGCATACTCTGCAGCATTAGTAGGTGACTTCAACAACTGGAACCCAAA 630
181 R S A E G I T Y R E W A P G A Y S A A L V G D F N N W N P N
TGCTGATGCTATGGCCAGAAATGAGTACGGCGTTTGGGAGATTTTCCTGCCTAACAATGCTGATGGTTCCCCTGCTATTCCTCATGGCTC 720
211 A D A M A R N E Y G V W E I F L P N N A D G S P A I P H G S
ACGTGTAAAGATACGGATGGACACACCATCTGGTGTTAAGGATTCCATTCCTGCCTGGATCAAGTTTTCTGTGCAGGCTCCAGGTGAAAT 810
241 R V K I R M D T P S G V K D S I P A W I K F S V Q A P G E I
ACCATACAACGGTATATATTATGACCCACCTGAAGAGGAGAAATATGTATTCAAACACCCTCAACCTAAGCGGCCCAAGTCACTGCGGAT 900
271 P Y N G I Y Y D P P E E E K Y V F K H P Q P K R P K S L R I
ATATGAATCACATGTTGGAATGAGTAGCCCGGAACCAAAGATAAATACATATGCTAACTTCAGAGATGAGGTGCTTCCAAGAATTAAAAA 990
301 Y E S H V G M S S P E P K I N T Y A N F R D E V L P R I K K
GCTTGGATACAATGCAGTACAGATAATGGCAATCCAGGAACACTCTTATTATGCAAGCTTTGGGTACCATGTTACGAATTTTTTTGCCCC 1,080
331 L G Y N A V Q I M A I Q E H S Y Y A S F G Y H V T N F F A P
AAGTAGCCGTTTTGGGACTCCAGAGGACCTAAAATCTCTTATTGATAAAGCGCATGAGCTTGGCTTGCTAGTGCTTATGGATATTGTTCA 1,170
361 S S R F G T P E D L K S L I D K A H E L G L L V L M D I V H
TAGTCATTCATCAAATAATACCTTGGATGGTTTGAATGGTTTCGATGGCACCGATACACATTACTTCCATGGTGGTCCACGAGGCCATCA 1,260
391 S H S S N N T L D G L N G F D G T D T H Y F H G G P R G H H
TTGGATGTGGGATTCTCGCCTATTCAATTATGGGAGTTGGGAAGTTTTGAGATTTCTATTGTCAAATGCGAGATGGTGGCTTGAAGAATA 1,350
421 W M W D S R L F N Y G S W E V L R F L L S N A R W W L E E Y
5' TGGATGTGGGATTCTCGCCTW 3' ——▶ RT-PCR Amplified segment
TAAATTTGATGGGTTTCGATTTGATGGGGTGACCTCCATGATGTATACTCACCATGGATTACAAGTGACATTCACTGGGAACTATGGCGA 1,440
451 K F D G F R F D G V T S M M Y T H H G L Q V T F T G N Y G E
GTATTTTGGATTTGCCACTGATGTTGATGCAGTAGTTTACCTAATGCTGGTAAACGATCTTATTCGTGGGCTTTATCCAGAAGCTGTATC 1,530
481 Y F G F A T D V D A V V Y L M L V N D L I R G L Y P E A V S
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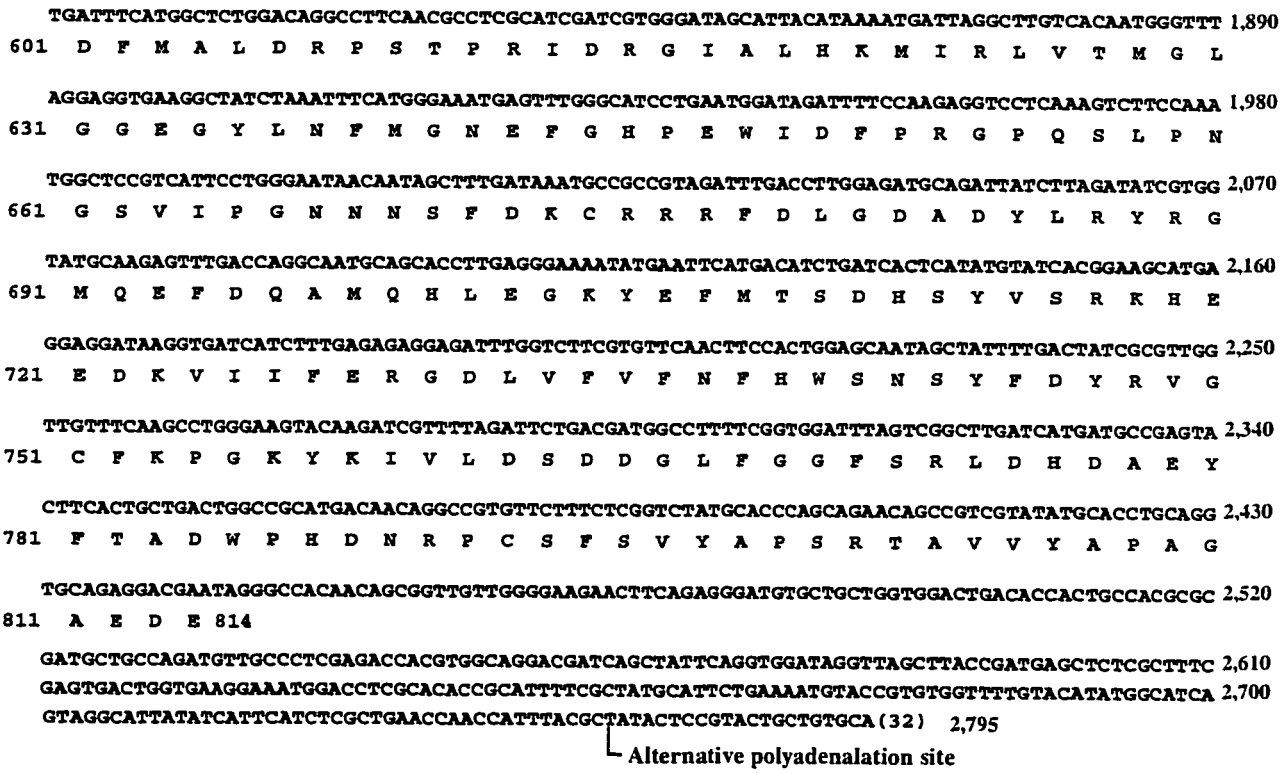
CCCAGACAAATGGATTGAACTTCTGAAGCAAAGTGACGAATATTGGGAAATGGGTGACATCGTGCACACCTTAACAAATAGAAGGTGGCT 1,710
541 P D K W I E L L K Q S D E Y W E M G D I V H T L T N R R W L
TGAAAAGTGTGTCACTTATTGTGAAAGTCATGATCAAGCTCTTGTTGGTGACAAGACAATTGCATTCTGGTTGATGGATAAGGATATGTA 1,800
571 E K C V T Y C E S H D Q A L V G D K T I A F W L M D K D M Y
RT-PCR Amplified segment ◀—— 3'ACCAACTACCTGTTCCTATACA 5'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601 D F M A L D R P S T P R I D R G I A L H K M I R L V T M G L
AGGAGGTGAAGGCTATCTAAATTTCATGGGAAATGAGTTTGGGCATCCTGAATGGATAGATTTTCCAAGAGGTCCTCAAAGTCTTCCAAA 1,980
631 G G E G Y L N F M G N E F G H P E W I D F P R G P Q S L P N
TGGCTCCGTCATTCCTGGGAATAACAATAGCTTTGATAAATGCCGCCGTAGATTTGACCTTGGAGATGCAGATTATCTTAGATATCGTGG 2,070
661 G S V I P G N N N S F D K C R R R F D L G D A D Y L R Y R G
TATGCAAGAGTTTGACCAGGCAATGCAGCACCTTGAGGGAAAATATGAATTCATGACATCTGATCACTCATATGTATCACGGAAGCATGA 2,160
691 M Q E F D Q A M Q H L E G K Y E F M T S D H S Y V S R K H E
GGAGGATAAGGTGATCATCTTTGAGAGAGGAGATTTGGTCTTCGTGTTCAACTTCCACTGGAGCAATAGCTATTTTGACTATCGCGTTGG 2,250
721 E D K V I I F E R G D L V F V F N F H W S N S Y F D Y R V G
TTGTTTCAAGCCTGGGAAGTACAAGATCGTTTTAGATTCTGACGATGGCCTTTTCGGTGGATTTAGTCGGCTTGATCATGATGCCGAGTA 2,340
751 C F K P G K Y K I V L D S D D G L F G G F S R L D H D A E Y
CTTCACTGCTGACTGGCCGCATGACAACAGGCCGTGTTCTTTCTCGGTCTATGCACCCAGCAGAACAGCCGTCGTATATGCACCTGCAGG 2,430
781 F T A D W P H D N R P C S F S V Y A P S R T A V V Y A P A G
TGCAGAGGACGAATAGGGCCACAACAGCGGTTGTTGGGGAAGAACTTCAGAGGGATGTGCTGCTGGTGGACTGACACCACTGCCACGCGC 2,520
811 A E D E 814
GATGCTGCCAGATGTTGCCCTCGAGACCACGTGGCAGGACGATCAGCTATTCAGGTGGATAGGTTAGCTTACCGATGAGCTCTCGCTTTC 2,610
GAGTGACTGGTGAAGGAAATGGACCTCGCACACCGCATTTTCGCTATGCATTCTGAAAATGTACCGTGTGGTTTTGTACATATGGCATCA 2,700
GTAGGCATTATATCATTCATCTCGCTGAACCAACCATTTACGCTATACTCCGTACTGCTGTGCA(32) 2,795
Alternative polyadenalation site

In order to isolate a full length cDNA, this fragment was used as a probe to screen 5.4 x 10^5 plaque forming units (pfu) of a maize leaf cDNA library (Stratagene). Of 11 independent clones isolated, the largest clone (designated as pL-5-2A) contained a 2.3 kb insert. As summarized in Table 3.1., another 15 independent clones were isolated by screening 2.5 x10^6 pfu in three rounds. Among them, two clones (pGl-17-1A and pLf-8-1A) of approximately 2.8 kb in size were further analyzed. Their size (2.8 kb) is very close to that of corresponding mRNA (2.8 to 2.9 kb) estimated from northern analysis of total RNA from various tissues (see below). The cDNA sequence and the deduced amino acid sequence from its longest open reading frame are shown in Fig. 3.1. A few clones have poly(A) tails extending from base 2,743, indicating as an alternative polyadenylation site.

3.2.2. The Putative *Sbe2a* cDNA Encodes a SBEII Very Similar to SBEIIb

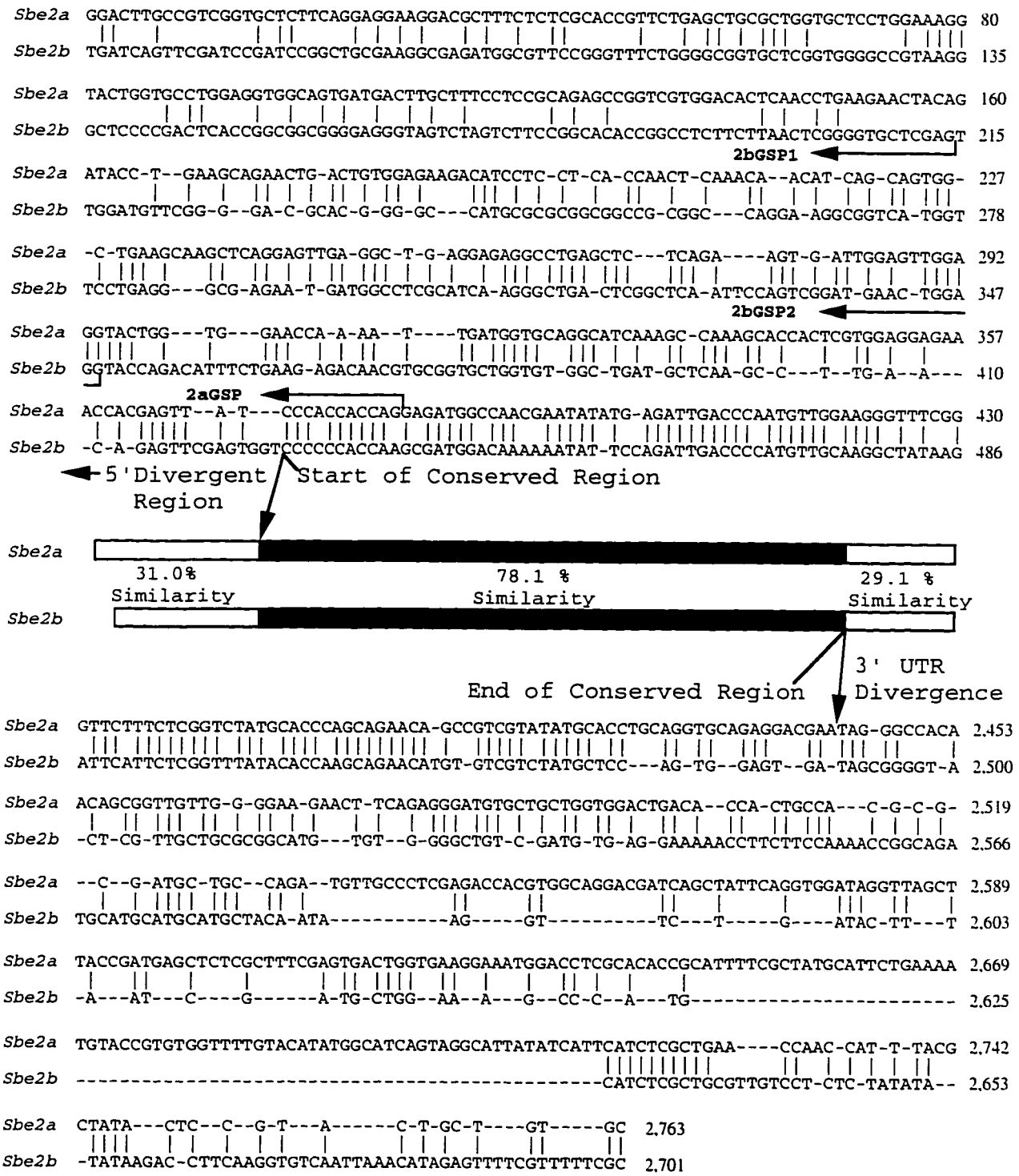
The sequence of the near-full-length putative *Sbe2a* cDNA (pLf-8-1A, 2,795 bp) showed about 67% similarity overall to that of the *Sbe2b* cDNA (Fig. 3.2.). The sequences between 380 bp to 2,445 bp are very highly conserved with the *Sbe2b* cDNA (78% sequence similarity). However, a short 5' end fragment (0-380) and the 3' UTR (untranslated region, 2,445-2,763) are quite divergent from their counterparts in *Sbe2b*, only 29% and 38% sequence similarity, respectively (Fig. 3.2.). 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 *A. thaliana*, i.e. a conserved region in the middle and two divergent regions at both ends [12]. These and other data (see below) indicate that while this cDNA is most similar to the *Sbe2* family, it is clearly distinct from *Sbe2b* in sequence.

Table 1. 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' EcoR I/Kpn I 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-17-1A, 2.8 kb

Fig. 3.2. Maize *Sbe2a* and *Sbe2b* cDNAs Are Highly Divergent at the 5' and 3' Ends.

Sequences which 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 which 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 Martinez-Needleman-Wunch algorithm (gap penalty: 1.10; gap length penalty: 0.33 and minimum match: 9) was implemented with Lasergene software (DNASTAR, Inc, Madison, WI).


Sbe2a GGACTTGCCGTCGGTGCTCTTCAGGAGGAAGGACGCTTTCTCTCGCACCGTTCTGAGCTGCGCTGGTGCTCCTGGAAAGG 80
Sbe2b TGATCAGTTCGATCCGATCCGGCTGCGAAGGCGAGATGGCGTTCCGGGTTTCTGGGGCGGTGCTCGGTGGGGCCGTAAGG 135
Sbe2a TACTGGTGCCTGGAGGTGGCAGTGATGACTTGCTTTCCTCCGCAGAGCCGGTCGTGGACACTCAACCTGAAGAACTACAG 160
Sbe2b GCTCCCCGACTCACCGGCGGCGGGGAGGGTAGTCTAGTCTTCCGGCACACCGGCCTCTTCTTAACTCGGGGTGCTCGAGT 215
2bGSP1
Sbe2a ATACC-T--GAAGCAGAACTG-ACTGTGGAGAAGACATCCTC-CT-CA-CCAACT-CAAACA--ACAT-CAG-CAGTGG- 227
Sbe2b TGGATGTTCGG-G--GA-C-GCAC-G-GG-GC---CATGCGCGCGGCGGCCG-CGGC---CAGGA-AGGCGGTCA-TGGT 278
Sbe2a -C-TGAAGCAAGCTCAGGAGTTGA-GGC-T-G-AGGAGAGGCCTGAGCTC---TCAGA----AGT-G-ATTGGAGTTGGA 292
Sbe2b TCCTGAGG---GCG-AGAA-T-GATGGCCTCGCATCA-AGGGCTGA-CTCGGCTCA-ATTCCAGTCGGAT-GAAC-TGGA 347
2bGSP2
Sbe2a GGTACTGG---TG---GAACCA-A-AA--T----TGATGGTGCAGGCATCAAAGC-CAAAGCACCACTCGTGGAGGAGAA 357
Sbe2b GGTACCAGACATTTCTGAAG-AGACAACGTGCGGTGCTGGTGT-GGC-TGAT-GCTCAA-GC-C---T--TG-A--A--- 410
2aGSP
Sbe2a ACCACGAGTT--A-T---CCCACCACCAGGAGATGGCCAACGAATATATG-AGATTGACCCAATGTTGGAAGGGTTTCGG 430
Sbe2b -C-A-GAGTTCGAGTGGTCCCCCCACCAAGCGATGGACAAAAAATAT-TCCAGATTGACCCCATGTTGCAAGGCTATAAG 486
5'Divergent Region
Start of Conserved Region
Sbe2a
31.0% Similarity
78.1 % Similarity
29.1 % Similarity
Sbe2b
End of Conserved Region
3' UTR Divergence
Sbe2a GTTCTTTCTCGGTCTATGCACCCAGCAGAACA-GCCGTCGTATATGCACCTGCAGGTGCAGAGGACGAATAG-GGCCACA 2,453
Sbe2b ATTCATTCTCGGTTTATACACCAAGCAGAACATGT-GTCGTCTATGCTCC---AG-TG--GAGT--GA-TAGCGGGGT-A 2,500
Sbe2a ACAGCGGTTGTTG-G-GGAA-GAACT-TCAGAGGGATGTGCTGCTGGTGGACTGACA--CCA-CTGCCA---C-G-C-G- 2,519
Sbe2b -CT-CG-TTGCTGCGCGGCATG---TGT--G-GGGCTGT-C-GATG-TG-AG-GAAAAACCTTCTTCCAAAACCGGCAGA 2,566
Sbe2a --C--G-ATGC-TGC--CAGA--TGTTGCCCTCGAGACCACGTGGCAGGACGATCAGCTATTCAGGTGGATAGGTTAGCT 2,589
Sbe2b TGCATGCATGCATGCTACA-ATA----------AG-----GT----------TC---T-----G----ATAC-TT---T 2,603
Sbe2a TACCGATGAGCTCTCGCTTTCGAGTGACTGGTGAAGGAAATGGACCTCGCACACCGCATTTTCGCTATGCATTCTGAAAA 2,669
Sbe2b -A---AT---C----G------A-TG-CTGG--AA--A---G--CC-C--A---TG---------- 2,625
Sbe2a TGTACCGTGTGGTTTTGTACATATGGCATCAGTAGGCATTATATCATTCATCTCGCTGAA----CCAAC-CAT-T-TACG 2,742
Sbe2b ----------CATCTCGCTGCGTTGTCCT-CTC-TATATA-- 2,653
Sbe2a CTATA---CTC--C--G-T---A------C-T-GC-T----GT-----GC 2,763
Sbe2b -TATAAGAC-CTTCAAGGTGTCAATTAAACATAGAGTTTTCGTTTTTCGC 2,701

Consistent with the DNA sequence comparison, the amino acid sequences deduced from the longest open reading frame of the putative *Sbe2a* cDNA share 77% similarity with that from the *Sbe2b* cDNA along their overall sequence (Fig. 3.3.). The amino acid sequences corresponding to the highly conserved region of the cDNA (aa 124 to 810) share 89% similarity with its SBEIIb counterpart. Thus, this leaf putative *Sbe2a* cDNA encodes a starch branching enzyme very similar, but not identical to the endosperm SBEIIb. Both amino acid and codon usage differences between the two cDNAs contribute to the overall nucleotide sequence difference of the two cDNAs.

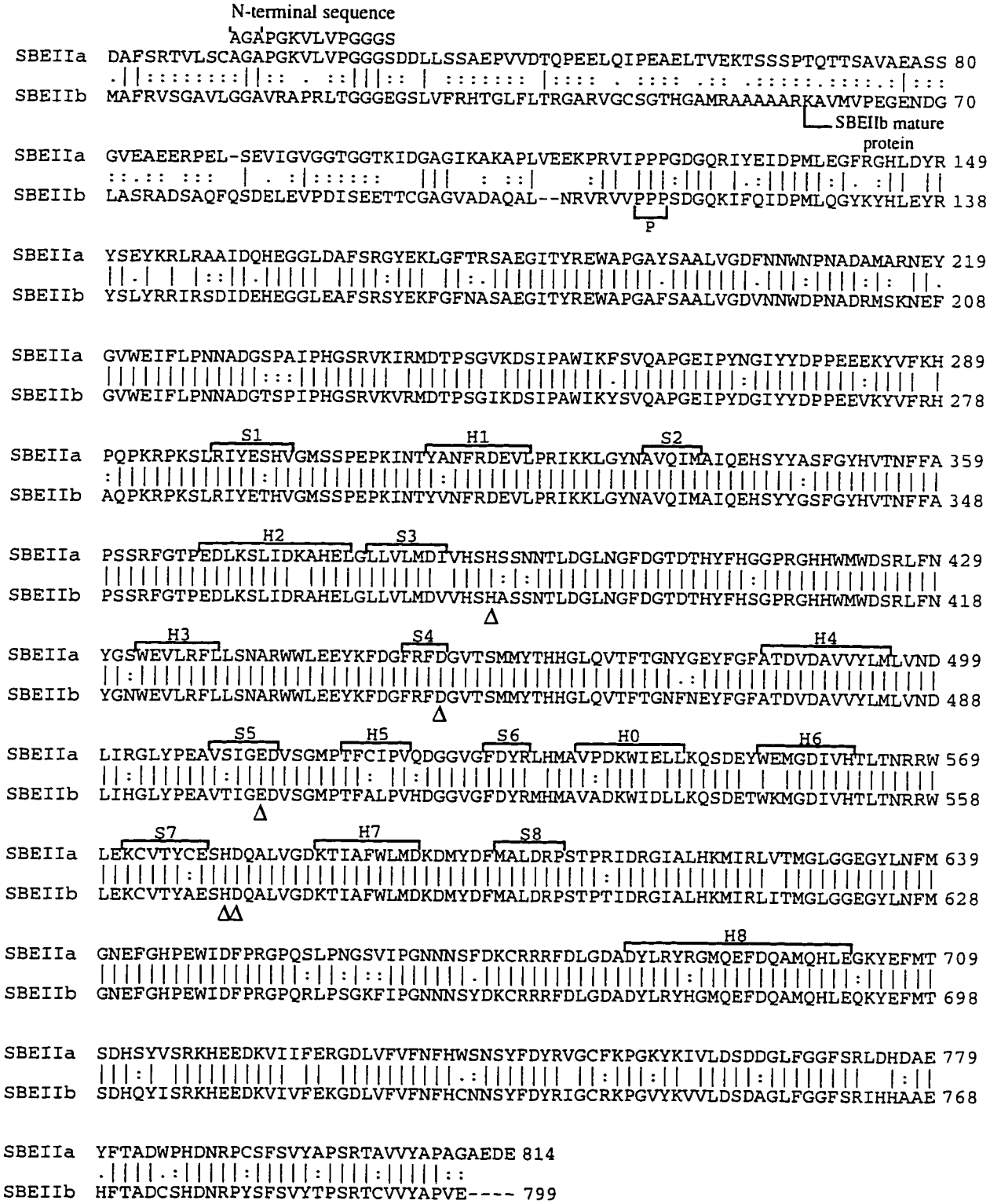
As in all other SBE isoforms in the SBEII family [6, 12], the central conserved region starts with a stretch of three consecutive prolines (SBEIIa positions, aa 124 to 126). This further supports the classification of this cDNA in the *Sbe2* family. Residues predicted to fold into the $(\beta\text{-}\alpha)_8$ barrel domain conserved in plant branching enzymes [6], are also highly conserved (Fig. 3.3.). Residues participating in the active sites of glucanases, which are conserved in all the other branching enzymes [6], are also completely conserved in the deduced amino acid sequence of the putative *Sbe2a* cDNA (Fig. 3.3.).

3.2.3. 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 to provide conclusive evidence that the putative *Sbe2a* cDNA encodes the SBEIIa isoform defined biochemically. SBEIIa protein was purified from *ae-B1* endosperm, which is devoid of potentially contaminating SBEIIb isoform, by a procedure modified from Guan and Preiss

Fig. 3.3. 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, [6]) are indicated with brackets. The residues participating in the active site of glucanases are denoted with Δ. The three prolines 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 as '' 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 [10]. Protein sequence comparison by Lipman-Pearson algorithm (gap penalty: 4; gap length penalty: 12 and kTuple: 2) was implemented by Lasergene software (DNASTAR, Inc, Madison, WI).


N-terminal sequence
AGAPGKVLVPGGGS
SBEIIa DAFSRTVLSCAGAPGKVLVPGGGSDDLLSSAEPVVDTQPEELQIPEAELTVEKTSSSPTQTTSAVAEASS 80
SBEIIb MAFRVSGAVLGGAVRAPRLTGGGEGSLVFRHTGLFLTRGARVGCSGTHGAMRAAAAARKAVMVPEGENDG 70
SBEIIb mature protein
SBEIIa GVEAEERPEL-SEVIGVGGTGGTKIDGAGIKAKAPLVEEKPRVIPPPGDGQRIYEIDPMLEGFRGHLDYR 149
SBEIIb LASRADSAQFQSDELEVPDISEETTCGAGVADAQAL--NRVRVVPPPSDGQKIFQIDPMLQGYKYHLEYR 138
P
SBEIIa YSEYKRLRAAIDQHEGGLDAFSRGYEKLGFTRSAEGITYREWAPGAYSAALVGDFNNWNPNADAMARNEY 219
SBEIIb YSLYRRIRSDIDEHEGGLEAFSRSYEKFGFNASAEGITYREWAPGAFSAALVGDVNNWDPNADRMSKNEF 208
SBEIIa GVWEIFLPNNADGSPAIPHGSRVKIRMDTPSGVKDSIPAWIKFSVQAPGEIPYNGIYYDPPEEEKYVFKH 289
SBEIIb GVWEIFLPNNADGTSPIPHGSRVKVRMDTPSGIKDSIPAWIKYSVQAPGEIPYDGIYYDPPEEVKYVFRH 278
S1 H1 S2
SBEIIa PQPKRPKSLRIYESHVGMSSPEPKINTYANFRDEVLPRIKKLGYNAVQIMAIQEHSYYASFGYHVTNFFA 359
SBEIIb AQPKRPKSLRIYETHVGMSSPEPKINTYVNFRDEVLPRIKKLGYNAVQIMAIQEHSYYGSFGYHVTNFFA 348
H2 S3
SBEIIa PSSRFGTPEDLKSLIDKAHELGLLVLMDIVHSHSSNNTLDGLNGFDGTDTHYFHGGPRGHHWMWDSRLFN 429
SBEIIb PSSRFGTPEDLKSLIDRAHELGLLVLMDVVHSHASSNTLDGLNGFDGTDTHYFHSGPRGHHWMWDSRLFN 418
Δ
H3 S4 H4
SBEIIa YGSWEVLRFLLSNARWWLEEYKFDGFRFDGVTSMMYTHHGLQVTFTGNYGEYFGFATDVDAVVYLMLVND 499
SBEIIb YGNWEVLRFLLSNARWWLEEYKFDGFRFDGVTSMMYTHHGLQVTFTGNFNEYFGFATDVDAVVYLMLVND 488
Δ
S5 H5 S6 H0 H6
SBEIIa LIRGLYPEAVSIGEDVSGMPTFCIPVQDGGVGFDYRLHMAVPDKWIELLKQSDEYWEMGDIVHTLTNRRW 569
SBEIIb LIHGLYPEAVTIGEDVSGMPTFALPVHDGGVGFDYRMHMAVADKWIDLLKQSDETWKMGDIVHTLTNRRW 558
Δ
S7 H7 S8
SBEIIa LEKCVTYCESHDQALVGDKTIAFWLMDKDMYDFMALDRPSTPRIDRGIALHKMIRLVTMGLGGEGYLNFM 639
SBEIIb LEKCVTYAESHDQALVGDKTIAFWLMDKDMYDFMALDRPSTPTIDRGIALHKMIRLITMGLGGEGYLNFM 628
ΔΔ
H8
SBEIIa GNEFGHPEWIDFPRGPQSLPNGSVIPGNNNSFDKCRRRFDLGDADYLRYRGMQEFDQAMQHLEGKYEFMT 709
SBEIIb GNEFGHPEWIDFPRGPQRLPSGKFIPGNNNSYDKCRRRFDLGDADYLRYHGMQEFDQAMQHLEQKYEFMT 698
SBEIIa SDHSYVSRKHEEDKVIIFERGDLVFVFNFHWSNSYFDYRVGCFKPGKYKIVLDSDDGLFGGFSRLDHDAE 779
SBEIIb SDHQYISRKHEEDKVIVFEKGDLVFVFNFHCNNSYFDYRIGCRKPGVYKVVLDSDAGLFGGFSRIHHAAE 768
SBEIIa YFTADWPHDNRPCSFSVYAPSRTAVVYAPAGAEDE 814
SBEIIb HFTADCSHDNRPYSFSVYTPSRTCVVYAPVE---- 799

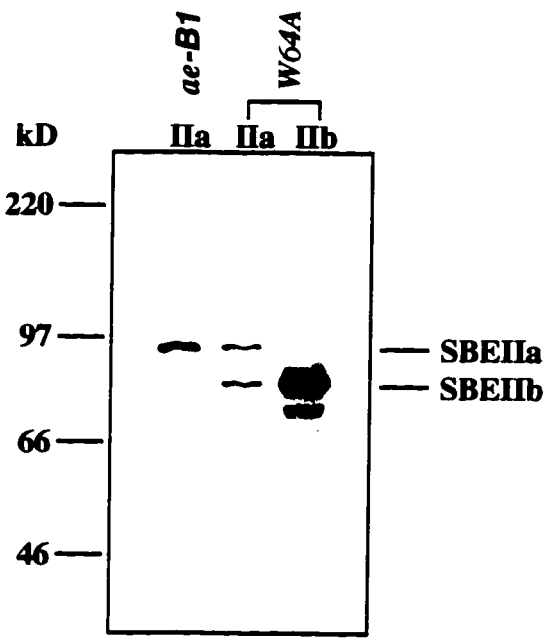
[18]. Enriched SBEIIa fractions from a DEAE-sepharose column were further purified through an amino ω-octo agarose column. A sharp, symmetrical peak of SBE activity was reproducibly eluted in fractions containing approximately 0.5 to 0.75 M KCl in buffer A. The identity of the enzyme was confirmed by western blot analysis of the fractions with an anti-SBEII antibody which recognizes both the 85 kD SBEIIb and the larger 89 kD SBEIIa [11, 31].

The SBEIIa protein was further purified through a Sephacryl S-300-HR column. An aliquot of pooled peak SBEIIa fractions essentially free of contaminants was compared by western blot analysis with wild-type SBEIIa and IIb of W64A endosperm in fractions from a DEAE-sepharose FF column (Fig. 3.4.). The purified SBEIIa protein migrated identically with the larger 89 kD band in the SBEIIa enriched fraction from W64A endosperm on the western blot. The 85 kD band detected in the SBEIIa enriched fraction is contaminating SBEIIb [11], and is not detected in SBEIIa fractions from *ae-B1* endosperm. The molecular weight of the mature SBEIIa calculated from the deduced amino acid sequences (aa 11-814, Fig. 3.3.) is 89.583 kD, matching very closely that of SBEIIa from endosperm determined by SDS-PAGE (89 kD) (Fig. 3.4., also see [11]). These results support the conclusion that the SBEII protein purified from the *ae-B1* endosperms was the previously identified SBEIIa [11, 25], rather than a novel SBEII type protein.

A portion of pooled SBEIIa peak fractions was fractionated by preparative SDS-PAGE, blotted on a PVDF membrane and subjected to N-terminal peptide sequencing. The N-terminal sequence of SBEIIa from *ae-B1* endosperm was 'AGA'PGKVLVPGGGS (' ' indicates the most prevalent amino acid of several alternatives in the first three cycles). The 14 N-terminal amino acids of the purified SBEIIa protein match perfectly the amino acid

Fig. 3.4. Immunological Comparison of SBEIIa Purified from *ae-B1* Endosperm with SBEIIa and SBEIIb Enriched Fractions from W64A Endosperm.

One μg of *ae-B1* endosperm SBEIIa of near homogeneity and 10 μg total protein from W64A endosperm SBEIIa and SBEIIb fractions of DEAE-Sepharose FF column were separated by SDS PAGE, blotted and detected with an anti-SBEII antibody [11, 31]. The SBEIIa fraction of W64A endosperm contains some contaminating SBEIIb as has been described before [11]. Positions of molecular weight markers (not shown) are indicated on the left.


ae-B1
W64A
kD
IIa
IIa
IIb
220
97
66
46
SBEIIa
SBEIIb

sequence deduced from the putative *Sbe2a* cDNA (Fig. 3.3.). The sequence at the predicted cleavage site RTVLSC↓A is similar to the proposed consensus ($R^{I}/_{V}X^{A}/_{C}$↓A) of chloroplast transit peptide [16], which suggest that the mature SBEIIa protein starts with the amino acid arginine. 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

3.2.4. 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 an *Sbe2a*-specific probe (2aGSP). This 2aGSP probe was derived from the highly divergent 380 bp 5' end of the *Sbe2a* cDNA (Fig. 3.2.). This probe does not hybridize to the *Sbe2b* cDNA under high stringency condition (Fig. 3.7A.). As shown in Fig. 3.5., a transcript of about 2.8-2.9 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 the tissues examined. The *Sbe2a* gene was also expressed in young stem and tassel tissues. A very weak *Sbe2a* transcripts level was detected in RNA extracted from roots of 9-days-old seedlings. The presence of the *Sbe2a* transcripts in seedling roots was also confirmed by RT-PCR (data not shown).

In order to unequivocally detect the two very similar *Sbe2a* and *Sbe2b* transcripts and evaluate their expression level, RNA samples were further analyzed by RNase protection assay (RPA). RPA has the advantage of higher sensitivity and specificity than northern analysis, and 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* used were derived from the 5' divergent regions of the two genes which were 455 and 244 bp in length respectively, including short stretches of transcribed vector sequences. Transcripts of the two genes in total RNA were expected to specifically protect the complementary 380 bp and 214 bp sequences of the antisense probes against RNase digestion as illustrated in Fig. 3.6A. Known amounts of sense RNA fragments *in vitro* transcribed from subcloned *Sbe2a* and *Sbe2b* cDNAs were used as standards.

The transcript levels of the *Sbe2a* and *Sbe2b* genes in various tissues measured by RPA are summarized in Table 3.2. A very low level of *Sbe2a* transcript was detected in W64A endosperm, and on a total RNA basis, it was approximately 10 times lower than in embryo, and 35 times lower than the *Sbe2b* transcript 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 for total RNA from endosperm and embryo was labeled with one fourth of the maximal Biotin-14-CTP concentration as used for labeling of the *Sbe2a* probe. The *Sbe2a* transcript level 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 non-allelic maize endosperm mutants reported by Giroux et al. (1994), possibly indicating a metabolite feedback control mechanism [17]. As in the northern analysis, embryos of both normal and *ae-B1* mutant plants have the highest levels of *Sbe2a* transcript on a total RNA basis (Table 3.2.). 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 [11]. Young roots of 2 DAE seedlings showed a *Sbe2a* transcript level similar to that in embryos. This is in contrast to the very low level of *Sbe2a* transcripts detected by northern analysis and RT-PCR in roots of 9 DAE seedlings. Whether the

Fig. 3.5. Expression of *Sbe2a* in Various Tissues of Normal W64A and *ae-B1* Maize Plants.

Thirty μg 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. 3.2.). Autoradiography was for 4 days.

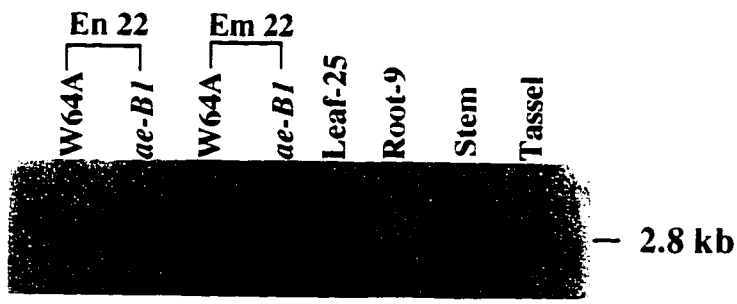

En 22
Em 22
W64A
ae-B1
W64A
ae-B1
Leaf-25
Root-9
Stem
Tassel
2.8 kb

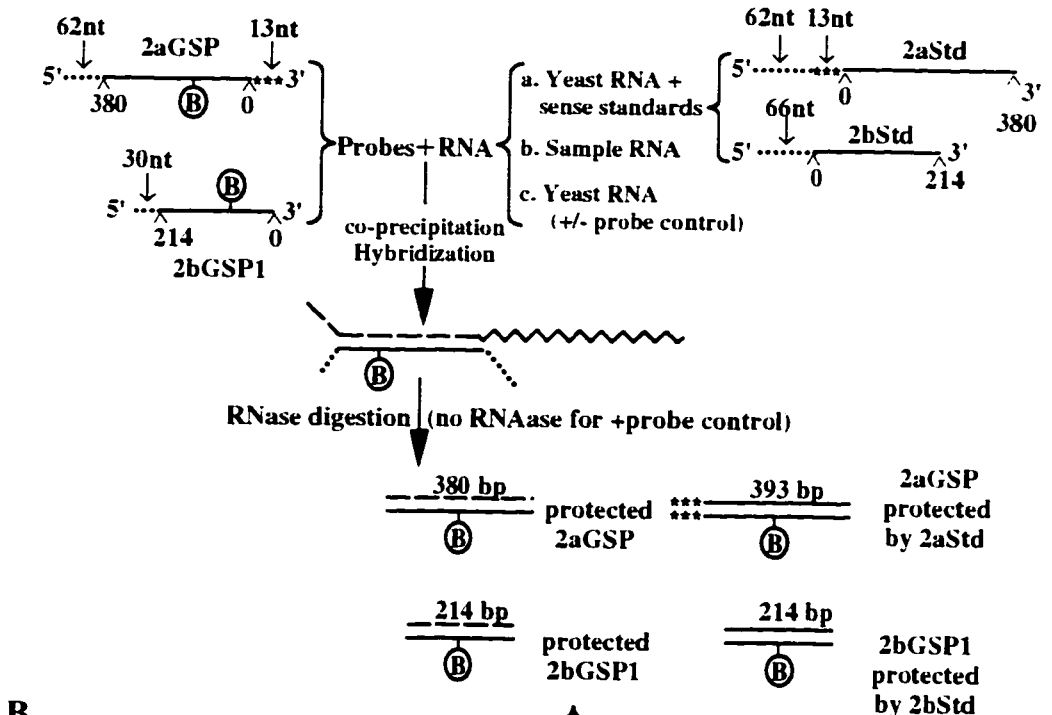
Fig. 3.6. RNase Protection Assay (RPA) of *Sbe2a* and *Sbe2b* mRNA in Various Tissues of *ae-B1* and Normal W64A Plants.

Antisense probes specific to *Sbe2a* (2aGSP) and *Sbe2b* (2bGSP1) were used in RPA of 20 μg of total RNA from various tissues except 2 DAE root (40 μg). Two ng 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 (Negative Control). 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 ρg to 14.4 ρg) 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 maximal Biotin-14-CTP concentration, and for RPA analysis of root and leaf total RNA one half maximal. 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 phosphotase was detected by exposure to an X-ray film for 2.5 min.

A


62nt
13nt
2aGSP
5'
3'
380
0
30nt
214
0
2bGSP1
Probes+RNA
co-precipitation
Hybridization
a. Yeast RNA + sense standards
b. Sample RNA
c. Yeast RNA (+/- probe control)
62nt 13nt
2aStd
66nt
2bStd
RNase digestion (no RNAase for +probe control)
380 bp
protected 2aGSP
393 bp
2aGSP protected by 2aStd
214 bp
protected 2bGSP1
214 bp
2bGSP1 protected by 2bStd

B

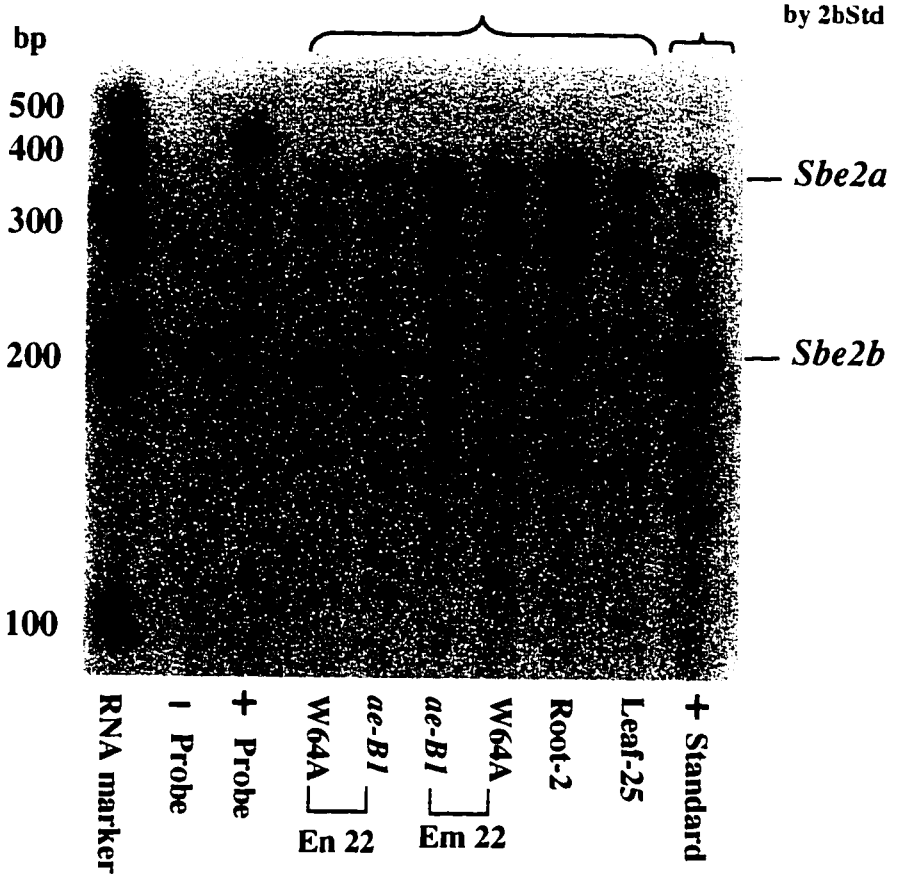

bp
500
400
300
200
100
Sbe2a
Sbe2b
RNA marker
- Probe
+ Probe
W64A
ae-BI
En 22
ae-BI
W64A
Em 22
Root-2
Leaf-25
+ Standard

Fig. 3.7. Maize *Sbe2a* and *Sbe2b* Genes Are Encoded at Different Genomic Loci.

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 days.

(A) Hybridization to the *Sbe2a*-specific probe.

(B) Hybridization to the *Sbe2b*-specific probe.

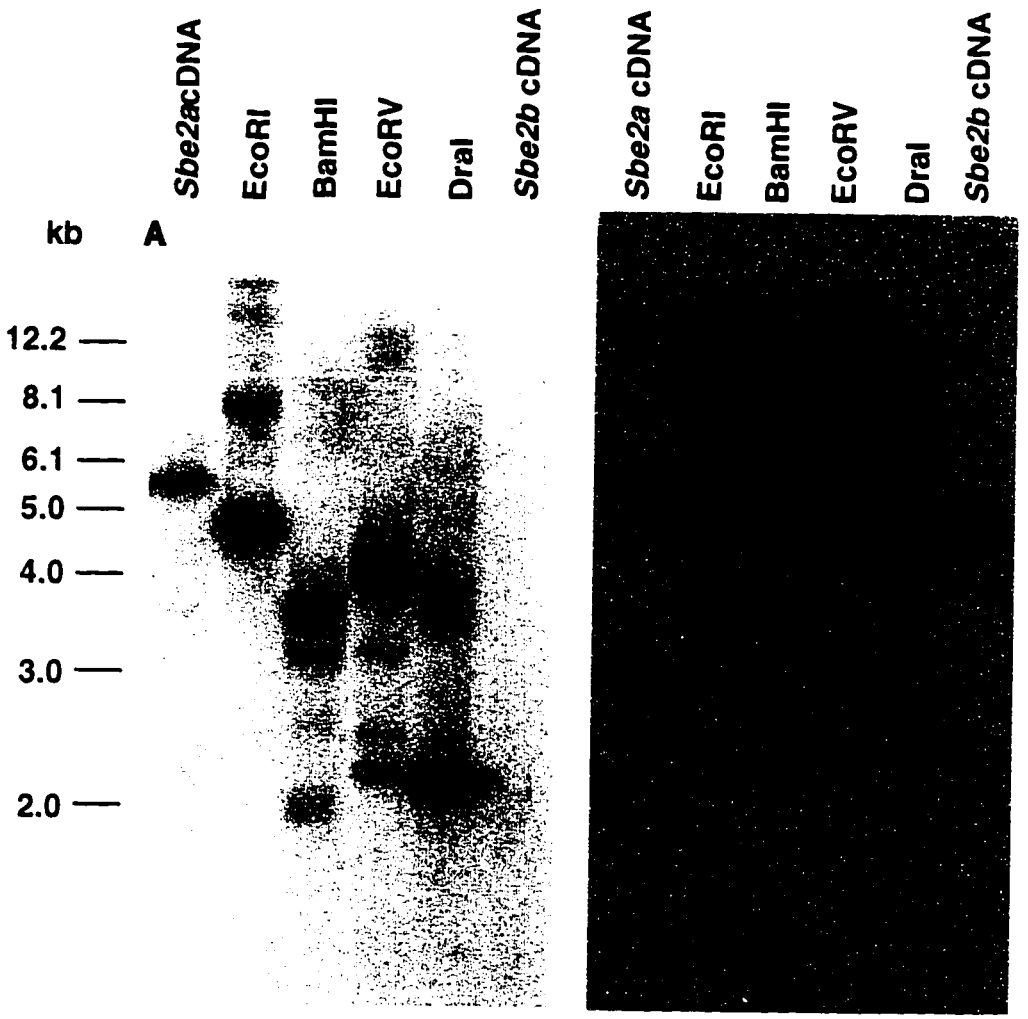

Sbe2acDNA
EcoRI
BamHI
EcoRV
DraI
Sbe2b cDNA
Sbe2a cDNA
EcoRI
BamHI
EcoRV
DraI
Sbe2b cDNA
kb
A
12.2
8.1
6.1
5.0
4.0
3.0
2.0

Table 3.2. 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 a standard error less that 10%. All quantitative measurements were made within the linear response range of the film. NA: Not applicable, '-': Below background.

RNA Samples		*Sbe2b* mRNA (fmol)	*Sbe2a* mRNA (x 10^{-1} fmol)	*Sbe2b* /*Sbe2a* Ratio
Endosperm (22 DAP)	Normal	1.59	0.45	35.3
	ae-B1	-	1.50	NA
Embryo (22 DAP)	Normal	1.3	4.70	2.8
	ae-B1	-	4.76	NA
Leaves (25 DAE)		-	2.49	NA
Roots (2 DAE)		-	4.40	NA

decrease of the *Sbe2a* transcript in developing roots has any physiological significance awaits further analysis. An intermediate level of *Sbe2a* transcript was observed in maize leaves.

There are several reasons why we believe that the residual *Sbe2b* transcripts in *ae-B1* endosperm detected by RPA were most likely truncated and non-functional. First, no SBEIIb protein nor its activity were detected in *ae-B1* endosperm [11]. Secondly, it is critical to consider that 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 [11]. 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 [11], no residual *Sbe2b* transcripts were detected by RPA in the embryo of *ae-B1* mutant kernels even with the *Sbe2b* probe labeled with half of maximal Biotin-14-CTP content. No *Sbe2b* transcripts were detectable by RPA in young roots and leaf tissues, also consistent with biochemical and northern analysis [8, 9, 15].

3.2.5. *Sbe2a* and *Sbe2b* Genes Reside at Different Genomic Loci

Southern genomic analysis was performed to investigate whether the putative *Sbe2a* and *Sbe2b* cDNAs are encoded at the same or different loci in the maize genome. 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. 3.2.). As shown in Fig. 3.7., 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. 3.7A.). The *Sbe2b* -specific probe detected one highly hybridizing band in genomic DNA cut with BamHI, EcoRV and DraI, and two bands in the genomic DNA cut by EcoRI (Fig. 3.7B.). None of the bands in corresponding lanes are of the same molecular weights. This evidence supports the conclusion that SBEIIa and SBEIIb are encoded at different loci in the maize genome. The high molecular weight band (about 11 kb) detected by the *Sbe2b* -specific probe in the EcoRI-cut genomic DNA could have resulted from incomplete digestion. Alternatively, the two bands may have resulted from an EcoRI cut in an intron as no EcoRI sites are found in the cDNA probe.

3.3. Discussion

Based on the data presented above and our previous molecular genetic analysis of *ae* mutants [11], we conclude that maize SBEIIa and SBEIIb are the products of two unique genes. First, while the sequence of the putative *Sbe2a* cDNA and the *Sbe2b* cDNA and their deduced amino acid sequences share high similarity over an extended region, they also have regions of substantial divergence (Fig. 3.2. and 3.3.). The amino acid sequences deduced from the putative *Sbe2a* cDNA also share common structural features with the amino acid sequences of all plant SBEII isoforms (Fig. 3.3.). Thus, we can conclude that the putative *Sbe2a* cDNA encodes an SBE belonging to the SBEII family, but distinct from SBEIIb.

Secondly, the N-terminal 14 amino acids of SBEIIa protein purified from *ae-B1* endosperm perfectly match the sequence deduced from the *Sbe2a* cDNA. Additionally, the

molecular weight (89,583 kD) calculated from the deduced SBEIIa mature protein sequences closely matches that of endosperm SBEIIa experimentally determined by SDS PAGE (Fig. 3.4.). SBEIIa extracted from *ae-B1* and normal W64A endosperm showed the same behavior on a DEAE-sepharose FF column, had identical mobility on a SDS-PAGE, and was recognized by the same antibody (Fig. 3.4.). Other than SBEIIa and IIb, no other SBEII type isoforms have ever been reported in maize leaf or kernel extracts. This suggests that the purified SBEIIa from *ae-B1* endosperm is the same as the previously biochemically described SBEIIa, rather than a novel SBEII.

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, endosperms and embryos of normal and *ae-B1* mutant plants by a combination of northern analysis, RPA and RT-PCR methods (Fig. 3.5. and 3.6.). SBEIIa enzyme activity was also reported in these tissues [8, 9]. 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, RPA or RT-PCR methods, consistent with the previous conclusion that SBEIIa is the only SBEII type isoform in maize leaves [8, 9]. Finally, the banding pattern of maize genomic DNA fragments detected by *Sbe2a* and *Sbe2b* specific probes are completely different, indicating that the two cDNAs are located at different loci 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 can not 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 [3, 21, 26]. Moreover, SBEIIb is not expressed in leaf tissues where transitory starch is produced, whereas SBEIIa is. It is also interesting to note that 10 times more *Sbe2a* mRNA was found in embryos than in endosperm. Thus, in embryos SBEIIa may play a more important role in amylopectin synthesis than does SBEIIb.

The SBEIIa and SBEIIb mature proteins share an 89% sequence similarity over 685 amino acids, accounting for approximately 85% of their total length (Fig. 3.3.). The predicted $(\beta\text{-}\alpha)_8$ barrel structure and amino acids participating in the active sites of plant SBEs are also highly conserved in the maize SBEIIa and SBEIIb. The loop size between β-strand 8 and α-helix 8, which was suggested to have impact on the size of branched glucosyl chains [6], are the same in SBEIIa and SBEIIb (Fig. 3.3.). In addition, the amino acids in the loop are highly conserved in the two isozymes. Both isozymes indeed seem to preferentially transfer shorter glucosyl chains than SBEI [30]. Other *in vitro* catalytic properties of SBEIIa and SBEIIb are also extremely similar [18, 25, 30]. These similarities in their structural and *in vitro* catalytic properties suggest that non-catalytic properties of SBEIIa and SBEIIb, such as their interaction with the glucan network or with starch synthases and/or debranching enzyme *in vivo*, may be responsible for their different qualitative contributions to the synthesis of starch in maize endosperm. It is also possible that the catalytic properties of SBEIIa and SBEIIb may be altered *in vivo* by their interactions with the glucan network or with starch synthases and/or debranching enzyme, or by regulatory proteins.

The only major structural difference between SBEIIa and SBEIIb mature proteins is a stretch of highly divergent amino acid sequences from their N-terminals to the three

highly conserved prolines. The N-terminal divergent region of SBEIIa mature protein has a 49 amino acid extension compared to 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 its counterpart of SBEIIa mature protein (Fig. 3.3.). A short highly divergent region at the N-terminal was also observed in the two members of the SBEII family in *A. thaliana* [12]. These short stretches of divergent amino acids at the N-terminal of SBEIIa and SBEIIb may define differences in their non-catalytic 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 3.2.). 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 FF column, considering some 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 the long-standing controversy as to the genetic control of SBEIIa and SBEIIb, and provides a new tool for studying the biochemical properties, genetic regulation and the roles of starch branching enzymes in starch synthesis.

3.4. Methods

3.4.1. RT-PCR

Primers for PCR amplification of *Sbe2* family cDNAs were derived from sequences almost perfectly conserved in maize *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 (GibcoBRL, Grand Island, NY) and Taq polymerase (Promega, Madison, WI). The Sbe2/reverse primer was used for first strand cDNA syntheses with 1 to 5 μg total RNA. The RT-PCR products were either blunt-ended with T4 DNA polymerase and cloned into Sma I cut pBluescript SK^- (Stratagene, Manaha, WI) [27], or directly cloned into pCR™ (version 2.0, Invitrogen, San Diego, CA) following the manufacturer's instruction.

3.4.2. Library Screening, DNA Sequencing and Sequence Analysis

A maize leaf (5 wk old, B73 inbred) Uni-Zap™ XR cDNA library (Stratagene, Cat. No. 937005) was screened by hybridization to radioactively labeled DNA fragments following the manufacturer's instruction. All DNA probes were labeled with ^{32}P dCTP by the random primed labeling method. All phage lifts membranes were hybridized and washed under high stringency conditions as described [7]. DNA sequencing was performed using Sequenase® (USB, Cleveland, OH 44122). Both strands of the cDNA clone (pLf8-1Aa) 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.

3.4.3. RNA Extraction, Northern Analysis and RNase Protection Assay

Preparation of plant materials, RNA extractions and northern analysis were performed as described [11, 15]. Template for synthesizing the *Sbe2a*-specific probe was the 393 bp 5' end fragment of pLf-8-1A (1 to 380 bp of the cDNA, plus 13 bp adapter sequence, Fig. 3.2.). The fragment was derived from EcoRI 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 EcoRV 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 EcoRI linearized plasmids containing the 5' end fragment in sense or antisense orientation relative to the T7 promoter. The antisense RNA (455 bp), containing 393 bp cDNA and 62 bp vector sequence, was maximally labeled with Biotin-14-CTP, and used as a probe in the RNase Protection Assay (RPA).

The *Sbe2b*-specific probe used in RPA was the 214 bp 5' end EcoRI to XhoI fragment of the full length *Sbe2b* cDNA (Fig. 3.2.). The fragment was subcloned into EcoRI, XhoI digested pBluescript SK$^-$ and pBluescript KS$^-$. The sense RNA (270 bp, including 66 bp vector sequence) was transcribed with T7 RNA polymerase from XhoI 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 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 for *Sbe2a* mRNA. This allows quantitation 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 bp and 270bp, respectively) used as standards were not labeled with biotin. RNA markers were *in vitro* transcribed from Century™ Marker Template (Ambion, Inc.) with maximal Biotin-14-CTP concentration. All *in vitro* transcribed RNA species were gel purified, and quantified spectrophotometrically. Preparation of probes and RNA standards and the RPA assay are illustrated in Fig. 3.6A.

RNase Protection Assay was carried out using The RPAII™ kit (Ambion, Inc.). Briefly, total RNAs from different tissues were co-precipitated with the *Sbe2a* and *Sbe2b* antisense probes (1 ng probe/ 20 μg total RNA), hybridized for 18 hr, 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 Hybond™-N+ nylon membrane (Amersham, Alington Heights, IL) with a semidry blotting apparatus (Fisher Scientific, Pittsburgh, PA). The protected biotinylated fragments were detected with streptavidin-alkaline phosphatase conjugates catalyzing a chemiluminescent reaction detected by X-ray film (BrightStar™ BioDetect™ system, Ambion Inc.). The films were scanned with a model 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 2b/2a RNA standard included in RPA were 225.5/14.4, 340.8/19.2, 113.6/4.8, 71.0/2.4, 51.1/1.4, 454.4/9.6 (ρg), respectively. The signal strengths of the probe fragments protected by the RNA standards were plotted against the amount of RNA standards in moles to construct a standard curve for calculations of the transcript levels in samples.

3.4.4. 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 Guan and Preiss [18]. Twenty-eight grams of frozen endosperm were extracted, ammonium sulfate fractionated and dialyzed according to Guan and Preiss [18]. The dialyzed SBE fraction was applied to a pre-equilibrated DEAE-Sepharose fast flow column (Pharmacia; 1.6 x 70 cm, 120 mL resin bed vol; about 10 mg protein per 1 mL of bed vol.) on a Pharmacia Gradi-Frac apparatus. 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), and SBEIIa was eluted with a linear gradient of 12 resin bed volumes of 0 to 0.4 M KCl in buffer A, following Guan and Preiss [18]. Fractions were assayed for branching enzyme activity as described [11]. To confirm the presence of a

single species of SBE in all SBEIIa fractions, aliquots were fractionated on an 8% polyacrylamide resolving gel, blotted and detected with an antibody recognizing both SBEIIa and SBEIIb [11, 31].

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 x 20 cm column, 30 mL resin bed vol and 0.1 mg per 1 mL resin bed vol) 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 Centriplus-30 filters (Amicon Inc., Beverly, MA).

The concentrated SBEIIa fraction was next applied to a Sephacryl S-300-HR column (Sigma; 1.6 x 40 cm column, 60 mL resin bed volume) and eluted with buffer A at a flow rate of 0.2 mL/ min. Aliquots from one mL column fractions containing SBEIIa activity were checked for purity with SDS PAGE (8% resolving gel) and silver staining [19]. Peak fractions essentially free of protein contaminants were pooled and concentrated with Centriplus-30 units. The SBEIIa protein of near homogeneity was further resolved by preparative SDS-PAGE, blotted onto a PVDF membrane (Trans-Blot®, Bio-Rad Laboratories), and visualized with Amido Black 10B staining [0.1 % Amido Black (Sigma) in 10 % acetic acid]. N-terminal sequencing of the blotted SBEIIa from *ae-B1* endosperm was performed by Wistar Protein Microsequencing Facility (Philadelphia, PA 19104).

3.4.5. Genomic Southern Blot Analysis

Maize genomic DNA was extracted from 16 DAP W64A kernels. Ten grams of frozen kernels were ground to fine powders in liquid nitrogen, added to 35 mL of extraction buffer (100 mM Tris-HCl, pH 8.8; 100 mM NaCl; 20 mM EDTA; 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 vortexed for 3 min then centrifuged at 10,000*g* for 10 min at 4^{0}C. The supernatant was extracted twice with an equal volume of chloroform:isoamylalcohol (50:1), and the genomic DNA was precipitated by adding 1/10 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, and digested with RNase A (50 μg/ mL) for 1 hr and then with Proteinase K (50 μg/ mL) for 1 hr at 37^{0}C. The DNA preparation was extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), precipitated again as before, wound on a glass rod and washed with 70% ethanol. The purified DNA was dissolved in 1 mL of TE buffer. Ten μg each of DNA was digested with restriction enzymes, fractionated on a 0.8% agarose gel, and alkaline blotted to a HybondN$^+$ membrane following the manufacturer's instruction. The blot was hybridized sequentially with single-stranded DNA (ssDNA) probes specific to *Sbe2b* or *Sbe2a* cDNAs generated as described by Konat, et al. [22]. 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 the PCR amplification of the *Sbe2a* ssDNA probe. The template specific to *Sbe2b* was the 5' end EcoRI and KpnI fragment (1 to 353 bp). The primer used for the amplification of *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^{0}C for 16 hr, and washed under high stringency conditions as described by Church and Gilbert [7].

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CHAPTER 4

CONCLUSIONS AND FUTURE RESEARCH

The cloning of a near full-length *Sbe2a* cDNA provides conclusive evidence for the independent genetic control of SBEIIa and SBEIIb in maize. This *Sbe2a* cDNA provides a powerful tool for further studies on the role of SBEIIa in the synthesis of amylopectin, the formation of starch granule structure, for *in vitro* studies of biochemical properties of SBEIIa and for potential genetic engineering of amylopectin synthesis in maize. The molecular characterization of the three SBE genes has shed some light on the individual roles of each SBE isoform in amylopectin synthesis, and their regulation in maize.

Branches in amylopectin have an organized cluster structure (reviewed in [13]). The outermost branches without further branches (A chains) are linked by α(1-6) bonds to inner branches (B chains) which may be branched at one or several glucose units. Each amylopectin molecule contains a single chain with one reducing end (C chains). Adjacent branches within branch clusters may form double helices that can be packed regularly, giving a crystallinity to starch granules [12, 13]. The chain length, the ratio of A to B chains, the degree of branching and the organization of these side chains may vary among different amylopectin molecules, which determines the degree of crystallinity of starch granules.

The two types of SBE (SBEI vs. SBEIIa and SBEIIb) in maize may be specialized in the formation of different branch structures, which consequently influence the crystallinity of starch granules. First, SBEI and SBEII (IIa and IIb) differ in their affinity for amylose, an *in vitro* substrate, and the glucan chain lengths they prefer to transfer *in*

vitro [7, 18, 19]. Likewise, it is possible that *in vivo* the two types of SBEs could yield branches of different lengths and branch types (A or B chains) because of difference in their catalytic properties. Secondly, the loss of SBEIIb activity in the endosperm of *ae* mutants results in the dramatic change in starch granule morphology [17], and a novel type of amylopectin with branches of longer average chain lengths [2, 8]. This indicates that SBEIIb may play a unique role in forming certain types of branch structures of amylopectin in normal maize endosperm, which is not able to be compensated for by SBEIIa or SBEI. Third, the expression patterns of the three isoforms indicate that both types of SBE may be essential for amylopectin synthesis (Chapter 2 and 3). Both *Sbe1* and *Sbe2a* are expressed essentially in all maize tissues at various developmental stages. Their expression also overlaps with that of *Sbe2b* in reproductive organs (kernels and tassels). This indicates that the one type of SBE activity, either SBEI or SBEII (IIa and/or IIb), is not sufficient for the synthesis of normal amylopectin. Finally, there is strong evidence that the amylopectin products of two types of SBE in pea embryos are qualitatively different [4].

To date no mutants deficient in SBEI or SBEIIa activity have been identified in maize or other higher plants. This could mean that the elimination of either of these two isozymes is lethal to maize plants or that phenotypes of kernels deficient in SBEIIa and SBEI are not different from normal. In leaves, SBEI and SBEIIa may be essential for synthesis of transitory starch in chloroplasts to maximize photoassimilation. In endosperm and embryos, they may be essential for synthesis of the minimum amounts of starch necessary to ensure survival of seedlings. No visible phenotype resulted from the antisense inhibition of SBEI type isoform in potato [9, 15]. Thus it is quite possible that the kernel phenotype of SBEI or SBEIIa mutants would not be different from normal. If the contribution of SBEI and SBEIIa to the synthesis of amylopectin and the crystallinity of starch granules is more qualitative than quantitative, the fine structural changes of

amylopectin and starch granules may not be readily discernible in mutants deficient in SBEI or SBEIIa.

In addition to their possible specialization in forming different branch structures of amylopectin, SBEI and SBEIIb may also serve as a genetic mechanism via their expression level regulating the quantity of different types of amylopectin being synthesized during kernel development. In contrast to a stable low level of expression in roots and leaves at different developmental stages, *Sbe1* expression in endosperm increased throughout much of the development of both W64A and B73 kernels (Chapter 2, Appendix). Moreover, the transcript level of *Sbe1* peaks at a developmental stage later than that of *Sbe2b* (Chapter 2). As discussed in the Appendix, the increase of *Sbe1* and *Sbe2b* transcripts closely match the increase of starch synthesis in developing kernels of the two inbreds. Thus, if the levels of SBEIIa and SBEIIb correspond to their transcript levels, it is possible that SBEI and IIb level is so regulated so that different amounts of two different types of amylopectin are formed at various developmental stages of the kernel.

In addition to forming α-1,6 linked branches, plant SBEs form more non-reducing ends which are the substrates for starch synthase. Indirect or direct interactions of SBE isoforms with debranching enzymes may determine the availability of non-reducing ends which could be rate-limiting for amylopectin synthesis. The loss of SBEIIb in *ae* mutants resulted in a reduction of total starch and the amylopectin content in kernels up to 20% and 70%, respectively [13]. This suggests that SBEIIb has a larger quantitative contribution to the synthesis of amylopectin than SBEI and SBEIIa.

In contrast to SBEIIb, SBEIIa may have a much less quantitative contribution to synthesis of similar or different type of amylopectin in maize. This is supported by the huge difference in transcript level between *Sbe2a* and *Sbe2b* in endosperm as reported in

Chapter 3. Moreover the activity of SBEIIa in maize endosperm is about one fourth to one half of that of SBEIIb as estimated from the SBE activity in the SBEIIa and SBEIIb fractions of the DEAE-Sepharose FF separation of maize endosperm SBE extracts (Data not shown).

SBEIIa and SBEIIb may or may not have different qualitative contributions to amylopectin synthesis in endosperm. The *in vitro* catalytic properties of SBEIIa and SBEIIb are extremely similar [7, 16, 19], as are their structural features except for the short N-terminal divergent sequences (Chapter 3). This suggests that SBEIIa and SBEIIb may not have different qualitative contributions to amylopectin synthesis. However, it is possible that the substrate specificity and other catalytic properties of SBEIIa and SBEIIb may be altered by their interactions with the glucan network and/or other starch synthetic enzymes, especially starch synthases and debranching enzymes.

It is also possible that the different qualitative contributions between SBEIIa and SBEIIb may directly result from their different interaction with other starch synthetic enzymes and/or with the growing glucan network because of their different non-catalytic properties. The divergent N-terminal regions in SBEIIa and SBEIIb may play a critical role in these non-catalytic properties. Analysis of mutants deficient in starch synthesis pathways supports that SBEIIa and SBEIIb interact differently with starch synthases and debranching enzyme (SU1). For example, the maize mutation *dull* (*du1*) affects the level of both starch synthase II and SBEIIa in endosperm [3]. Elimination of SBEIIb prevents accumulation of phytoglycogen in *ae1su1* double mutant kernels [17], suggesting a close interaction between SBEIIb and the debranching enzymes in amylopectin synthesis.

The individual roles of the three isoforms in the formation of amylopectin fine structures is an important area for future research on SBE in order to better understand amylopectin synthesis. Based on the above discussion, addressing the following

questions in future studies may lead to a better understanding of the individual roles of SBEIIa and IIb in amylopectin synthesis. 1. Does the N-terminal divergent regions of SBEIIa and SBEIIb influence their catalytic properties *in vivo* ? Do these two divergent regions play any role in the interaction between the two isozymes and debranching enzyme (SU1), or starch synthases ? 2. Is the expression level of *Sbe2b* in endosperm relative to that of *Sbe2a* or are unique non-catalytic properties responsible for the large quantity of amylopectin synthesis in endosperm? A combination of molecular and biochemical methods will allow us to address these questions in the future.

The two questions could be addressed by a hybrid protein approach. The two divergent domains in SBEIIa and SBEIIb could be exchanged by molecular manipulations of the two cDNAs and over-expression of the hybrid proteins *in vitro*. The exchange of the divergent N-terminal domains may alter the *in vitro* catalytic properties of the two SBE isoforms by influencing their tertiary structures. The *in vitro* catalytic properties of the two hybrid SBEs should be compared to those of their parental SBE isoforms. The *in vivo* effect of these two divergent domains on SBEIIa and SBEIIb in amylopectin synthesis can then be assessed by the effects of the hybrid cDNAs on the complementation of the *ae* mutation. Four recombinant constructs of the hybrid cDNAs separately under the control of either the *Sbe2a* or the *Sbe2b* promoter can be used to transform *ae* mutant genotypes to evaluate the influence of the two divergent region in starch biosynthesis. In addition, two constructs with the *Sbe2a* cDNA controlled by the *Sbe2b* promoter, and *Sbe2b* cDNA controlled by the *Sbe2a* promoter can be used to transform *ae* mutants to see whether the difference in the expression level of the two isoforms, or in their *in vivo* catalytic or non-catalytic properties are responsible for the synthesis of the large quantity of amylopectin with normal branching in endosperm. Although the types of transformation experiments in maize are technically difficult, they could be very rewarding for a better understanding of

the roles of SBEIIa and SBEIIb in starch synthesis. Moreover, novel types of starch with potential applications could be produced in endosperm of transformants.

An alternative to transformation of maize *ae* mutants, the transformation experiment could be performed in a model system, in the alga *Chlamydomonas.* Starch in growth-arrested *Chlamydomonas* is similar in structure to maize endosperm storage starch by many criteria [5, 6, 10, 11]. An amylose-free mutant of *Chlamydomonas* equivalent to the maize *wx* mutant has been isolated and used to study starch synthesis [5]. A *Chlamydomonas* mutant reminiscent of maize *ae1*, *du1*, and *su2* accumulating high-amylose starch was also recently reported [10]. This suggest that the starch synthesis machinery of growth-arrested *Chlamydomonas* is very similar to that of maize endosperm. Moreover, methods for efficient and stable nuclear and chloroplast transformation of *Chlamydomonas* have been well established [1, 14]. This makes *Chlamydomonas* an excellent model system to study the individual roles of each maize SBE isoforms and their interactions with other maize starch synthetic genes by recombinant DNA methods.

The *Chlamydomonas* mutant accumulating high-amylose starch could be used for the transformation experiments to evaluate the effects of the N-terminal divergent domains of maize SBEIIa and SBEIIb on amylopectin synthesis through the hybrid protein approach. *Chlamydomonas* double mutants equivalent to *ae wx*, and triple mutants equivalent to *ae su wx* could be very useful to molecular studies on the interactions among starch synthases, branching enzymes and debranching enzymes in starch synthesis. Studies of starch biosynthesis in such a mono-cellular model system can overcome many technical difficulties encountered in complex systems such as maize endosperm and potato tubers, and provide important insights into starch biosynthesis in plants.

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APPENDIX 1

ANALYSIS OF THE EXPRESSION OF STARCH SYNTHETIC GENES IN ENDOSPERM AND KERNELS OF THE B73 INBRED

Materials and Methods

All plant materials were prepared as described in the materials and methods section of Chapter 2. However, the B73 inbred kernels were from a later planting such that the sampling of B73 kernels commenced approximately one month later than that of W64A kernels at the same DAP (Days After Pollination). Extraction of total RNA, RNA blotting and hybridization with the five cDNA probes of starch synthetic genes were performed as described in Chapter 2. The relative transcript level (R.L.) of the five genes are the average of two independent measurements. They were calculated as described in Chapter 2.

Results and Discussion

The general pattern of *Sbe1* and *Sbe2b* expression during kernel development in the B73 inbred was similar to that reported in Chapter 2 for the W64A inbred. The peak expression of *Sbe2b* was detected earlier than that of *Sbe1* (30 DAP, vs. 40 DAP, Fig. A1). The maximal expressions of both genes and the other three starch synthetic genes in the B73 inbred occurred significantly later in development than those in the W64A inbred. This suggests that starch synthesis should be delayed significantly in endosperm of the later maturity B73 inbred than in W64A endosperm. This lag of starch synthesis in the later maturity B73 inbred was recently confirmed by the comparison of starch synthesis in

developing B73 and W64A kernels [6]. It was shown that increases in the number of starch granules and the total volume of starch granules in developing kernels were delayed significantly in the later maturity B73 inbred by comparison to the early W64A inbred [6].

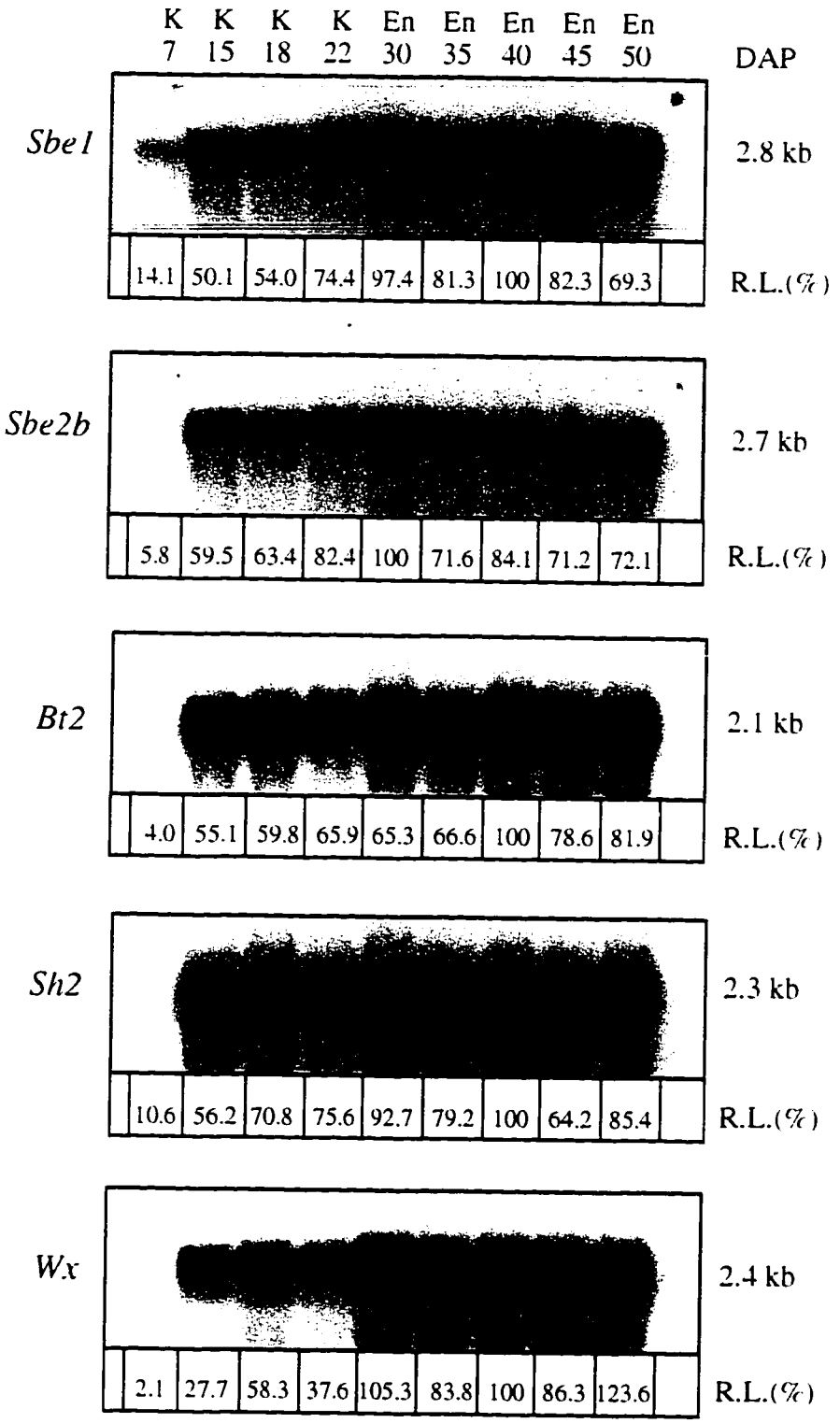
The high level of *Sbe1* transcripts in B73 endosperm at 30 DAP was presumably an overestimation due to experimental error because the *Sbe1* transcripts in endosperm of 35 DAP was much lower. It may also be due to the size difference of the sampled kernels on an ear. Kernels of different size on an ear are at different developmental stages as reflected by the composition and starch granule size [8], which could have potentially obscured a gradual increment of transcript level. The expression pattern of *Sbe1* during endosperm development in the B73 inbred observed here was also substantially different from that in the Oh43 inbred, in which *Sbe1* mRNA was reported to peak as early as 10 DAP, and decrease slowly thereafter [1]. However the data reported by Baba, et al. [1] was not quantitative, and might not accurately reflect the expression pattern of *Sbe1* in Oh43 kernels.

The differential expression of *Sbe1* and *Sbe2b* during endosperm development of both W64A and B73 plants may have a role in controlling the structure of starch granules synthesized at different developmental stages. The degree of crystallinity is partially determined by the branch length in amylopectin [7]. The two isozymes indeed differ in the preferred branch length transferred *in vitro* [10]. SBEI preferentially transfers longer chains than SBEIIa and IIb [10]. Thus, the ratio of SBEI and SBEIIb in endosperm at a particular developmental stage may play a central role in determining the degree of crystallinity of the starch granules synthesized at the developmental stage.

The developmental timing for peak expression of *Bt2* and *Sh2* matched that of *Sbe1*, rather than *Sbe2b* as in W64A. The difference could have been due to a larger experimental error for the measurements of gene expression in the B73 inbred because the

data are the average of only two repeats. Alternatively it might be biologically significant. Since starch synthesis and kernel development are delayed significantly in the B73 inbred compared those in the W64A inbred [6], it would not be surprising to see ADPG pyrophosphorylase encoded by *Bt2* and *Sh2*, a key regulatory enzyme for starch synthesis, reach its peak later in B73 inbred than in W64A because of the developmental delay. The match of the maximal expression of *Bt2* and *Sh2* with that of *Sbe1*, rather than *Sbe2b* might be needed by this particular inbred to produce more amylopectin with longer branches. The composition of starch granule indeed varies among different inbreds [8]. It awaits further investigation whether this difference is biologically significant. The expression of *Wx* seemed to reach its peak later in endosperm development consistent with its expression in W64A endosperms, although a large variance of the expression level was observed. This variance may be due to experimental errors.

Fig. A1. Northern blot analysis of the expression of starch biosynthetic genes during development of B73 maize kernels. 7.5 μg of total RNA extracted from kernels (K) and endosperm (En) at various days after pollination (DAP) was fractionated, blotted and probed consecutively with radioactively labeled *Sbe1* [5], *Sbe2b* [4], *Sh2* [3], *Bt2* [2], and *Wx* [9] cDNAs. The relative mRNA level (R.L.) of each gene in a sample was expressed as the percentage of its transcript level in samples of either 30 or 40 DAP endosperm, calibrated for the 26S rRNA levels in the two samples. The R.L. of various genes were an average of two independent measurements.


K 7
K 15
K 18
K 22
En 30
En 35
En 40
En 45
En 50
DAP
Sbe1
2.8 kb
14.1 50.1 54.0 74.4 97.4 81.3 100 82.3 69.3
R.L.(%)
Sbe2b
2.7 kb
5.8 59.5 63.4 82.4 100 71.6 84.1 71.2 72.1
R.L.(%)
Bt2
2.1 kb
4.0 55.1 59.8 65.9 65.3 66.6 100 78.6 81.9
R.L.(%)
Sh2
2.3 kb
10.6 56.2 70.8 75.6 92.7 79.2 100 64.2 85.4
R.L.(%)
Wx
2.4 kb
2.1 27.7 58.3 37.6 105.3 83.8 100 86.3 123.6
R.L.(%)

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