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Diurnal oscillation of $SBE$ expression in sorghum endosperm

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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. Xxxxxxxxx.
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 C₄ 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 C₄ 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°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 Pharamcia, 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’
untranslated 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\textsuperscript{R} II - TOPO\textsuperscript{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
(http://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, KpnI 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 32P-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 °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 SBEIB 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**


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.