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ORIGINAL ARTICLE

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Starch branching enzyme IIb in wheat is expressed at low levels in the endosperm compared to other cereals and encoded at a non-syntenic locus

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Abstract Studies of maize starch branching enzyme mutants suggest that the *amylose extender* high amylose starch phenotype is a consequence of the lack of expression of the predominant starch branching enzyme II isoform expressed in the endosperm, SBEIIb. However, in wheat, the ratio of SBEIIb and SBEIIa expression are inversely related to the expression levels observed in maize and rice. Analysis of RNA at 15 days post anthesis suggests that there are about 4-fold more RNA for SBE IIa than for SBE IIb. The genes for SBE IIa and SBE IIb from wheat are distinguished in the size of the first three exons, allowing isoform-specific antibodies to be produced. These antibodies were used to demonstrate that in the soluble fraction, the amount of SBE IIa protein is two to three fold higher than SBIIb, whereas in the starch granule, there is two to three fold more SBE IIb protein amount than SBE IIa. In a further difference to maize and rice, the genes for SBE IIa and SBE IIb are both located on the long arm of chromosome 2 in wheat, in a position not expected from rice–maize–wheat synteny.

Keywords Amylopectin · Amylose · Cereal · Starch branching enzyme · Wheat

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List of symbols

CS	Chinese spring
dNTP	Deoxynucleotide triphosphate
DPA	Days post anthesis
GST	Glutathione S-transferase
NT	Nullisomic tetrasomic
ORF	Open reading frame
PBS	Phosphate buffered saline

Introduction

Starch is composed of two types of glucose polymer: amylose and amylopectin. While amylose is a predominantly linear chain of α 1,4-glucan, amylopectin is highly branched with α-1,6 glycosidic bonds that link linear chains. Starch biosynthesis is known to occur through the organised activity of a sequence of enzymes: ADP glucose pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), starch branching enzyme (EC 2.4.1.18) and debranching enzyme (EC 2.4.1.41). Enzymes such as starch phosphorylase and D-enzyme may also have roles in starch biosynthesis (Mengel and Judel 1981; Colleoni et al. 1999).

The extent of branching in starch is a key determinant of its functionality. Branching of starch is catalysed by starch branching enzymes (SBEs). Two different classes of SBEs are reported in plants, SBE I and SBE II, which differ in their substrate specificity and mode of action (Morell et al. 1997). Specific roles of these two classes of enzymes are known from mutational and gene suppression analyses in both cereal and non-cereal crops. In cereals, two different isoforms of SBE II are reported, SBE IIa and SBE IIb (Boyer and Preiss 1978; Mizuno et al. 1993; Morell et al. 1997; Sun et al. 1998).

Lack of SBE IIb leads to a high amylose phenotype in both maize and rice, although the effect reported in rice is less pronounced than in maize (Stinard et al. 1993; Nishi et al. 2001). No significant phenotype has yet been observed in SBE I deficient potato, maize or wheat (Safford et al. 1998; Baga et al. 1999; Blauth et al. 2002; Regina et al. 2004), although some apparent alteration in the amylopectin fine structure was recently reported for rice (Satoh et al. 2003). It has been shown in potato that a simultaneous reduction of SBE I and SBE II can result in a very high amylose phenotype (Schwall et al. 2000).

In maize, barley and rice, SBE IIa and SBE IIb, though closely related, are encoded by different genes. cDNAs for both SBE IIa and SBE IIb were cloned from maize, rice and barley (Mizuno et al. 1993; Gao et al. 1997; Sun et al. 1998). Maize SBE IIa and SBE IIb cDNAs show 85% nucleotide sequence identity over the central region with divergent 5′ and 3′ ends (Gao et al. 1997). The predicted protein sequences of SBE IIa and SBE IIb of barley showed an amino acid identity of 80% (Sun et al. 1998). The only complete gene of SBE IIb characterised to date in cereals is from maize (Kim et al. 1998) and spans a region of 17 kb of DNA with 22 exons. The SBE IIa gene was characterised from *Aegilops tauschii*, the D-genome donor of wheat (Rahman et al. 2001). The structures of the SBE IIa gene from wheat and SBE IIb gene from maize are similar in that both have 22 exons and exons 3–21 are identical in size (Kim et al. 1998; Rahman et al. 2001).

The relative expression levels of SBEIIa and SBEIIb differ amongst species. SBE IIa in maize is predominantly expressed in leaves, whereas the predominant form in the endosperm is SBE IIb (Gao et al. 1997). Similarly, in rice SBE IIb is the major isoform in the endosperm (Yamanouchi and Nakamura 1992). In barley the activities of SBE IIa and SBE IIb isoforms are roughly equal within the endosperm (Sun et al. 1998). In wheat, however, the predominant isoform in the endosperm soluble phase has an N-terminal sequence characteristic of the IIa isoform which is also found associated with starch granule (Rahman et al. 2001). While SBE IIb has been noted to be present in the wheat endosperm (Tetlow et al. 2004), the work focussed on the identification of post-translational modification and complexation state of the enzyme and did not explore questions of gene structure, localisation or expression.

In this paper we report the quantitation and characterisation of SBE IIb from wheat endosperm that was achieved through the isolation of a cDNA encoding SBE IIb in wheat (GenBank accession no. AY740401). Antisera distinguishing the SBE IIa and IIb isoforms were produced using synthetic peptides, based on deduced discriminatory sequences, and was used to show that SBE IIb is the predominant isoform in the starch granule in wheat endosperm in terms of the amount of protein but the minor isoform in the amyloplast stroma, in which SBE IIa predominates in terms of protein. The gene encoding SBE IIb from the D genome donor of wheat (GenBank accession nos. AY740398, AY740399 and AY740400) was characterised and localised to the long arm of chromosome 2 (as was the gene encoding SBE IIa earlier).

Materials and methods

Plant material

The wheat cultivar Chinese Spring (*Triticum aestivum* cv CS) and *A. tasuchii* var. *strangulata*, accession CPI 110799 were used to create cDNA and genomic libraries, respectively. Chromosome engineered lines of CS, nullisomic-tetrasomic (NT) lines that are nullisomic for a pair of chromosomes from one genome but tetrasomic for a homoeologous chromosome from another genome, ditelosomic lines that lack a whole chromosomal arm and microdeletion lines having various sized terminal deletions on specific chromosomal arms (Endo and Gill 1996) were used for locating the gene for SBE IIb in wheat. The wheat cultivar, Fielder at 8, 15, 25 DPA was used for measuring the expression levels of SBEs in endosperms.

Isolation of cDNAs and genomic clones of SBE IIb

The construction of cDNA and genomic libraries used to isolate cDNA and genomic clones of SBE IIb is described in Rahman et al. (1997, 1999) and in Li et al. (1999). Primers 1 and 2 designed based on the sequence from the divergent 5′ region of barley SBE IIb cDNA (Sun et al. 1998) were used to PCR amplify a fragment from barley cDNA library using conditions described in Sun et al. (1998). The identity of this fragment was confirmed by sequencing and was used as a probe to hybridise a cDNA library constructed from CS (Li et al. 1999). A cDNA, designated wBEIIb-cDNA was thus isolated. The sequence from the positions 537–890 of SBE IIa cDNA, cDNA 1 (Rahman et al. 2001) was amplified by PCR and used to screen *A. tauschii* genomic library to isolate clones referred to as G1, G2 and G5. Conditions of library screening were hybridisation in 25% (v/v) formamide, 5X SSC, 0.1% (v/v) SDS, 10X Denhardt's solution, 100 μg mL^{-1} salmon sperm DNA at 42°C for 16 h followed by washing in 2X SSC, 0.1% (v/v) SDS at 65°C three times, for 1 h each.

Sequencing and sequence analysis

Sequencing was performed on an ABI 377 sequencer (Perkin Elmer) using a dye terminator technology following protocols recommended by the manufacturer. Sequences were analysed using the GCG package within ANGIS (Australian National Genomic Information System).

Northern- blot analysis

RNA from endosperm at 15 days after flowering was obtained from wheat grown in the green house as described in Li et al. (1999). RNA extraction was done following the method of Higgins et al. (1976). After separation on denaturing formamide gels RNA was blotted onto Hybond N+ paper (Amersham, Buckinghamshire, UK), essentially as described in Maniatis et al. (1982) and hybridised to probes prepared from the extreme 3′ end of wheat SBE IIa cDNA and wSBEIIb-cDNA. The experiment was conducted twice using two separate RNA blots (experiment a and b, see Fig. 3) with different amounts (10 and 5 µg respectively) of RNA loading. The probes used for hybridisation were reversed each time, that is, blot 1 was hybridised with SBE IIa probe at first and following stripping off the signal, the blot was probed with SBE IIb probe. The blot 2 on the other hand, was hybridised with SBE IIb probe at first followed by SBE IIa probe. In both cases uniform amount of ^{32}p labelled probes of SBE IIa and SBE IIb, estimated using a scintillation counter were used for hybridisation.

RT-PCR

Reverse transcription reaction was conducted on total RNA from wheat endosperm at 15 days after flowering using SuperScript Choice System for cDNA synthesis (Invitrogen) according to manufacturer's protocol to determine the relative level of transcripts of SBE IIa and SBE IIb. Primers used to amplify SBE IIa transcripts were AR2a1/2EF (5′-GAAGGACTCCTCTCGCGCC-3′) and AR2a3Ra (5′-GGACAACTCGCGGTTTC-TCCC-3′). Primers used to amplify SBE IIb transcripts were AR2bE1/2F (5′-GTTCACCCCGTGCCGTCG-3′) and AR2b3R (5′-TCTTCACCTCCATCCTGTAGGC-3′). Wheat ubiquitin primers ARBTF1 (5′-CA-ACGTGAAGGCGAAGATCC-3′) and ARBT22 (5′-TAGTTCACCAAAGCTG CTCC-3′) were used as internal control.

Preparation of SBE IIa and IIb antibodies

SBE IIa antibody (anti-wBEIIa) was produced as reported previously (Morell et al. 1997). To generate antiserum against wheat SBE IIb (anti-wBEIIb), a N-terminal synthetic peptide was synthesised (Australian National University Biomolecular Resources Facility, Canberra, Australia) with an additional glycine-cysteine at the carboxamide terminus for coupling purposes. It was coupled to ovalbumin (Sigma) using the heterobifunctional cross-linker m-maleimidobenzoyl-*N*- hydroxysuccinimide ester (MBS, Pierce). Peptide (5 mg) was dissolved in 10 mM phosphate buffer pH 7 and mixed with MBS-activated ovalbumin (5 mg) for 3 h at room temperature. Coupled peptides were dialysed exhaustively against phosphate buffered saline (PBS, pH 7) and stored at 4°C with 0.1% (w/v) sodium azide as preservative.

New Zealand white rabbits were immunised using 1 mg coupled peptide (in 0.5 ml PBS plus an equal volume of Freund's Complete Adjuvant). Booster immunisations (0.5 mg coupled protein in 0.5 ml PBS plus 0.5 ml Incomplete Freund's Adjuvant) were administered at monthly intervals. Blood was collected from the ear vein for serological testing 7 days after the second booster immunisation.

Production of SBEIIa and SBEIIb fusion proteins

The DNA fragments encoding the SBE IIa and SBE IIb N-termini (157 and 166 bp respectively) were amplified from the cDNA encoding SBE IIa (Nair et al. 1997) and SBEIIb (this study) by PCR using primer combinations BEIIaN5′ (5′ GGATCCGCGCGGCCTCTCCAGG-GAA3′) and BEIIaN3′ (5′GAATTCGAAGA TTCA-AGTTTTTCTGCAGTCC 3′) for SBE IIa or BEIIbN5′ (5′GGATCCGCGCGGGGGG GCCGTCC 3′) and BEIIbN3′ (5′ GAATTCGAAGACCAAATACTATC-TTCACCTC 3′) for SBE IIb. The amplification was performed using a FTS-1 thermal sequencer (Corbett, Australia) for 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 2 min and 1 cycle of 25°C for 1 min. The PCR products were first cloned into pGEM-T vector (Promega) and then cloned into a glutathione *S*-transferase fusion vector (pGEX-3X) according to manufacturer's protocol (Pharmacia-Biotech). The expressed proteins in *E. coli* cells were purified using Glutathione Sepharose 4B affinity chromatography (SIGMA).

Protein extraction, gel electrophoresis and immunoblotting

SDS-PAGE, non-denaturing PAGE and immunoblotting were carried out as described previously (Morell et al. 1997). Soluble proteins were extracted by homogenising the endosperms in 50 mM phosphate buffer containing 5 mM EDTA, 5 mM DTT, 1 mM pefabloc and 20% glycerol. After centrifugation at 14,000 g for 10 min, protein concentration in the extracts was estimated, using Coomassie Plus Protein Assay Reagent from Pierce. Starch granule associated proteins were extracted according to Rahman et al. (1995) using 250 µl of extraction buffer per 10 mg of starch.

SDS-PAGE, non-denaturing PAGE and immunoblotting were carried out as described previously (Morell et al. 1997). For quantification of the level of expression of the proteins, 10% SDS-PAGEpolyacrylamide gels were used. For analysis of soluble proteins,

16 μg of endosperm soluble proteins were loaded per lane. Starch granule associated proteins were extracted according to Rahman et al. (1995) using 250 μl of extraction buffer per 10 mg of starch and for the analysis of starch granule bound proteins, 16 μl of the extract was loaded per lane.

The immunoblots were probed with anti-wBEIIa and anti-wBEIIb antibodies and immunoreactive bands were revealed using an Amersham ECL detection system according to the manufacturer's instructions. The immunoblots were scanned and the band intensity of the images measured using the Phoretix software package (NonLinear Dynamics Ltd, Newcastle, UK).

PCR amplification of chromosome engineered lines

DNA was isolated from leaves of CS and its chromosome engineered lines using Qiagen small scale DNA extraction kit. The primers used to locate SBE IIb gene are ARA 12F (5′ CCGTCCTACATGACACCTGGCCG 3′), ARA 10R (5′ CCGCCGGA TCGAGGAGCCGACGG 3′), AR2b19cF (5′ CTATGCCAATTGAACAACA ATGC3′) and AR2b23cR (5′ CGTGTTCATCAATGTCTGAACG 3′). PCR amplification was conducted in a 20 μl reaction containing 2.5 units Hotstar Taq, 1X buffer supplied with the enzyme containing 1.5 mM $MgCl_2$, 0.125 mM each deoxynucleotide triphosphate (dNTPs), 1 μM each of the forward and reverse primers and 100 ng DNA. The PCR programme included an initial denaturation step of 95°C for 5 min, followed by 36 cycles of 95°C for 30 s, 57°C for 1 min and 72°C for 2 min, and a final cycle of 72°C for 5 min. PCR product of AR2b19cF/AR2b23cR (20 μl) was further digested with the restriction enzyme *Rsa*1 (5 units) at 37°C overnight in 1X NE buffer 1 (New England Biolabs Inc). The digested PCR products were then separated on a 2% agarose gel and visualized after staining with ethidium bromide.

Fluorescence in situ hybridisation (FISH)

Fluorescence in situ hybridisation with the genomic clone G1 was performed on chromosome squashes from CS essentially as described in Turner et al. (1999). The plasmid clone pAs1 was used for the identification of the D genome chromosomes of wheat. G1 and pAs1 were labelled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively, by Nick Translation Mix kit (Roche Diagnostics). The digoxigenin-labelled and biotin labelled probes were detected with rhodamine conjugated sheep anti-digoxigenin Fab fragment and fluorescein conjugated streptavidin (Roche Diagnostics), respectively. The chromosomes were counterstained by DAPI.

Results

Isolation of wheat SBE IIb cDNA

A probe specific for the 5′ end of the SBE IIb cDNA from barley was generated as described by Sun et al. (1998), using primers that amplified the extreme 5′ end of the mRNA for barley SBE IIb. A cDNA clone, designated wSBEIIb-cDNA, was isolated using this probe from a library constructed from wheat cv CS developing endosperm (Li et al. 1999). Nucleotide sequence comparison with SBE II cDNAs from other cereals revealed that this cDNA encoded a SBE IIb type enzyme. The open reading frame (ORF) of wSBE IIb-cDNA was 2,511 bp which is 50 bp longer than the wheat SBE IIa cDNA (Rahman et al. 2001) due to an extension at the 5′ end. The ORFs of cDNAs encoding SBE IIa and SBE IIb show an overall nucleotide identity of 78% which increases to ~83% when the first 450 bp sequence from the 5′ region is not included. The 5′ region of SBE IIa and SBE IIb cDNAs are significantly divergent with an identity of only 49% (from positions 1 bp to 500 bp of wSBEIIb-cDNA and 1–433 bp of wheat SBE IIa cDNA). Although the cloned wSBEIIb-cDNA does not include the polyA tail, sequences identical to consensus polyadenylation signal, AATAA, are found 85 bp downstream from the stop codon. Sequence comparison of the deduced N-terminal amino acids of SBE IIb and SBE IIa pre-proteins from different species are shown in Fig. 1. Wheat SBE IIa has an overall identity of 87% to maize SBE IIa and 79% to maize SBE IIb which reduces to 62 and 46%, respectively, when approximately the first 200 amino acids are compared (Table 1). Wheat SBE IIb has an identity of 82% to maize SBE IIb and 79% to maize SBE IIa. This identity also reduces to 53 and 44% when the first 200 amino acids are compared. The phylogenetic relationship between various SBE IIs are shown in Fig. 2.

Expression analysis at the mRNA level

Probes from the 3′ untranslated region of wheat SBE IIa cDNA and wSBE IIb-cDNA were hybridised to RNA from wheat endosperm at 15 days post anthesis (DPA) in two separate biological experiments. The genes share low identity in this region (~35%) and the probes did not cross-hybridise (data not shown). An RNA species of ~2.9 kb was hybridised to each of the probes (experiment 1, Fig. 3a and experiment 2, Fig. 3b). The intensity of hybridisation was determined by densitometry and compared in both experiments, using uniformly labelled probes in each of the experiments. The results indicated that RNA hybridising to the probe from wSBE IIb-cDNA was present at 4 to 4.5-fold lower concentration than RNA hybridising to the probe from wheat SBE IIa cDNA. No hybridising signal was detected in the case of leaf using either SBE IIa or SBE

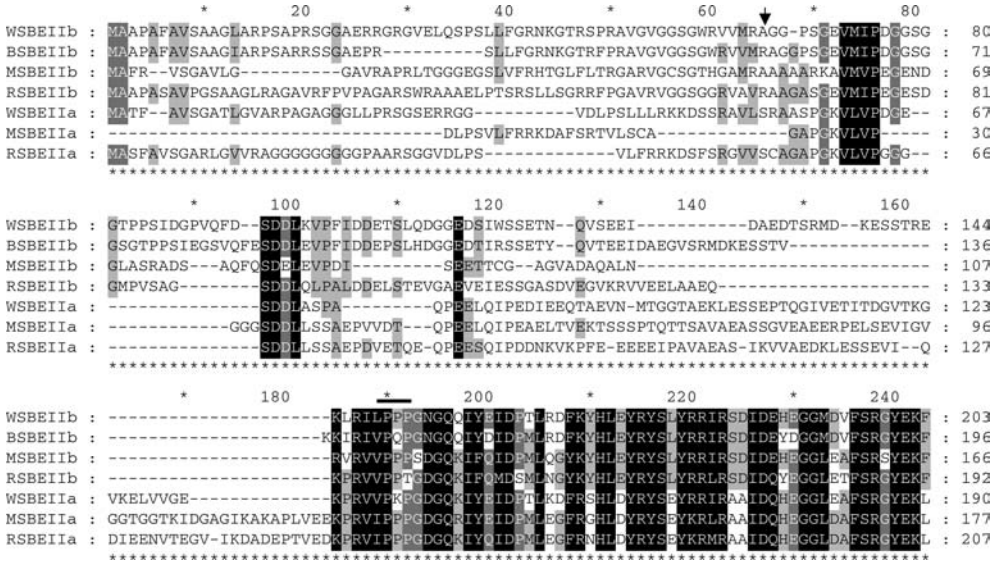

Fig. 1 Comparison of deduced amino acid sequences of the N-terminal third of SBE II proteins. Sources of SBE II sequences and Genbank accession numbers are as follows: WSBEIIb—wheat SBE IIb (this paper), BSBEIIb—barley SBE IIb (AAC69754); MSBEIIb—maize SBE IIb (AAC33764); RSBEIIb—rice SBE 3 (BAA03738) and WSBEIIa - wheat SBE IIa (CAA72154); MSBEIIa – maize SBE IIa (AAB67316); RSBEIIa – rice SBE 4 (E14723). Conserved residues are shaded, the intensity ranging from black for identical residues to white depending on the level of similarity. The proline-rich motif in wSBE IIb is indicated by a *bar* over the sequence. The predicted cleavage site for wSBE IIb is indicated by an *arrow*

IIb probe (data not shown). Further evidence for the greater abundance of SBE IIa RNA compared to SBE IIb RNA in wheat endosperm was provided by semi-quantitative RT-PCR after 20, 25 and 35 cycles of amplification. This showed that SBE IIa RNA was present at approximately two times the level of SBE IIb RNA. Further experiments to more precisely compare the levels of SBE IIa and SBE IIb RNA are in progress.

Expression of SBEII in the endosperm soluble fraction and starch granules

Antiserum against wheat SBE IIb (anti-wBEIIb) was generated using the N-terminal synthetic peptide, AG-GPSGEVMI. This peptide represents the N-terminal sequence of the putative mature peptide and is also identical to the N-terminus of the barley SBEIIb protein (Sun et al. 1998). In order to confirm that anti-wBEIIb did not cross react with wheat SBE IIa (Morell at al. 1997; Rahman et al. 2001) and vice versa, Glutathione *S*-transferase (GST) fusion proteins of both SBE IIa and SBE IIb were produced and probed with both the antibodies. The results shown in Fig. 4 clearly demonstrate that anti-wBEIIb did not cross react with wheat SBE IIa and similarly anti-wBEIIa did not cross react with wheat SBE IIb. The anti-sera, anti-wBEIIa and anti-wBEIIb, were then used to investigate the levels of expression of SBE IIa and SBE IIb in the endosperm during development in the wheat cv CS (Fig. 5). Both SBE IIa and SBE IIb are expressed from an early stage of endosperm development. An estimate of the relative amounts of SBEIIa and IIb was made for the soluble and the granule-bound endosperm fractions using SDS-PAGE. Known amounts of GST fusion proteins with SBE IIa or SBE IIb sequences were run on these gels as standards and the amounts of SBE IIa or SBE IIb were calculated as described in Figure 5. These experiments suggest that at all stages of development SBE IIa is the major SBE II isoform in protein amount in the endosperm soluble fraction, on an average contributing to ~82% of the total protein, while SBE IIb is the major isoform in the granules, comprising of approximately ~72% of the granule bound SBE II protein. Our studies have shown that there is ~10 times more of SBE II in terms of protein amount in the amyloplast stroma compared to the granule (data not shown).

Structure of wheat SBE IIb gene

Three λ phage clones, G1, G2 (Rahman et al. 2001) and G5 were isolated from a library prepared from *A. tauschii*, the donor of the D genome to wheat. These were

Table 1 Amino acid sequence identity of wheat and maize SBE II isoforms

		Maize [a]		Maize [b]	
		SBE IIa(%)	SBE IIb(%)	SBE IIa(%)	SBE IIb(%)
Wheat	SBE IIa	87	79	62	46
	SBE IIb	79	82	44	53

[a] overall protein sequence
[b] approximately first 200 amino acids are compared

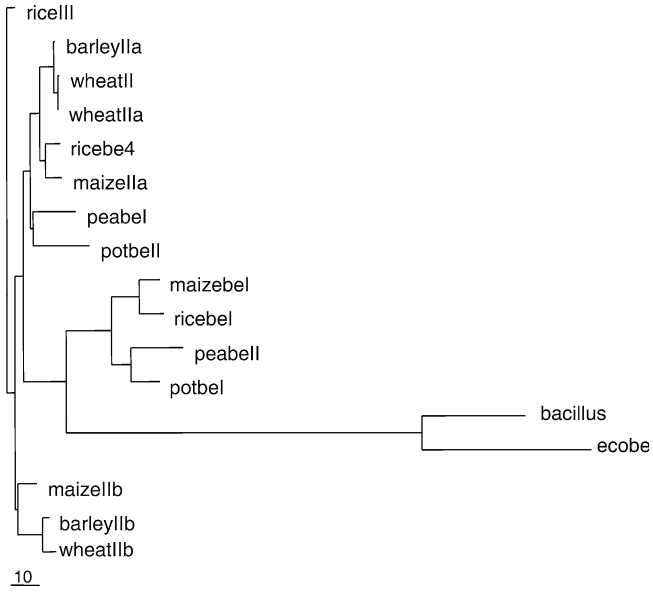

Fig. 2 Dendogram of the relationship between the various deduced amino acid sequences of branching enzymes generated by the PILEUP program. The sequences are wheat II (wheat SBE IIa, Genbank accession no. Y11282, Nair et al. 1997), wheat IIa (Genbank accession no.CAA72514, Rahman et al. 2001), barley IIa and IIb (Sun et al. 1998), maize IIa (Gao et al. 1997), maize IIb (Fisher et al. 1993), maize BE I (Baba et al. 1991, 1993), rice III (rice SBE IIb, Mizuno et al. 1993), rice be 4 (rice, SBE IIa, Genbank accession no. E14723), pot be I (Potato SBE I, Khoshnoodi et al. 1996), pot be II (potato SBE II, Cangiano et al. 1993), pea be I and be II (Burton et al.1995), *E.coli* be (Baecker et al. 1986), and *Bacillus* (Kiel et al. 1992). Note that pea SBEI and pea SBEII sequences correspond to maize SBEII and SBEI, respectively, through differences in nomenclature conventions

shown by sequencing to contain identical SBE IIb type genes designated as *wSBE II-DB1*. The sequence obtained from the G1 clone completely matched wSBEIIb-cDNA, indicating that *wSBE II-DB1* is the structural gene encoding SBE IIb from the D genome of wheat. Partial sequence of *wSBE II-DB1* was obtained by sequencing the clone G5 which contained the longest insert based on restriction mapping (data not shown). Further sequence information was obtained by direct PCR amplification from *A. tauschii* DNA using primers based on wSBE IIb-cDNA.

The *wSBE IIb-DB1* gene contained 22 exons spanning a region of more than 16 kb. The entire gene was sequenced except for parts of exons 21 and 22, and introns 16 and 20. The size of the exons sequenced ranged from 43 bp to 230 bp. The first two exons were high in GC content (65–75%) compared to the others (< 53%). These two exons were also characterised by higher frequency of CpG dinucleotide (12–15 per 100 bp). The size of introns sequenced varied from 74 bp to 2491 bp. Intron 16 (sequencing yet to be completed) appears to be the largest containing over 3,800 bp.

A putative TATA box was located 150 bp upstream of a putative translation initiation site. Sequences characteristic of enhancer element, GC box, were also detected in the promoter region. Several motifs commonly associated with genes expressed in endosperm are also present in this region. The GCN 4 box, known to regulate expression according to nitrogen availability, was observed at a position 411 bp upstream of the translation initiation site. Some of the introns (introns 2, 14 and 16) were found to contain sequences similar to the prolamin box, conserved in cereal seed protein gene promoters. Gibberellin regulated Myb motifs were also located both in the promoter region and within several introns.

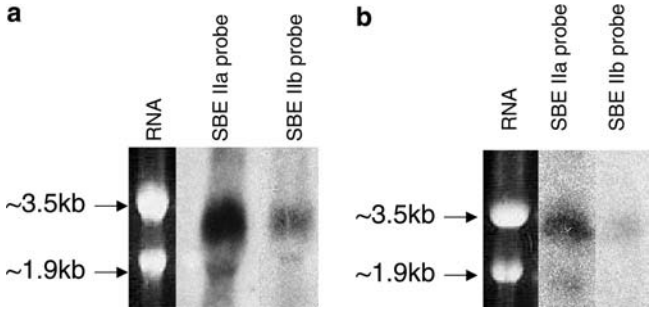

Fig. 3 Northern blot hybridisation of RNA from developing endosperm (15 DPA) using probes from the 3′ untranslated region of wheat SBE IIa cDNA (left lane) and wSBEIIb-cDNA (right lane). The results from two different hybridisation experiments with different biological replicates are shown in panel a and b (see Materials and Methods). Lane one in both panel a and b represents RNA loading (10 µg and 5 µg respectively) on agarose gel. The second and third lanes in each panel represent the same lane on the agarose gel blot after hybrisdisation with specific probes for either SBE IIa or SBE IIb (see Materials and methods for further details)

Localisation of SBE IIb gene in wheat

The D genome-homeoallele of SBE IIb was specifically amplified from CS when the primers ARA 12F and ARA 10R that targeted the promoter region of *wSBE II-DB1* were used. Analysis of nullisomic-tetrasomic lines of CS using this pair of primers resulted in the localisation of the gene on to chromosome 2D (Fig. 6a). Localisation of the gene to the long arm of homoeologous group 2 chromosomes was obtained by PCR amplification of the intron 3 region of *wSBE II-DB1* from deletion lines of CS using the primers AR2b19cF and AR2b23cR followed by digestion using the restriction enzyme *Rsa*1 (Fig. 6b). This was made possible by exploiting the sequence polymorphism between the alleles from the different genomes. Southern blot hybridisation using a probe from the intron 3 region of *wSBE II-DB1* gene also confirmed this localisation (data not shown).

In situ hybridisation

In situ hybridisation was conducted on CS chromosome squashes using G1, the genomic clone containing part of

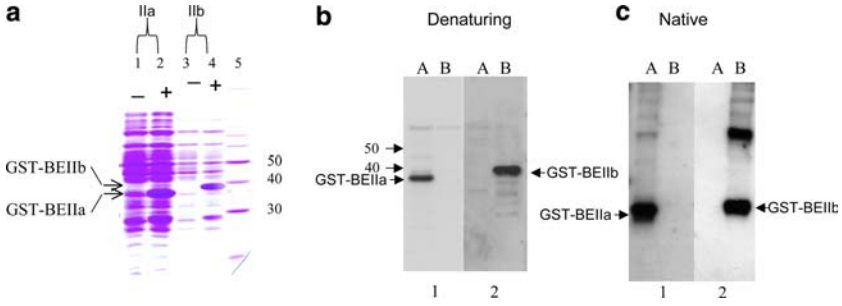

Fig. 4 GST-SBEII fusion proteins. **a** SDS-PAGE of proteins extracted from *E. coli* cells containing the fusion vector for SBE IIa (lanes 1 and 2) and SBE IIb (lanes 3 and 4). Lanes 1 and 3 are un-induced cells. Lane 5 contains the molecular weight marker. **b** and **c** Immunoblots of fusion proteins under denaturing and native conditions, respectively. Lane A contains GST-SBE IIa and B contains GST-SBE IIb fusion protein crude extract. Panel 1 was probed with anti-wBEIIa and panel 2 was probed with anti-wBEIIb

wSBE II-DB1. The D genome chromosomes of CS were identified by double labelling with pAs1, a repetitive sequence used for chromosome identification (Mukai et al. 1990). The result revealed that SBE IIb gene in wheat was located in the interstitial region of the long arm of chromosome 2 in agreement with the results of PCR and Southern blotting (Fig. 7).

Discussion

Plants contain two broad types of starch branching enzymes and in maize they are commonly referred to as SBE I and SBE II. Characterisation of the SBE II type of branching enzyme from cereals has revealed that this is composed of two related isoforms, a and b. In this paper we report the characterisation of the gene encoding SBE IIb from wheat and show that it is a minor isoform in protein amount in the soluble phase of developing endosperm, in contrast to maize, but appears to be the major isoform bound to the starch granule in terms of the amount of protein. This was achieved through the use of specific antisera which were raised against the peptide sequence at the amino terminal region of SBE IIa and SBE IIb as deduced from the cognate cDNA sequences. Our results suggest that these antibodies detect the respective products from the A, B and D genomes (data not shown) and there is no evidence of any preferential binding. Earlier efforts to separate the SBE IIa and SBE IIb proteins from the soluble phase of the endosperm on the basis of activity were unsuccessful. Only one N-terminus sequence was obtained and this corresponded to the SBE IIa isoform (Morell et al. 1997). It is possible that no N-terminus sequence for SBE IIb was obtained since this isoform is only a minor form in the soluble phase in terms of the amount of protein. The specific activity of SBEIIa and SBE IIb were not quantified and it does not necessarily follow that the activity of SBE IIa is the predominant activity in the endosperm. The situation in wheat therefore is in sharp contrast to maize where the amount of SBE IIb is about 50 times greater than that of SBE IIa and high amylose is obtained by disruption of the SBE IIb gene but not of the SBE IIa gene.

The cDNA for SBE IIb encodes a polypeptide of 831 amino acid residues which would be expected to have a molecular mass of 84 kDa after N-terminal processing. However, we have not yet been able to determine the N-terminus of the processed SBE IIb isoform by direct sequencing. Since SBEs are coded by nuclear genes and located in amyloplasts and chloroplasts an appropriate transit peptide is expected as part of the protein. The SIGCLEAVE application of the GCG package identified a cleavage site for the transit peptide between Ala-23

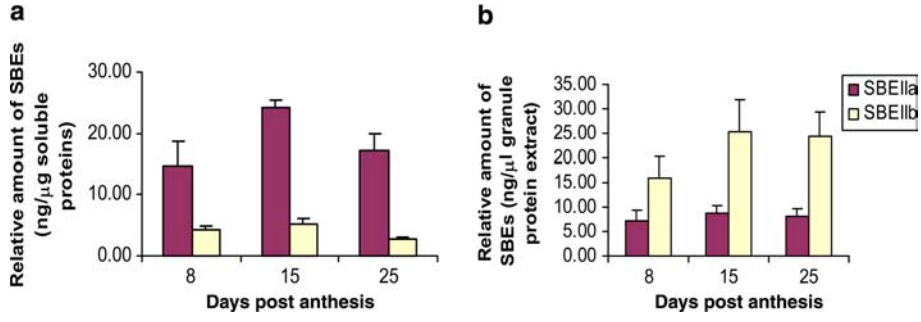

Fig. 5 The level of expression of SBE II isoforms in the **a** soluble proteins and **b** starch granule-bound proteins. Protein samples were run on SDS gels together with purified GST-BEIIa (30–320 ng) and GST-BEIIb (19–200 ng) fusion proteins as standards. Immunoblots of SDS-PAGE were scanned and the band intensities of the images were measured. The amounts of soluble and granule-bound SBE IIa and SBE IIb were estimated from the standard curve generated from the purified fusion proteins. The graphs show the result of three independent experiments for soluble and granule-bound proteins

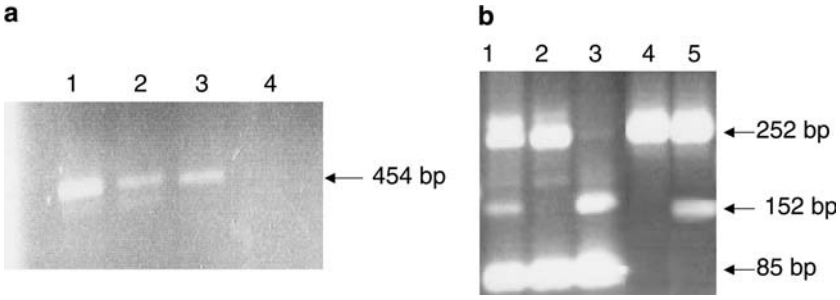

Fig. 6 **a** PCR amplification of the promoter region of *wSBE II-DB1* gene using the primers ARA 12F and ARA 10R from different wheat lines. Lanes (1) CS, (2) N2AT2D, (3) N2BT2D and (4) N2DT2A. The primers amplified a 454 bp product from all the lines except NTDT2A. **b** PCR amplification of intron 3 region of *wSBE II-DB1* gene using the primers AR2b19cF and AR2b23cR. Following digestion of the PCR product with the restriction enzyme *Rsa*1, the bands from the three different genomes in wheat were separated into the A genome (85 bp), B genome (152 bp) and the D genome (252 bp) bands. Lanes (1) CS, (2) CSDT2BL-9, a CS deletion line from which a considerable portion of the long arm of chromosome 2B is deleted, (3) *T. durum* (tetraploid, AB genomes), (4) *Ae.tauschii* (diploid, D genome) and (5) CSDT2AS, a CS deletion line which is missing the whole long arm of chromosome 2A

and Arg-24. However this programme, based on Heijne (1986), provides an accuracy of only 75–80% in identifying the site of cleavage. Comparison with the N-terminal amino acids of mature barley SBE IIb (Sun et al. 1998), maize SBE IIb (Fisher et al. 1993) and rice SBE 3 (Mizuno et al. 1993) revealed the more probable cleavage site to be between Arg-73 and Ala-74. A proline-rich motif characteristic of the C-terminal extremity of the N-terminal arm is detected from which the possible length of the N-terminal arm of wheat SBE IIb is expected to be 88 amino acid residues. The N-terminal arm is a characteristic feature of SBE II isoforms that distinguishes them from SBE I group (Sun et al. 1998). Sequence alignment of deduced N-terminal amino acids of SBE IIb pre-protein revealed the highest degree of identity with that of barley SBE IIb (87%) followed by rice RBE 3 (57%) and maize SBE IIb (44%) (Fig. 1). The corresponding regions from wheat SBE IIa had an overall identity 64%.

The tissue distribution of SBE IIa and SBE IIb in wheat is clearly different from that in maize. Northern blot analysis indicated that the RNAs encoding SBE IIa and SBE IIb are both expressed in wheat endosperm. In maize, SBE IIa showed high expression levels in the vegetative tissues and moderate expression in developing endosperm, but SBE IIb expression is confined to very high levels in developing endosperm (Gao et al. 1997). Similar to maize, SBE IIb is restricted to endosperm in barley while SBE IIa is expressed in both vegetative and reproductive tissues (Sun et al. 1998). The spatial distribution of the different isoforms of SBE II, at least in maize, appears to be indicative of the target tissue in which each isoform acts. Analysis of maize mutants revealed that a mutation in the SBE IIb gene yielded a high amylose phenotype in endosperm starch (Hedman and Boyer 1982), whereas mutation in SBE IIa altered the phenotype of only the leaf starch (Blauth et al. 2001). It would be interesting to compare the effects of muta-

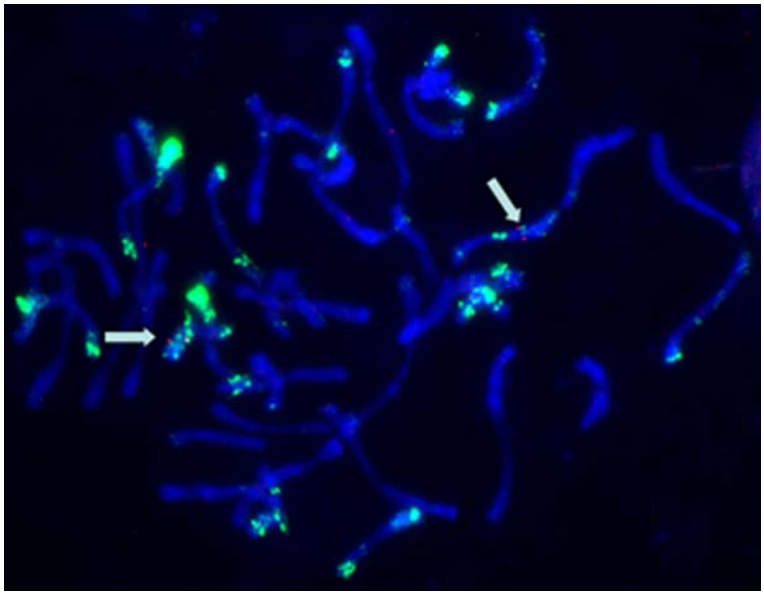

Fig. 7 FISH of a *wSBEII-DB1* probe to CS chromosomes. An entire genomic lambda clone (*G1*) encoding *wSBE II-DB1* (approximately 18 kb) and the repetitive sequence pAs1 were used as probes. The D genome chromosomes were identified based on green fluorescence signal from the hybridisation of pAs1 probe. The SBE IIb signals (red fluorescence) are indicated with *arrows*. *Bar* represents 10 μm

tions in SBE IIa and SBE IIb in wheat and maize; however, no such mutants have been described so far from wheat. Another point of difference with maize appears to be in the accumulation of each isoform in the amyloplast of wheat. Our study shows that the SBE IIb isoform is the predominant polypeptide in the starch granule and the SBE IIa isoform is the predominant form in the soluble phase in wheat endosperm. In contrast, in maize the SBE IIb isoform is the predominant form in the soluble phase. However, the temporal distribution of SBE I and SBE IIs in the endosperm remain similar to maize. Expression analysis of both SBE IIa and SBE IIb in wheat by immunoblotting revealed that both these enzymes are expressed earlier in wheat endosperm development (Fig. 5) than is reported for SBE I (Morell et al. 1997).

The gene structure of *wSBE II-DB1* is very similar to that of *wSBE II-DA1* in that there are 22 exons. The sizes of the exons of these two genes are identical to each other except for the first three exons. The sizes of exon 21 and 22 of *wSBE II-DB1* are still not determined. The sizes of exons 4 to 21 are also conserved in the maize gene for SBE IIb (Kim et al. 1998) and Arabidopsis SBE II 2.2 gene (GenBank accession no. AL162506). It appears that the presence of large introns of >2 kb is a characteristic of SBE IIb genes as evidenced in maize (Kim et al. 1998), wheat (this study) and barley (Sun et al. 1998).

Analysis of the promoter region revealed the presence of motifs associated with genes expressed in the endosperm in both *wSBE II-DA1* and *wSBE II-DB1*. The endosperm specific expression of *wSBE II-DA1* has been confirmed through transformation studies in rice using the SBE IIa promoter (Rahman et al. 2001). While the importance of the endosperm box element and GCN4 like motifs in endosperm specific expression has been demonstrated in several cereal storage protein genes (Muller and Knudson 1993; Norre et al. 2002), none of the genes for starch biosynthetic enzymes previously reported (including the gene for SBE IIa) contains the GCN4 motif (Li et al. 1999; Rahman et al. 1999, 2001). However, the presence of motifs like GCN4 and GAmyb in the promoter of the gene for SBE IIb reported in this paper may be indicative of its nutritional and hormonal regulation apart from tissue specific and developmental regulation. In barley the presence of a sequence similar to the B-box motif of the patatin promoter in the intron 2 of the SBE IIb gene was demonstrated to confer endosperm specific expression (Ahlandsberg et al. 2002). This intronic element is absent in the recently reported SBE IIb sequence from sorghum (Mutisya et al. 2003). Further investigation is needed to find out whether the prolamin box like sequences observed in some of the introns of *wSBE II-DB1* have a role in gene regulation.

Our study localised the SBE IIb gene in wheat to the long arm of chromosome 2. The SBE IIa gene in wheat has also been localised to the same chromosomal arm (Rahman et al. 2001). In rice and barley the situation appears to be different. Rice SBE IIa (RBE 4) is located to chromosome 4 (syntenic to wheat chromosome 2, Sorells et al. 2003) and SBE IIb (RBE 3) is located to chromosome 2 (TIGR database, http://www.tigr.org/), syntenic to wheat chromosome 6 (Sorells et al. 2003). In barley chromosomes 5 (1H) and 2 (2H) contain the SBE IIb and SBE IIa loci, respectively (Sun et al. 1998). In maize the location of the SBE IIb gene is reported to be on chromosome 5 (Neuffer et al. 1997), the region is syntenic to rice chromosome 2 (http://www.tigr.org/). SBE IIa in maize is located to long arm of chromosome 8 which is syntenic to rice chromosome 1 (http://www.maizegdb.org/). In general, SBE IIb gene in wheat is present in a non-syntenic genomic location. The localisation of the SBEIIa and SBEIIb genes in wheat is unusual and there is insufficient data available to suggest whether the co-location of the genes in wheat pre-dates, or post-dates, the situation in other species. A complex locus for SBE I genes has already been reported from wheat (Suzuki et al. 2003) but this reflects local duplication and re-arrangement effects rather than translocation. It is tempting to speculate that the differences in gene expression observed between species are a consequence of the different locations in the genomes occupied by the SBEIIa and SBEIIb genes in wheat and maize, respectively. It would be of interest to define in future experiments whether the differences were due to local order effects such as promoter sequences, or were a consequence of higher order effects such as histone organisation or the presence (or absence) of adjacent genes.

Starch biosynthesis requires the complex interplay of a number of enzymes and the reduction of one enzyme has been observed to lead to the reduction or increase of other enzymes in many cases. For example, in the SGP-1 mutation in wheat, loss of the SS II polypeptide (which is found in both the granule and the soluble phase) led to decreases in the amount of other granule bound proteins (GBSS, SBE II and SS I) (Kosar-Hashemi, personal communication; Yamamori et al. 2000) in the granule and an altered amylose content. A similar observation is made for the *Sex-6* mutation in barley (Morell et al. 2003) but in this case the elevation of amylose was much higher. In contrast, in lines lacking SBE I (which is entirely in the soluble phase) from maize, rice and wheat, no change in amylose content could be detected and alterations in other starch biosynthetic enzymes were not reported (Blauth et al. 2002; Satoh et al. 2003; Regina et al. 2004). No wheat lines lacking SBE IIb or SBE IIa have been reported to date so the effect of such a lack on other starch biosynthetic enzymes cannot be investigated. However, Tetlow et al. (2004) have recently reported the formation of complexes of phosphorylated starch biosynthetic enzymes within the wheat amyloplast. Both SBEIIa and SBEIIb were found to be targets for phosphorylation, and there was evidence that each isoform, when phosphorylated, had higher activity than when unphosphorylated. Both isoforms were present in complexes isolated by immunoprecipitation, however, while SBEI and SBEIIb were co-immunoprecipated,

there was no evidence to suggest that SBEIIa was present in the same complex. Additional analysis of phosphorylation of branching enzymes and complex formation is required in different species, and in wheat, would be of interest to study in the different mutant background. Furthermore, the type and nature of interaction between the different starch biosynthetic enzymes is likely to be influenced by the partitioning of the isoforms between the starch granules and amyloplast stroma as well as variation in expression among different species. This is a focus for future investigations.

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