Diurnal oscillation of *SBE* expression in sorghum endosperm

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Running title: Regulation of *SBE* expression

Abstract

Spatial and temporal expression patterns of the sorghum *SBEI*, *SBEIIA* and *SBEIIB* genes, encoding, respectively, starch branching enzyme (SBE) I, IIA and IIB, in the developing endosperm of sorghum (*Sorghum bicolor*) were studied. Full-length genomic and cDNA clones for sorghum was cloned and the *SBEIIA* cDNA was used together with gene-specific probes for sorghum *SBEIIB* and *SBEI*. In contrast to sorghum *SBEIIB*, which was expressed primarily in endosperm and embryo, *SBEIIA* was expressed also in vegetative tissues. All three genes shared a similar temporal expression profile during endosperm development, with a maximum activity at 15-24 days after pollination. This is different from barley and maize where *SBEI* gene activity showed a significantly later onset compared to that of *SBEIIA* and *SBEIIB*. Expression of the three *SBE* genes in the sorghum endosperm exhibited a diurnal rhythm during a 24-h cycle.

Key words: Barley; Diurnal regulation; Endosperm; Oscillation; SBE; Sorghum; Starch

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; GBSSI, Granule-bound starch synthase I; SBE, starch branching enzyme

The cDNA sequence of sorghum *SBEIIA* will appear in GenBank under the accession nr. XXXXXXXX.

Introduction

Starch synthesis is the main process that determines yield in cereal grains. The pathway in starch synthesis involves conversion of sucrose to ADP-glucose and subsequent conversion of this precursor into the polyglucan molecules amylose and amylopectin. The process of starch synthesis is governed by several groups of enzymes, i.e. ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE). These enzymes exist in different isoforms and the biochemical characteristics of the enzymes and the expression profiles of the corresponding genes specify the structural organization of starch molecules in plant organs such as the endosperm (see Tomlinson and Denyer, 2003, for a review on starch synthesis and structure in cereals; see also Ball et al., 1998; Buléon et al., 1998; Myers et al., 2000; Nakamura, 2002; Smith, 2001, for other reviews on starch synthesis).

Regulation of starch synthesis is exercised by an intricate network of sugar signaling and hormonal transduction pathways, the nature of which is poorly understood (Jansson 2004; León and Sheen, 2003; Rolland et al., 2006; Sheen et al., 1999; Smeekens, 2000).

Furthermore, it has been shown that starch synthesis in source organs is under diurnal and circadian control (Cheng et al., 2002; Dian et al., 2003; Geigenberger and Stitt, 2000; Sehnke et al., 2001). Circadian regulation of the *GBSSI* gene, encoding granule-bound SSI (GBSSI), in leaves has been reported for Arabidopsis (Tenorio et al., 2003), sweet potato (Wang et al., 2001) and snapdragon (Merida et al., 1999). In three instances, diurnal oscillations of starch synthesis gene expression has been observed also in sink organs; for the growth ring formation starch granules in potato tubers (Pilling and Smith, 2003), for the gene encoding the catalytic subunit of AGPase in potato tubers (Geigenberger and Stitt, 2000), and the *SBEI* and *SBEII* genes, encoding, respectively, SBEI and SBEII, in cassava storage roots (Baguma et al., 2003).

Sorghum is the fourth most important cereal crop trailing behind rice, maize and wheat. However, it is ranked second to maize in supply of grain requirement within sub-Saharan Africa. In most of these countries, sorghum is the main source of starch for human diet. To date, over 500 million people in the developing countries depend on sorghum as the main staple food. In other countries,

sorghum starch is mainly used in livestock feed formulations and as a cheap source of raw material for industrial applications.

The value of sorghum, a C_4 plant, is derived from its ability to grow in marginal areas lacking sufficient moisture and fertility unfeasible to support production of maize, wheat or rice. Consequently, sorghum holds the potential to supply a greater share of the world's grain demand. This congruency makes expansion of sorghum starch production and utility feasible as a main alternative to maize starch for food and non-food products. Furthermore, as a C_4 grass with a relatively small genome (735 Mb), sorghum can also serve as a model plant for potential bioenergy grasses such as Miscanthus.

We have previously reported on the temporal and spatial expression profiles for the sorghum *SBEI* and *SBEIIB* genes (Mutisya et al., 2003). In the present study, we wanted to compare the expression profiles for the *SBEI*, *SBEIIA* and *SBEIIB* genes in sorghum and assess whether they are subject to diurnal control. Since sorghum *SBEIIA* had not yet been cloned, we also describe the isolation and characterization of this gene and the similarity between the sorghum SBEIIA and SBEIIB proteins.

Materials and methods

Plant materials

Sorghum (*Sorghum bicolor* L. Moench) and barley (*Hordeum vulgare*) plants were grown in greenhouse under controlled 16-h light/8-h dark cycles as described (Mutisya et al., 2003). For analysis of the spatial *SBE* expression profiles, seeds were harvested at 9 days after pollination (d.a.p.). For the temporal expression profiles during endosperm development, seeds were harvested at indicated intervals after d.a.p. For analysis of diurnal expression, seeds were harvested at 9-12 d.a.p. Samples for analyses were immediately frozen and stored at – 80^{o}C until use.

Molecular cloning and DNA sequence analysis

Screening of the sorghum genomic library (SB-BBc; Mutisya et al., 2003) for *SBEIIA* was performed with heterologous barley probes. To identify all candidate clones for *SBEII*, we used a full-length barley *SBEIIA* cDNA probe. The probe was labeled with (^{32}P)-dCTP (Amersham Phamarcia, Biotech., UK) according to instructions by

the manufacturer. Hybridization was performed as described (Mutisya et al., 2003). To identify clones specific for *SBEIIA*, further screening was performed using a unique 5-´end region of the barley *SBEIIA* cDNA.

Total RNA was isolated from developing sorghum endosperm according to Sun et al. (1999). Primers were designed from genomic sequences within the first 9 exons of sorghum *SBEIIA* and the 3' unstranslated region of maize *SBEIIA*. The first strand cDNA was synthesized as per manufacturers instruction (Amersham Pharmacia Biotech., UK). Reverse transcriptase (RT) PCR was performed according to standard protocols. The PCR products were cloned into the PCR^{R} II - TOPO^{R} cloning vector (Invitrogen, USA) and sequenced.

Sequencing of DNA inserts of clones was carried out on both strands using a DNA sequencer. Database searches were carried out using the BLAST programs available at NCBI (hptt://www.NCBI.nlm.nih.gov/Blast). Sequence alignment was performed using the MacVector program (Accelrys Software Inc., France).

DNA and RNA blot analyses

Total genomic DNA was isolated from young sorghum leaves as described by Mutisya et al. (2003). To determine the *SBEIIA* gene copy number, approximately 20 μg DNA was digested with the restriction enzymes HpaI, Kpn1 and SacI that cut only once within the probe. The digests were subjected to DNA gel blot analysis as described (Mutisya et al., 2003) using a 5'-labelled *SBEIIA* probe.

For examination of *SBE* expression, total RNA was isolated from sorghum and barley and purified as described (Mutisya et al., 2003). RNA gel blot analyses were performed as described by Sun et al. (2003) using ^{32}P-labelled gene-specific cDNA fragment for sorghum *SBEI* and *SBEIIB* (Mutisya et al., 2003), sorghum *SBEIIA* (this work) and barley *SBEIIB* (Sun et al., 1998). The membranes were striped of the radioactive probes in a boiling 0.5% (w/v) SDS solution and re-hybridized with 18S rRNA-labeled probes.

Protein extraction and analysis

Developing sorghum endosperms at 15 d.a.p. were harvested at 6-h intervals and ground in a mortar into fine powder and homogenized

with 2 volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mm DTT and 5 mM EDTA). The homogenate was centrifuged at 10, 000 g for 15 min at 4 ^{o}C. The supernatant was re-centrifuged at 12, 000 g to remove all debris. Protein gel blot analyses were performed as described (Mutisya et al., 2003). Zymogram assays for SBE activity was carried out as described by Sun et al. (1996).

Results and discussion

Isolation and analysis of *SBEIIA* cDNA and genomic DNA

A sorghum BAC library (Mutisya et al., 2003) was screened using a heterologous barley *SBEIIA* probe. Out of a total of 105,592 clones, we found 22 that were specific for *SBEIIA*. One of the clones that hybridized strongly to the probe was subjected to restriction digest and re-probed with the same *SBEIIA* probe. The hybridizing DNA fragments were isolated, sub-cloned and sequenced. A 4.2 kb long sequence distributed over two overlapping fragments (2.5 and 2.0 kb, respectively) from the 5′ region was sequenced. A BLAST search using the longest fragment revealed that the sorghum *SBEIIA* clone shared a high degree of homology with *SBEIIA* from maize, wheat, barley and rice, in a descending order. Based on the

alignment with maize and wheat *SBEIIA*, it was evident that the sequence from the sorghum clone contained the first 9 exons and 8 introns.

Based on the 5' sequence of sorghum *SBEIIA* and the 3' sequence of maize *SBEIIA*, primers were designed for RT-PCR amplification of a sorghum *SBEIIA* cDNA clone using total RNA isolated from developing sorghum endosperm 21 d.a.p. Only one PCR product with the expected size was obtained. The PCR product was cloned and sequenced. The *SBEIIA* cDNA clone was 2835 nucleotides long and encompassed the entire coding region.

Sequence analysis of sorghum SBEIIA

The deduced amino acid sequence of the *SBEIIA* cDNA suggests that it encodes a polypeptide of 677 amino acids. A comparison of the primary structures of sorghum SBEIIA and SBEIIB revealed that they share 84% sequence identity (Fig. 1). The four regions implicated in the catalytic site of amylolytic enzymes (Jespersen et al., 1993) are conserved in sorghum SBEIIA (data not shown; see Mutisya et al., 2003, for a discussion on sorghum SBEIIB). The principal difference between the two enzymes is the 130 amino

acids-long N-terminal sequence of SBEIIB (Fig. 1). This is similar to the situation in barley and might reflect a differential partitioning of the SBEIIA and SBEIIB isoforms (Sun et al., 1998).

Sorghum *SBEIIB* gene copy number

DNA gel blot analysis was performed to determine the gene copy number of *SBEIIA*. Using three restriction enzymes with a single recognition site within the probe consistently yielded two hybridizing bands, strongly indicating a single copy of *SBEIIA* in the sorghum genome (data not shown). A single-copy *SBEIIA* gene is in agreement with the situation for *SBEIIB* in sorghum (Mutisya et al., 2003) and for *SBEIIA* and *SBEIIB* in barley, wheat and rice (Kim et al., 1998; Rahman et al., 2001; Sun et al., 1998; Yamanouchi and Nakamura, 1997).

Spatial and developmental expression of sorghum *SBEIIB*

We noted previously (Mutisya et al., 2003) that the sorghum *SBEI* and *SBEIIB* genes were predominantly expressed in endosperm and embryo tissues. The spatial expression pattern of sorghum *SBEIIA* was investigated and compared to that of *SBEI* and *SBEIIB*. Total RNA was extracted from different tissues and subjected to RNA gel

blot analyses using gene-specific probes. The results demonstrated that both *SBEIIA* and *SBEIIB* were expressed predominantly in the embryo and endosperm at the time point examined, however, transcripts hybridizing to *SBEIIA* were also detected in the leaves, stems and roots (Fig. 2B). The differential expression of the sorghum *SBEIIA* and *SBEIIB* genes is in agreement with the patterns in barley and maize, where *SBEIIB* is exclusively or preferentially expressed in the endosperm while *SBEIIA* is expressed in all tissues analyzed (Gao et al., 1996; Sun et al., 1998). The size of the detected sorghum *SBEIIA* transcript was approximately 2.8 kb, similar to what has been reported for barley (Sun et al., 1998), maize (*SBE2b*; Fisher et al., 1993) and rice (*SBE3*; Mizuno et al., 1993).

During the grain filling period, expression of *SBEIIA* gene was detected around 10 d.a.p. (Fig. 2A). Steady-state levels of *SBEIIA* transcripts peaked around 22 days after pollination and then drastically reduced to undetectable levels until grain maturity. This temporal expression profile is similar to that of sorghum *SBEIIB* and *SBEI* (Mutisya et al., 2003). Thus in contrast to barley, where *SBEI* activity shows a considerably later onset as compared to *SBEIIA* and *SBEIIB* (Mutisya et al., 2003; Sun et al., 1998), the activity for all three *SBE* genes in sorghum appears to peak at the same time. If,

and how, that translates to differences in starch structure between the two cereals during endosperm development remains to be elucidated.

Diurnal oscillations of the sorghum *SBEII* genes

To further investigate the temporal expression of the sorghum *SBE* genes we monitored transcript accumulation in endosperms of seeds harvested at 9 d.a.p. from plants grown under two different light/dark (LD) regimes. Interestingly, the *SBE* expression levels showed a diurnal fluctuation with an induction in the light and decline in the dark (Fig. 3A, B). A similar behavior in expression was observed also for barley *SBEIIB* (Fig. 3 C). Whether *SBE* transcript accumulation in sorghum and barley also exhibited an oscillation within the light periods is difficult to assess at this time and is a question that should be addressed by further experiments.

Analyses of SBE protein levels and activity

Protein gel blot assays with an antiserum against SBEIIB was employed to examine the levels of SBE proteins in seeds harvested from LD or DD sorghum plants at different time of the day. We noted that SBEIIB protein levels in the endosperm were relatively

constant throughout the sampling period for both LD and DD plants (Fig. 4A). Zymogram analysis of SBE activity in endosperm from LD sorghum plants also revealed no overt fluctuation in branching enzyme activity during a 24-h period (Fig. 4B). However, careful examination of the gel points to the possibility of a low-amplitude 12-h oscillation. In addition to the SBE activities, another activity band, probably corresponding to endogenous starch phosphorylase *a*, was visible on the zymogram.

Conclusion

We have isolated the *SBEIIA* gene from sorghum, characterized its expression, and compared it to that of sorghum *SBEI* and *SBEIIB*. Most notably, we found that the expression for all three *SBE* genes exhibited a diurnal rhythm. Possibly, the rhythmicity in *SBE* expression serves a means for the endosperm cells to anticipate the diurnal flux of sucrose from the source. Oscillation in *SBE* expression was observed also in barley endosperm and thus it might be a general phenomenon for starch synthesis in sink organs.

The oscillation in *SBE* expression did not translate to a matching fluctuation in SBE protein levels of SBE activity, although a weak 12-h oscillation in SBE activity cannot be excluded. That

rhythmicity in mRNA levels operates without downstream effects on the accumulation of the corresponding protein products have been demonstrated before. For example, in Arabidopsis leaves it was reported that certain genes encoding enzymes involved in starch degradation were subject to circadian regulation although the abundance of corresponding enzymes remained constant during the circadian cycle (Lu et al., 2005).

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References

Baguma Y, Sun C, Ahlandsberg S, Mutisya J, Rubaihayo RP, Magambo MJ, Egwang TG, Larsson H, Jansson C. Expression patterns pf the gene encoding starch branching enzyme II in the storage roots of cassava (*Manihot esculenta* Crantz). Plant Sci 2003*;* 164: 833-839.

Ball S, van de Wal MHBJ, Visser RGF. Progress in understanding the synthesis of amylose. Trends Plant Sci 1998; 3:462-467.

Buléon A, Colonna P, Planchot V, Ball S. Int J Biol Macromol Starch granules: structure and biosynthesis. 1998; 23:85-112.

Cheng L-S, Lin Q, Nose A. A comparative study on diurnal changes in metabolite level in the leaves of three crassulacean acid metabolism (CAM) species, *Ananas comosus*, *Kalanchoe daigremontiana* and *K. pinnata*. J Exp Bot 2002; 53:341-350.

Dian W, Jiang H, Chen Q, Liu F, Wu P. Cloning and characterization of the granule-bound starch synthase II gene in rice: gene expression is regulated by the nitrogen level, sugar and circadian rhythm. 2003; 218:261-268.

Fischer DK, Boyer CD, Hannah LC. Starch branching enzyme II from maize endosperm. Plant Physiol 1993; 102:1045-1046.

Gao M, Fisher DK, Kim K-N, Shannon JC, Guiltinan MJ. Evolutionary conservation and expression patterns of maize

starch branching enzyme I and IIb genes suggests isoform specialization. Plant Mol Biol 1996; 30:11223-32.

Geigenberger P, Stitt M. Diurnal changes in sucrose, nucleotides, starch synthesis and AGPS transcript in growing potato tubers that are suppressed by decreased expression of sucrose phosphate synthase. Plant J 2000; 236:795-806.

Jansson C. Sugar signaling mutants in Arabidopsis. In: Esser K et al, ed. Progress in Botany. Springer Verlag, Berlin, 2004: 42-52.

Jespersen HM, MacGregor EA, Henrissat B, Sierks MR, Svensson B. Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic $(b/a)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. J Protein Chem 1993; 12:791-805.

Kim K-N, Fisher DK, Gao M, Guiltinan MJ. Molecular cloning of the Amylose-Extender gene encoding starch branching enzyme IIb in maize. Plant Mol Biol 1998; 38: 945-956.

León P, Sheen J. Sugar and hormone connections. Trends Plant Sci 2003; 8:110-116.

Lu Y, Jackson PG, Sharkey TD. Daylength and circadian effects on starch degradation and maltose metabolism. Plant Physiol 2005; 138:2280-2291.

Merida Al, Rodriguez-Galan JM, Vincent C, Romero JM. Expression of the granular-bound starch synthase I (*Waxy*) gene from snapdragon is developmentally and circadian clock regulated. Plant Physiol 1999; 120:401-409.

Mizuno K, Kamura K, Arai Y, Kawasaki T, Shimada H, Baba, T. Starch branching enzymes from immature rice seeds. J Biochem 1992; 112:643-51.

Mutisya J, Sathish P, Sun C, Andersson L, Ahlandsberg S, Baguma Y, Palmqvist S, Odhiambo B, Åman P, Jansson C. Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): Comparative analyses of enzyme structure and gene expression. J Plant Physiol 2003; 160:921-930.

Myers AM, Morell MK, James MG, Ball SG. Recent progress toward understanding biosynthesis of the amylopectin crystal. Plant Physiol 2000; 122:989-997.

Nakamura Y. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiol 2002; 43:718-725.

Pilling E, Smith AM. Growth ring formation n the starch granules of potato tubers. J Plant Physiol 2003; 132:365-371.

Rahman S, Regina A, Li Z, Mukai Y, Yamamoto M, Kosar-Hashemi B, Abrahams S, Morell MK. Comparison of starch-branching enzyme genes reveals evolutionary relationships among isoforms. Characterization of a gene for starch-branching enzyme IIa from the wheat D genome donor *Aegilops tauschii.* Plant Physiol 2001; 125: 1314-1324.

Ral J-P, Colleoni C, Wettebled F, Dauvillee D, Nempont C, Deschamps P, Li Z, Morell M, Chibbar R, Purton S, d'Hulst C, Ball SG. Circadian clock regulation of starch metabolsim establishes GBSSI as a major contribtor to amylopectin synthesis in *Chlamydomonas reinhardtii*. Plant Physiol 2006; 142:305-317.

Rolland F, Baena-Gonzalez E, Sheen J. Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annual Review of Plant Biology 2006; 57:675-709.

Sheen J, Zhou L, Jang JC. Sugars as signaling molecules. Curr Opinions Plant Biol 1999. 2:410-418.

Sehnke PC, Chung H-J, Wu K, Ferl RJ. Regulation of starch accumulation by granule-associated plant 14-3-3 proteins. Proc Natl Acad Sci USA 2001; 98:765-770.

Smeekens S. Sugar-Induced Signal Transduction in Plants. Ann Rev Plant Physiol Plant Mol Biol 2000; 51:49-81.

Smith AM. The biosynthesis of the starch granule. Biomacromol 2001; 2:335-341.

Smith SM, Fulton DC, Chia T, Thorneycroft D, Chapple A, unstan H, hylton C, Zeeman SC, Smth AM. Diurnal changes in the transcrptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. Plant Physiol 2004; 136:2687-2699.

Sun C, Palmqvist S, Olsson H, Borén M, Ahlandsberg S, Jansson C. A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the *iso1* promoter. Plant Cell 2003; 15:2076-2092.

Sun C, Sathish P, Ahlandsberg S, Jansson C. The two genes encoding starch branching enzymes IIa and IIb are differentially expressed in barley. Plant Physiol 1998; 118:37-49.

Sun C, Sathish P, Ek B, Jansson C. Demonstration of in vitro starch branching enzyme activity for a 51/52-kDa polypeptide isolated from developing barley (*Hordeum vulgare*) caryopses. Physiol Plant 1996; 96:474-483.

Tenorio G, Orea A, Romero JM, Merida A. Oscillation of mRNA level and activity of granular-bound starch synthase I in Arabidopsis leaves during the day/night cycle. Plant Mol. Biol 2003; 51:949-958.

Tomlinson K, Denyer K. Starch synthesis in cereal grains. Adv Bot Res 2003; 40:2-61.

Wang SJ, Yeh KW, Tsai CY. Regulation of starch granule-bound starch synthase I gene expression by circadian clock and sucrose in source tissue of sweetpotato. Plant Sci 2001; 161:635-644.

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

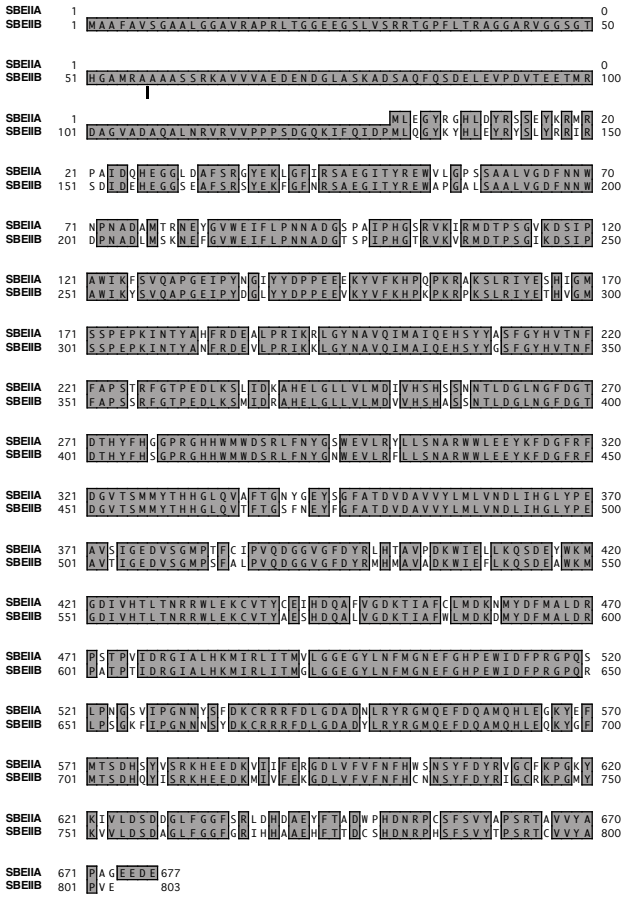
Figure legends

Figure 1. Alignment of the sorghum SBEIIA and SBEIIB sequences. Identical amino acids are indicated as grey boxes. The postulated transit peptide cleavage site for SBEIIB (Mutisya et al., 2003) is shown as a vertical line below the sequence.

Figure 2. Temporal and spatial expression profiles for sorghum *SBEIIA* and *SBEIIB*. **(A)**. Steady state levels of transcripts in endosperm at indicated days after pollination (d.a.p.). **(B)**. Steady state levels of transcripts in endosperm (En), embryo (Em), leaves (Lv), stem (St), or root (Rt).

Figure 3. Diurnal expression profiles of *SBE* genes in sorghum and barley endosperm. Steady state levels of *SBE* transcripts in sorghum **(A, B)** or barley **(C)** plants grown under light/dark cycles, with light switched on at 6 am **(A)**, 12 noon **(B)**, or 4 am **(C)** were analyzed. In **(C)** only data for *SBEIIB* are shown but results were similar for *SBEI* and *SBEIIA*. Times are indicated as follows: 3, 3 am; 6, 6 am; 9, 9 am; 12, noon; 15, 3 pm; 18, 6 pm; 21, 9 pm; 24, midnight. The horizontal bars indicate transitions between light (white) and darkness (black). Levels for 18S rRNA are shown as controls.

Figure 4. SBE protein levels and activity in sorghum endosperm during a 24-h cycle. Total endosperm protein was extracted and subjected to protein gel blot analysis with an SBEIIB antiserum **(A)** or zymogram analysis of SBE activity **(B)**. Each lane was loaded with 100 g protein extract. Other conditions as in Fig. 3.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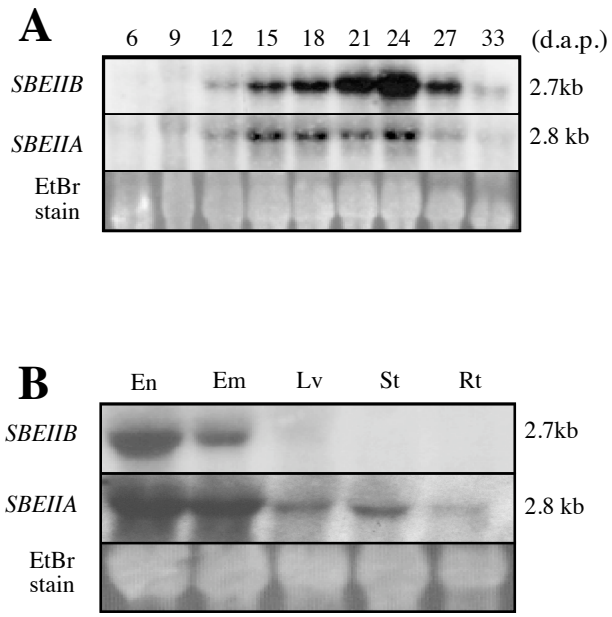
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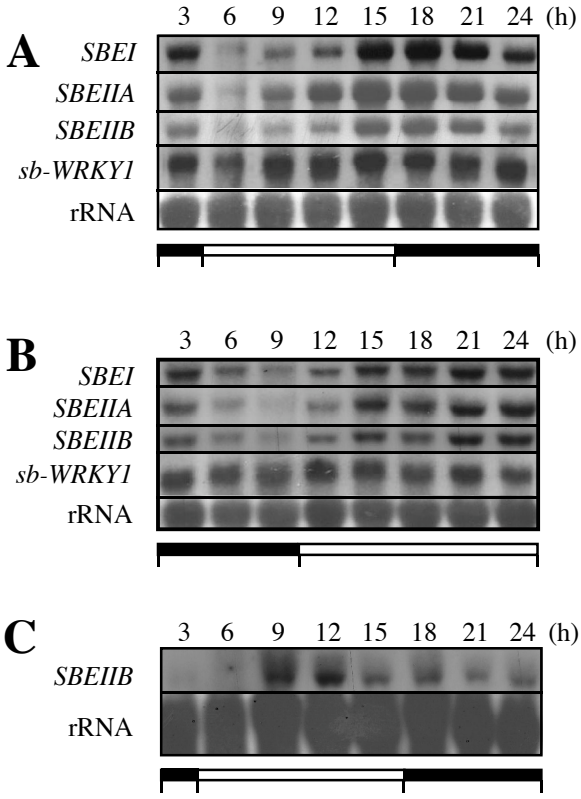
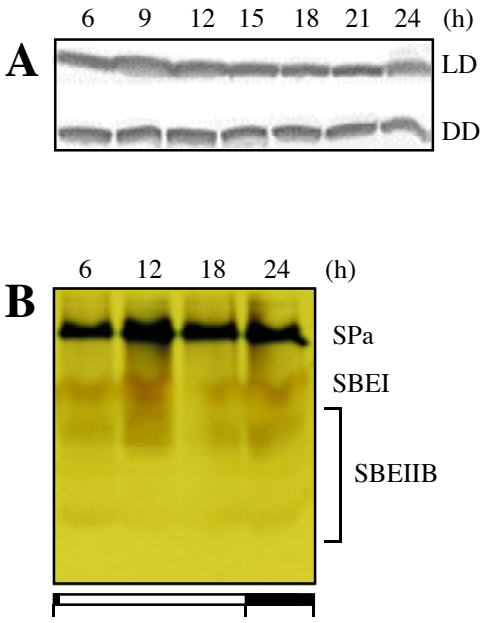
EtBr stain


A
3 6 9 12 15 18 21 24 (h)
SBEI
SBEIIA
SBEIIB
sb-WRKY1
rRNA
B
3 6 9 12 15 18 21 24 (h)
SBEI
SBEIIA
SBEIIB
sb-WRKY1
rRNA
C
3 6 9 12 15 18 21 24 (h)
SBEIIB
rRNA


6 9 12 15 18 21 24 (h)
A
LD
DD
6 12 18 24 (h)
B
SPa
SBEI
SBEIIB