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Wheat Granule-Bound Starch Synthase I and II Are Encoded by Separate Genes That Are Expressed in Different Tissues[1]

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Studies of waxy mutations in wheat and other cereals have shown that null mutations in genes encoding granule-bound starch synthase I (GBSSI) result in amylose-free starch in endosperm and pollen grains, whereas starch in other tissues may contain amylose. We have isolated a cDNA from waxy wheat that encodes GBSSII, which is thought to be responsible for the elongation of amylose chains in non-storage tissues. The deduced amino acid sequences of wheat *GBSSI* and *GBSSII* were almost 66% identical, while those of wheat *GBSSII* and potato *GBSSI* were 72% identical. *GBSSII* was expressed in leaf, culm, and pericarp tissue, but transcripts were not detected in endosperm tissue. In contrast, *GBSSI* expression was high in endosperm tissue. The expression of *GBSSII* mRNA in pericarp tissue was similar at the midpoints of the day and night periods. The *GBSSII* genes were mapped to chromosomes 2AL, 2B, and 2D, whereas *GBSSI* genes are located on group 7 chromosomes. Gel-blot analysis indicated that genes related to *GBSSII* also occur in barley, rice, and maize. The possible role of *GBSSII* in starch synthesis is discussed.

Starch is composed of two distinct polymers; amylopectin, which consists of long chains of (1–4)-linked α-D-glucopyranosyl units with extensive branching resulting from (1–6) linkages, and amylose, which is a relatively linear molecule of (1–4)-linked α-D-glucopyranosyl units (Whistler and Daniel, 1984). Both types of chains are elongated by starch synthases that transfer α-D-Glc from ADP-Glc to the growing chain, and specific starch synthases are active in the synthesis of each type of polymer. Whereas a number of starch synthases are thought to catalyze amylopectin synthesis (Dry et al., 1992; Baba et al., 1993; Edwards et al., 1995, 1996; Abel et al., 1996; Knight et al., 1998; Cao et al., 1999), granule-bound starch synthase I (GBSSI) is believed to be the sole starch synthase responsible for the formation of amylose (for review, see Smith et al., 1995).

Waxy or GBSSI mutants have been identified or produced in a number of species, including rice (Murata et al., 1965), maize (Weatherwax, 1922), wheat (Nakamura et al., 1995), barley (Ishikawa et al., 1994), potato (Hovenkamp-Hermelink et al., 1987), and pea (Denyer et al., 1995a). While studies of such mutants have clearly indicated that GBSSI is responsible for amylose synthesis in storage tissues, starch granules are also found in tissues such as pericarp, leaf, stem, and root. The starch granules of these tissues may show different biochemical and physical characteristics than those of storage starch. For example, wheat pericarp starch has a different amylose to amylopectin ratio than that of endosperm starch, and while endosperm starch consists of a bimodal population of large and small granules, pericarp starch granules are small and relatively uniform in size (Nakamura et al., 1998). Leaf starches from pea (Tomlinson et al., 1997) and potato (Hovenkamp-Hermelink et al., 1988) have lower amylose contents than those of embryo starches, and the distribution of branch lengths in pea leaf amylopectin differs from that of embryo amylopectin (Tomlinson et al., 1997).

In several cases, starch from non-storage tissues of GBSSI mutants was observed to stain blue-black with iodine, indicating the presence of amylose. In waxy maize (Hixon and Brimhall, 1968; Badenhuizen, 1969), starch from pollen, endosperm, and embryo sac lacked amylose, whereas starch in other tissues, including leaves and pericarp, stained blue-black, and in waxy rice (Igaue, 1964) amylose levels of leaf and stem tissues were comparable to levels in non-waxy types. This suggested that a second GBSSI isoform is responsible for the synthesis of amylose in non-storage starch in cereals.

In a waxy wheat line lacking functional *GBSSI* genes (Vrinten et al., 1999), pericarp starch granules contained amylose and showed significantly higher GBSS activity than did endosperm starch granules (Nakamura et al., 1998). A 59-kD protein, distinct from the 61-kD GBSSI or waxy protein, was found in pericarp starch granules but not in granules from endosperm. Since this isoform was largely limited to the granule-bound fraction and was involved in the synthesis of amylose, it was designated GBSSII (Nakamura et al., 1998). Novel GBSS isoforms have also recently been reported to be responsible for the production of amylose in the pods of pea (Denyer et al., 1997) and in the pericarp of *Triticum monococcum* (Fujita and Taira, 1998). However, it is not yet known whether these isoforms are encoded by genes separate from those encoding GBSSI.

We present the characterization of a *GBSSII* cDNA from wheat, and compare the sequence, expression pattern, and chromosomal location of *GBSSII* with that of *GBSSI*.

MATERIALS AND METHODS

Plant Material

The waxy wheat (*Triticum aestivum*) variety CD-1479 (Hoshino et al., 1996), which lacks all GBSSI proteins in

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endosperm starch, and the non-waxy wheat variety Chinese Spring were used in investigations of *GBSSII* expression. To determine the chromosomal location of the *GBSSII* genes, 19 nullisomic-tetrasomic and four ditelosomic lines of Chinese Spring wheat obtained from U.S. Deparment of Agriculture-Agricultural Research Service (Beltsville, MD) and the Kihara Institute of Biological Research (Yokohama, Japan) were used. Barley (cv Igri), maize (cv Honeydent 125Z), rice (cv Hitomebore), potato (cv May Queen), tomato (cv Natsunokoma), pea (cv Tsurunashi endou), soybean (cv Tachiyutaka), and taro (cv Dodare) plants were used to determine whether genes corresponding to wheat *GBSSII* were present in other species.

RNA Extraction

RNA was extracted using Triazol reagent (Life Technologies/Gibco-BRL, Cleveland) essentially according to the manufacturer's instructions. Tissue was frozen in liquid nitrogen and ground to a fine powder, then homogenized using a polytron homogenizer. Insoluble material was removed by centrifugation at 12,000*g* for 10 min, and the supernatant was extracted with chloroform and precipitated with isopropyl alcohol. For precipitation of RNA from seed and pericarp tissues a 1:1 mixture of isopropyl alcohol: high-salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) was used.

Isolation of a *GBSSII* cDNA

Poly(A^+) RNA was isolated from waxy wheat seed collected at 5 d post-anthesis (DPA), and cDNA synthesis and library construction was performed using a ZAP cDNA synthesis kit (Stratagene, La Jolla, CA). The library was plated at a density of approximately 500 pfu per 60-mm plate, and antiserum to potato GBSSI at a dilution of 1:2,000 was used for immunoscreening. Screening and detection were performed using the ProtoBlot II AP System (Promega, Madison, WI) according to the manufacturers' instructions. After a second round of screening at a density of approximately 60 pfu per 60-mm plate, five positive clones were identified and sequenced.

Isolation of a *GBSSI* cDNA

Poly(A^+) RNA extracted from 10-DPA seed of Chinese Spring wheat was used to construct adaptor-ligated cDNA using a Marathon kit (CLONETECH Laboratories, Palo Alto, CA). *GBSSI* cDNAs were amplified using the AP1 primer included in the kit, and a primer (5′-TTGCTGCAGGTAGCCACACCCTG-3′) designed using the sequences from the 5′ untranslated regions of the barley (Rohde et al., 1988) and wheat (Clark et al., 1991) *waxy* clones. The cDNA amplification products were cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

DNA Sequence Analysis

Sequencing was carried out with a DNA sequencer (model 373A, PE-Applied Biosystems, Foster City, CA). Inserts of clones were sequenced on both strands, and sequence analysis was performed using the Genetyx-Mac (Software Development, Tokyo) program.

SDS-PAGE and Protein Sequencing

Preparation of starch granules and separation of starch granule-bound proteins by low-bis acrylamide SDS-PAGE was performed as described by Nakamura et al. (1992). To avoid cross-contamination of Wx-B1 and Wx-D1 proteins, which run close together on SDS-PAGE gels, the Wx-A1 and Wx-D1 proteins were extracted from the Chinese Spring nullisomic-tetrasomic line N4AT4B, and the Wx-B1 protein from the N7DT7B line. After blotting, the proteins of interest were identified by staining with Coomassie Brilliant Blue R, and bands corresponding to the proteins of interest were excised and applied to a gas-phase protein sequencer (model 490A, Perkin-Elmer Applied Biosystems, Foster City, CA).

Northern Analysis

Total RNA samples were heat denatured, then separated by electrophoresis in 1% (w/v) agarose gels containing 2.2 M formaldehyde, and transferred to GeneScreen Plus membrane (NEN Research Products, Boston) by capillary transfer. The blots were prehybridized at 42°C in buffer containing 50% (v/v) formamide, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 0.2% (w/v) bovine serum albumin, 50 mM Tris, pH 7.5, 1.0 M NaCl, 0.1% sodium pyrophosphate, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 100 μg/mL denatured salmon sperm DNA, then hybridized for 1 d in the same buffer containing ^{32}P-labeled probe. The membranes were washed twice for 30 min in 2× SSC and 1% (w/v) SDS at 65°C, and once in 0.1× SSC at 65°C for approximately 10 min, or until background radioactivity had dropped to near zero.

DNA Isolation and Southern Analysis

DNA was extracted from young leaves of plants using the Nucleon PhytoPure system (Amersham-Pharmacia Biotech, Uppsala). Genomic DNA (15 μg per lane) was digested with the appropriate restriction enzyme and subjected to electrophoresis on 0.8% (w/v) agarose gels. DNA was transferred to GeneScreen Plus (NEN Research Products) membrane by capillary transfer. The blots were prehybridized at 42°C in 50% (v/v) formamide, 1% (w/v) SDS, 2× SSC, 10% (w/v) dextran sulfate, 0.5× Denhardt's solution, and 250 μg/mL denatured salmon sperm DNA, and hybridized in the same solution containing [^{32}P]dCTP-labeled probe. The membranes were washed twice for approximately 30 min at 65°C in 2× SSC and 1% (w/v) SDS, and once for 5 to 30 min at 65°C in 0.2× SSC, 0.1% (w/v) SDS.

RESULTS

Isolation and Characterization of a *GBSSII* cDNA Clone

Five clones that reacted with the potato anti-GBSSI antibody were isolated. Sequencing indicated that these clones

were identical except for variation in the lengths of their 5′ untranslated regions. The lack of variation among the clones was somewhat unexpected since common wheat is a hexaploid plant, and suggests either that the transcripts from the A, B, and D genome are identical, or that one gene has a particularly high expression level. The longest of these clones, which is referred to as wheat *GBSSII* (Fig. 1), has been deposited in GenBank under the accession no. AF109395. The *GBSSII* cDNA is 2,081 bp in length, including a 17-bp poly(A^+) tail. A 1,799-bp open reading frame begins with an ATG initiation codon at position 120 and ends with a TGA stop codon at position 1,917. The open reading frame is flanked by 5′ and 3′ untranslated regions of 119 and 148 bp, respectively. A putative polyadenylation signal (AATAA) was found at nucleotide positions 1,960 to 1,964.

To allow comparisons between wheat *GBSSI* and *GBSSII* sequences, a *GBSSI* cDNA was also isolated. The sequence of this clone is shown in Figure 1, and has been deposited in GenBank under the accession no. AF163319. We were able to determine that this cDNA originated from the D genome by comparing the deduced amino acid sequence (Fig. 2) with the N-terminal amino acid sequences of waxy proteins originating from the A, B, and D genomes (Fig. 3). A wheat GBSSI cDNA, pcSS22, which appears to have originated from the A genome, was previously isolated by Clark et al. (1991). However, pcSS22 included a 33-bp insertion, which we could not detect in *GBSSI* cDNAs originating from the A, B, or D genomes (P.L. Vrinten and T. Nakamura, unpublished data). *GBSSI* and *GBSSII* had an overall identity of 56.9% at the nucleotide level, with the 5′ and 3′ non-translated regions and the areas encoding the transit peptides showing the least similarity (Fig. 1).

The deduced amino acid sequence of GBSSII, shown in Figure 2, predicts a 599-amino acid polypeptide. The presence of an N-terminal transit peptide including amino acids 1 to 78 was predicted using the Genetyx-Mac program. The amino acids following the predicted transit peptide correspond well to the N-terminal amino acid sequence of the mature GBSSII protein (Nakamura et al., 1998; Fig. 3), but did not match with N-terminal peptides of GBSSI proteins originating from the A, B, or D genomes (Fig. 3). The deduced mature protein has a calculated molecular mass of 58.4 kD, which is reasonably close to the apparent molecular mass (59 kD) of pericarp GBSSII as estimated on SDS-PAGE gels (Nakamura et al., 1998). Eight sequence blocks, which are conserved among 28 plant starch synthases and procaryotic glycogen synthase (Cao et al., 1999), were also present in wheat GBSSII (Fig. 2). Thirty-two of the 33 residues Cao et al. (1999) identified as invariant were found in GBSSII, and the single exception at position 466 involves a conservative substitution within one of the functional groups defined by Dayhoff and Orcutt (1979). The KXGG consensus sequence, which is believed to be the ADP-Glc binding site (Furukawa et al., 1990, 1993), is found at amino acids 86 to 89 of the deduced GBSSII

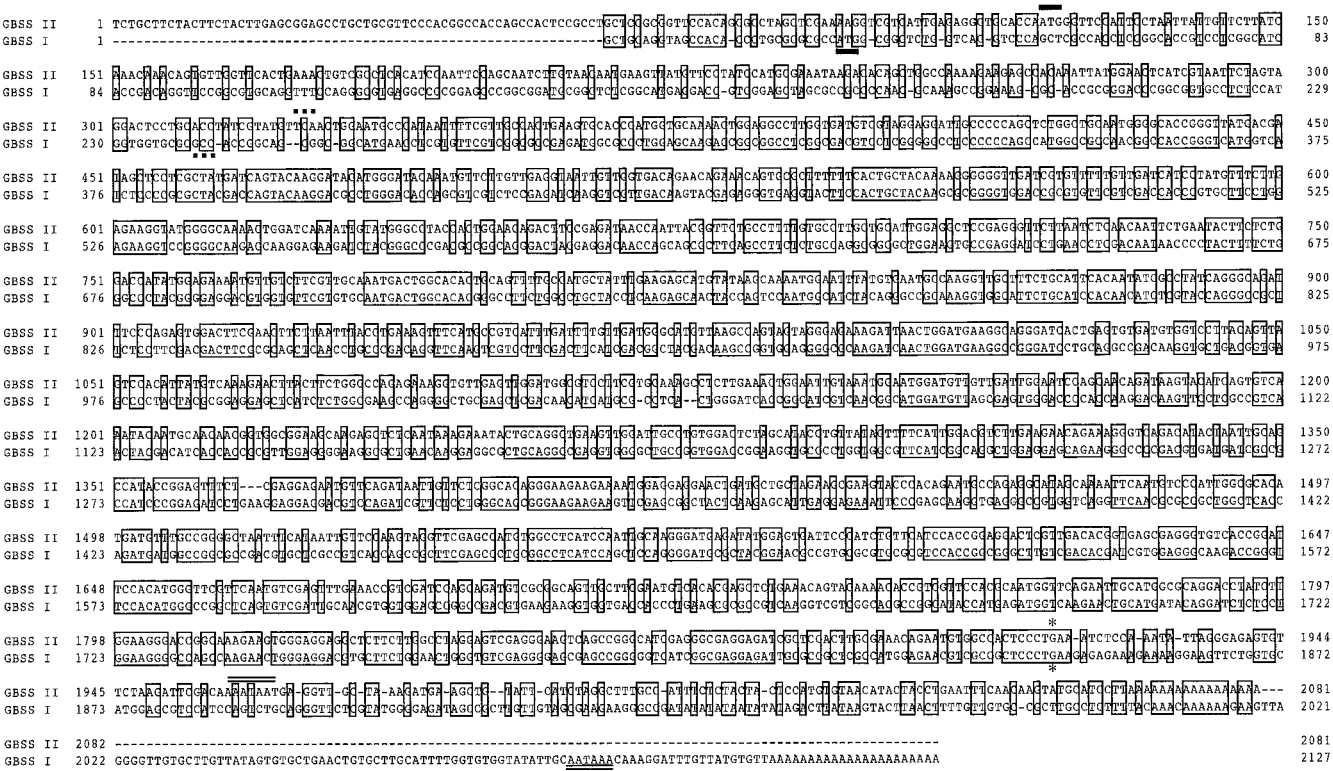

Figure 1. Alignment of wheat *GBSSII* and *GBSSI* cDNA sequences. Sequences were aligned using the Gentyx-Mac program. Identical nucleotides are boxed. The translational start codons are indicated with solid lines, the first codons of the mature proteins with dotted lines, the stop codons with stars, and the putative polyadenylation signals with double lines.

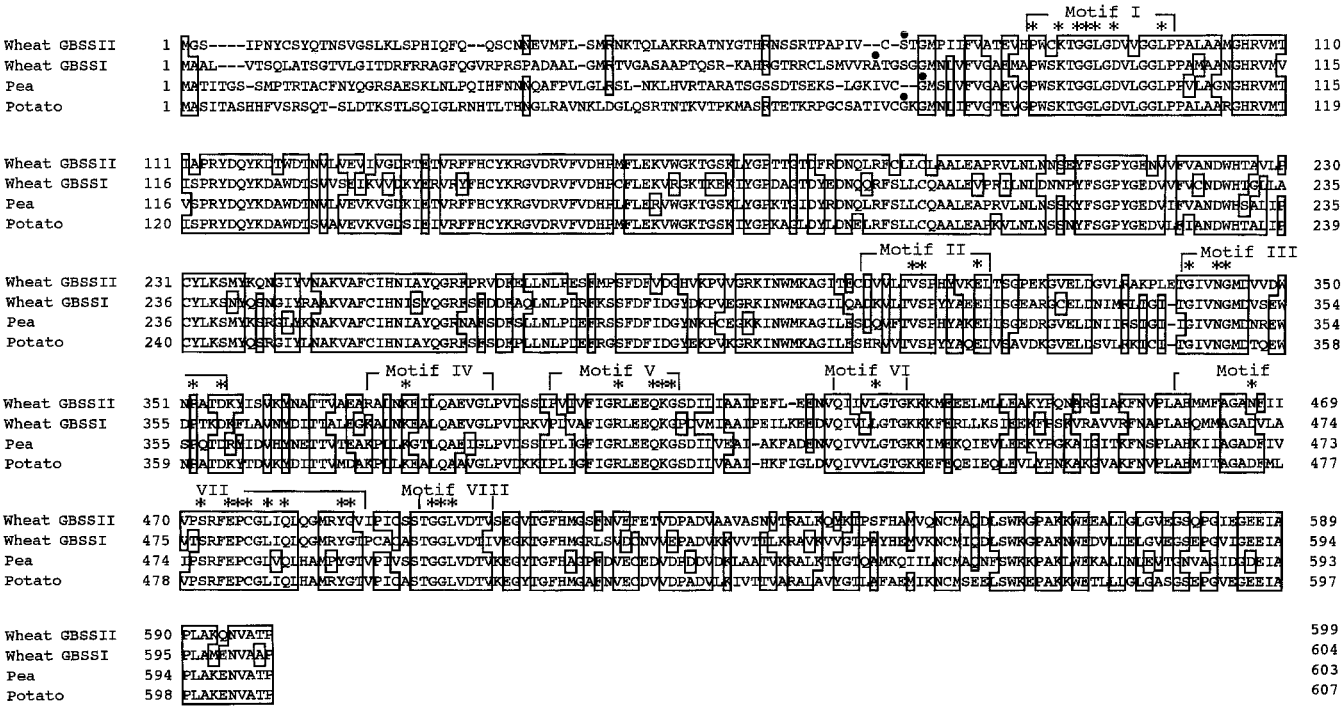

Figure 2. Alignment of the primary structures of GBSS from several plant species. Sequences were aligned using the Genetyx-Mac program. The wheat GBSSII sequence was deduced from the cDNA. Sources of other sequences are as follows: wheat GBSSI, accession number AF163319; potato, accession number X58453; and pea, accession number X88789. The first amino acid of the mature protein is indicated with a dot. The eight sequence motifs conserved among procaryotic glycogen synthase and plant starch synthase sequences (Cao et al., 1999) are shown, and the 33 conserved residues within these motifs are indicated by asterisks.

protein. The deduced mature wheat GBSSII sequence showed 65.6%, 69.9%, and 72.1% identity with GBSSI sequences from wheat, pea, and potato, respectively.

A dendogram clustered GBSSs from several species into two groups (Fig. 4), and wheat GBSSI clustered with the GBSSIs or waxy proteins from monocotyledonous species, as has been previously observed (Harn et al., 1998). In contrast, wheat GBSSII clustered loosely with the GBSSIs from pea, potato, cassava, and sweet potato.

Expression of *GBSSII* in Wheat Tissues

GBSSII expression was detected in pericarp, leaf, and culm tissues (Fig. 5). Expression was highest in pericarp from seed harvested at approximately 3 DPA, at which stage endosperm development is not yet detectable and most or all starch is found as transient starch in the pericarp tissue. *GBSSII* transcripts were not detectable in endosperm tissue from 20-DPA seed. The *GBSSII* signal detected in leaf tissue from waxy plants was stronger than that from Chinese Spring plants, but a signal was clearly visible in a blot of poly(A^+) RNA from Chinese Spring leaf tissue (not shown). The difference in transcript levels between the two cultivars may have been due to a slight difference in leaf maturity at the time of harvest, or it may reflect small varietal differences in transcript sequences, since the *GBSSII* cDNA was isolated from waxy wheat.

A high level of *GBSSI* transcripts was found only in endosperm tissue (Fig. 5), in agreement with results obtained by Ainsworth et al. (1993a). Expression was highest in Chinese Spring wheat, although low levels of transcript were detectable in whole seeds of waxy wheat (visible on overexposure; results not shown) as previously described (Vrinten et al., 1999). Very low levels of transcript were also detected in Chinese Spring 3-DPA pericarp after overexposure of blots (results not shown), which may indicate either that GBSSI is expressed at a low level in pericarp, or that some development of endosperm is already occurring at 3 DPA.

At low wash stringencies, a low level of cross-hybridization between the *GBSSI* cDNA and the *GBSSII* transcript occurred (data not shown), but the transcripts

Figure 3. N-terminal sequences of GBSSII (Nakamura et al., 1998) and GBSSIs from the A (Wx-A1), B (Wx-B1), and D (Wx-D1) genomes. Amino acids that differ between the GBSSI proteins are shown in outline. X, Undetermined residue.

GBSSII	**STGMPIIFVATEVHPWXKTGGLGDVVGXLPPA**
GBSSI (Wx-A1)	**ATGSGGMNLVFVGAEMAPWSKTGGLGDVLGGLPAAMAANGHRVMVI**
GBSSI (Wx-B1)	**ATGSAGMNLVFVGAEMAPWSKTGGLGDVLGGLPPAMAANGHRVMVI**
GBSSI (Wx-D1)	**ATGSGGMNLVFVGAEMAPWSKTGGLGDVLGGLPPAMAANGHRVMVI**

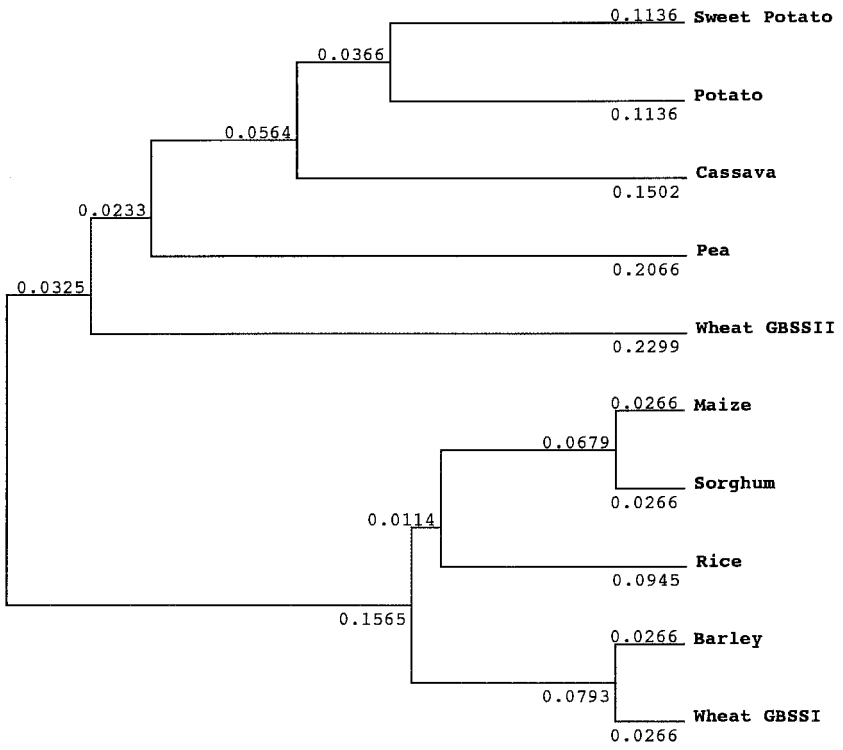

Figure 4. Dendogram representation of the phylogenetic relationships between GBSS from several plant species. The sources of wheat GBSSI and GBSSII, pea, and potato sequences are as given in Figure 2. Sources of other sequences are as follows: sweet potato, accession number U44126; cassava, accession number X74160; maize, accession number X03935; sorghum, accession number U23945; rice, accession number X62134; and barley, accession number X07932.

were readily distinguishable because the *GBSSII* transcript was approximately 2,200 nt and *GBSSI* was approximately 2,400 nt. Much of this size difference appears to be due to the shorter 3′ untranslated region of *GBSSII* (Fig. 1).

In snapdragon, a circadian regulation of *GBSSI* expression occurs in leaf tissue, and mRNA could not be detected during the night even when plants were maintained under constant illumination (Merida et al., 1999). Since wheat *GBSSII* and snapdragon *GBSSI* share a high level of homology and are expressed in similar tissues, we thought the regulation of these genes might be similar. However, similar amounts of *GBSSII* mRNA were present in 0-DPA ovary/pericarp tissue collected at the midpoints of the day and night (Fig. 6). Although green tissue was visible in young pericarp, circadian regulation of *GBSSII* clearly did not occur.

Chromosomal Location of *GBSSII* Genes in Wheat

Digestion of genomic DNA from Chinese Spring nullisomic-tetrasomic and ditelosomic lines with *Knp*I produced three DNA fragments (15, 12, and 5 kb) that hybridized to the *GBSSII* cDNA insert (Fig. 7). The 12-kb fragment was missing from digests of N2OT2A, while N2BT2A and DT2AS lacked the 5- and 15-kb fragments, respectively. Therefore, the *GBSSII* genes are located on chromosomes 2B, 2D, and on the long arm of chromosome 2A. Since only three fragments were produced, with one fragment originating from each of the A, B, and D genomes, the *GBSSII* genes in wheat represent a set of single-copy homeoloci. The genes encoding wheat *GBSSI* are located on chromosomes 7AS, 7DS, and 4AL (Chao et al., 1989; Ainsworth et al., 1993a; Nakamura et al., 1993; Yamamori et al., 1994);

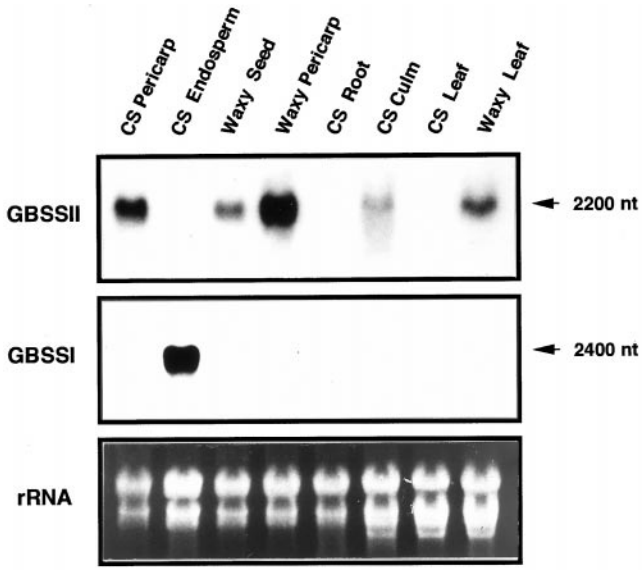

Figure 5. Northern-blot analysis of *GBSSI* and *GBSSII* mRNA accumulation in wheat tissues. Total RNA (10 μg) was loaded in each lane. After probing with the radiolabeled insert of the *GBSSII* cDNA (top), the blot was stripped and reprobed with the radiolabeled insert of the *GBSSI* cDNA (middle). The ethidium-bromide-stained gel is shown at the bottom. CS, Chinese Spring; Pericarp, seed harvested at 3 DPA. Since endosperm development could not be detected at this time, whole seeds were used. Endosperm, Endosperm tissue removed from 20-DPA seed; Waxy Seed, whole seeds of waxy wheat harvested at 20 DPA.

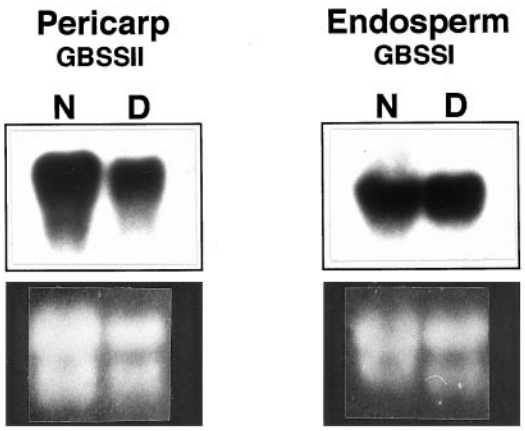

Figure 6. Northern-blot analysis of *GBSSI* and *GBSSII* mRNA accumulation at the midpoints of day and night. Total (10 μg) RNA was loaded in each lane. The ethidium-bromide-stained gels are shown at the bottom. Day and night samples were harvested from the same group of Chinese Spring plants at the midpoint of the day, and 12 h later at the midpoint of the night. Pericarp, Seed harvested at approximately 0 DPA (flowering). Since endosperm development could not be detected at this stage, whole seed was used. Endosperm, Endosperm tissue removed from 15-DPA seed.

thus, the localization of *GBSSII* genes to the group 2 chromosomes provides clear evidence that *GBSSI* and *GBSSII* are encoded by separate genes.

Presence of *GBSSII* Genes in Other Plant Species

Hybridization of the GBSSII probe with DNA from a number of plant species produced clear bands in wheat, barley, rice, and maize (Fig. 8). Reprobing of this blot with a wheat *GBSSI* cDNA indicated that different fragments were produced by *GBSSI* and *GBSSII* in all four species (Fig. 8), suggesting that a second *GBSS* gene related to *GBSSII* also occurs in these species. The presence of blue-black-staining starch containing amylose in non-storage organs of waxy mutants has been reported in both maize (Hixon and Brimhall, 1968; Badenhuizen, 1969) and rice (Sano, 1985); therefore, the presence of genes related to *GBSSII* in these species is not surprising. In potato, a single *GBSSI* appears to be responsible for the presence of amylose in tuber, leaf, root, and pollen starch (Jacobsen et al., 1989), but at least two isoforms occur in pea (Denyer et al., 1997; Tomlinson et al., 1998). However, although wheat *GBSSII* showed closer homology to *GBSSI* from pea and potato than to *GBSSI* from wheat at both the nucleotide and amino acid levels, neither wheat *GBSSI* nor *GBSSII* produced clear bands with the dicotyledonous species pea, potato, tomato, soybean, or taro.

DISCUSSION

Several lines of evidence indicate that the cDNA characterized here encodes a GBSS isoform, GBSSII, found in the pericarp of wheat (Nakamura et al., 1998). First, the *GBSSII* cDNA was isolated from pericarp tissue by immunoscreening with anti-potato GBSSI, which clearly recognizes the pericarp GBSSII, and the N-terminal amino acid sequence of GBSSII closely matches the start of the deduced GBSSII mature protein (Figs. 2 and 3). Second, the deduced GBSSII protein is closely related to GBSSI from several species (Figs. 3 and 4). Third, *GBSSII* transcript is found predominantly in pericarp tissue and is not present in endosperm tissue, corresponding with the distribution of GBSSII protein (Nakamura et al., 1998). *GBSSII* transcript is also found in leaf and culm tissues, suggesting that amylose production in all or almost all transient starches is conditioned by GBSSII.

Although the cloning of a second *GBSS* or *waxy* gene has not been described in any other plant species, several of the enzymes involved in starch synthesis appear to have multiple forms encoded by separate genes and expressed in a tissue-specific manner. For example, in barley and maize, SBEIIa and IIb are encoded by different genes showing

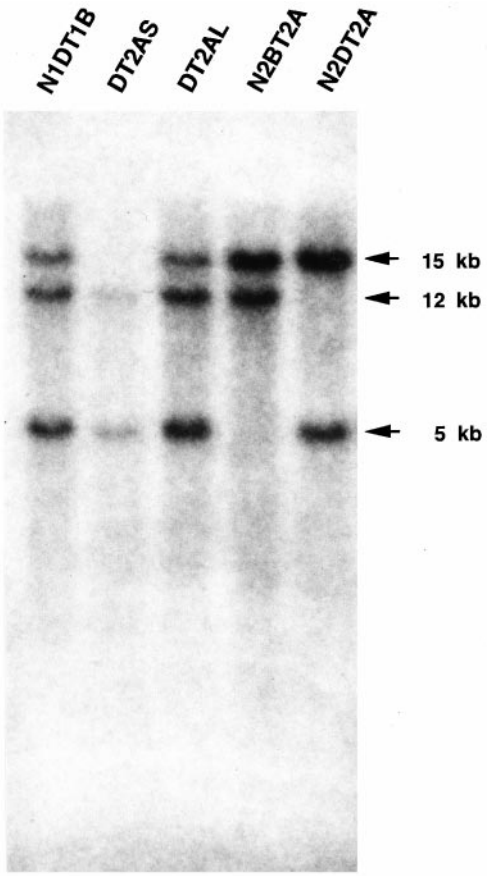

Figure 7. Southern analysis of DNA from nullisomic-tetrasomic and ditelosomic lines of Chinese Spring wheat. DNA was digested with *Knp*I and 15 μg was analyzed by electrophoresis. The approximate size of fragments are shown on the right. N1DT1B, Chinese Spring with no 1D chromosomes but with four copies of chromosome 1B; DT2AS, Chinese Spring with no copies of the long arm of chromosome 2A but with four copies of the short arm; DT2AL, Chinese Spring with no copies of the short arm of chromosome 2A but with four copies of the long arm; N2BT2A, Chinese Spring with no copies of chromosome 2B but with four copies of chromosome 2A; N2DT2A, Chinese Spring with no copies of chromosome 2D but with four copies of chromosome 2A.

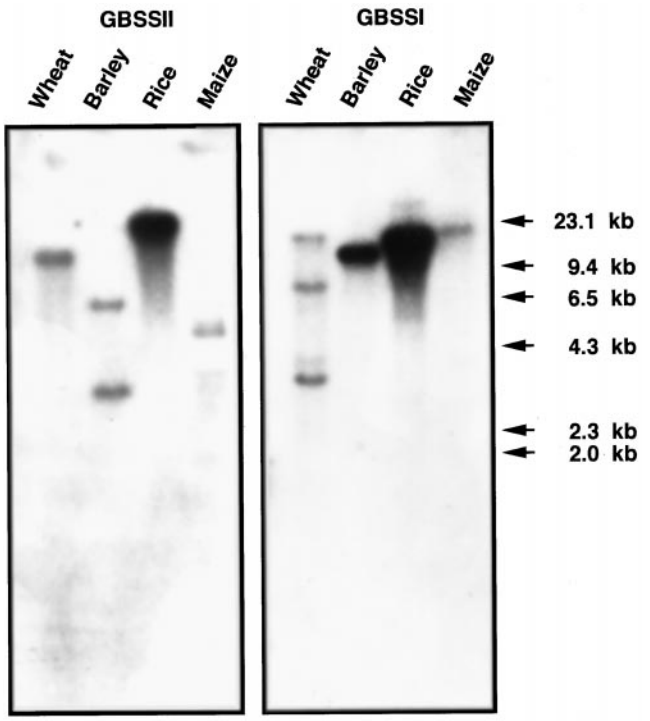

Figure 8. Southern analysis of *GBSSII* and *GBSSI* in various plant species. DNA was digested with *Hin*dIII, and 15 μg was analyzed by electrophoresis. Lanes containing taro, pea, potato, soybean, and tomato DNA were also included on the blots, but are not shown since bands hybridizing to *GBSSII* or *GBSSI* were not detected.

tissue-specific expression, with *SBEIIa* being expressed in vegetative tissues, while *SBEIIb* is not (Fisher et al., 1996; Gao et al., 1996, 1997; Sun et al., 1998). Similarly, transcripts of the maize starch synthase IIa (*SSIIa*) gene were detected mainly in the endosperm, while *SSIIb* was expressed primarily in leaf tissue (Harn et al., 1998). A model recently put forth by Force et al. (1999) suggests that complementary mutations in duplicated genes that result in the partitioning of original gene functions may increase the chances of both duplicates being maintained. In line with this, the tissue-specific expression of *GBSSI* and *II* in higher plants may represent subfunctions of the single *GBSS* present in the monocellular photosynthetic algae *Chlamydomonas reinhardtii*.

The location of the *GBSSII* genes on group 2 chromosomes clearly shows that wheat *GBSSI* and *GBSSII* are encoded by different loci. Most starch synthesis enzymes and starch granule-associated proteins in wheat, including *GBSSI* (Chao et al., 1989; Nakamura et al., 1993; Yamamori et al., 1994), *Agp1*, which encodes the small subunit of ADP Glc pyrophosphorylase in endosperm (Ainsworth et al., 1993b), starch granule proteins 1 and 3 (Yamamori and Endo, 1996), branching enzyme I (Morell et al., 1997; Rahman et al., 1997; Rahman et al., 1999), and soluble starch synthase (Devos and Gale, 1997), have been localized to the group 7 chromosomes. However, genes for one class of starch-branching enzymes, which apparently represent SBE-II genes (Rahman et al., 1999) have been localized to group 2 chromosomes (Sharp, 1997). In barley, two genes encoding isozymes of SBE-II have been identified, one of which (*SBE-IIb*) is specific to endosperm tissue, while *SBE-IIa* is also expressed in vegetative tissues (Sun et al., 1998). It seems likely that a similar situation occurs in wheat, and it will be interesting to determine if the wheat *SBE-II* genes on chromosome 2 are of the *SBE-IIa* type. Although the significance of the co-localization of starch synthesis enzymes on group 7 chromosomes is not known, the possible co-localization of a duplicated set of starch synthesis genes on group 2 chromosomes that show expression in vegetative tissue is worth examining.

Although extensive attempts were made to detect activity of recombinant GBSSII expressed in a glycogen-synthase-deficient *E. coli* line, recombinant enzymes with or without signal peptides failed to show significant activity (P.L. Vrinten and T. Nakamura, unpublished data). Recent experiments have indicated that in *C. reinhardtii*, amylose is synthesized by extension of amylopectin (van de Wal et al., 1998). Further experiments using *C. reinhardtii* mutants lacking debranching enzyme suggested that provision of a primer alone is not sufficient for GBSSI activity, since unbound GBSSI appeared to be capable of only a very low amount of amylose synthesis in debranching enzyme mutants, with the amylose-like material produced amounting to only 0.4% of the normal starch amount in wild-type algae (Dauvillee et al., 1999). Glycogen-like polysaccharides were also produced in these mutants, but GBSSI was not involved in their synthesis, leading to the suggestion that an organized crystalline amylopectin matrix, as well as a primer, is required to activate GBSSI (Dauvillee et al., 1999). These requirements may explain the lack of ADP-glucosyl transferase activity of the recombinant GBSSII enzyme expressed in *E. coli*. Although starch synthase activity has been demonstrated for other recombinant plant starch synthases expressed in *E. coli* (Edwards et al., 1995, 1996; Knight et al., 1998; Cao et al., 1999), such activity has not been demonstrated to date with recombinant GBSSI or waxy proteins. Wheat GBSSI or waxy protein, whether solubilized in a native state or renatured from denatured protein, shows very little starch-synthase activity in vitro (Denyer et al., 1995b). The normally granule-bound wheat GBSSII may resemble GBSSI in requiring attachment to the starch granule for optimum activity, since the presence of GBSSII protein in pericarp starch granules resulted in the production of amylose in the absence of GBSSI (Nakamura et al., 1998).

The potato *GBSSI* gene is inducible by sugars (Visser et al., 1991; Kossmann et al., 1999), and the similar mRNA accumulation under light and dark conditions observed with both *GBSSII* in pericarp and *GBSSI* in endosperm appears consistent with the use of imported sugars for starch synthesis in these tissues. However, although pericarp appears to function as a storage tissue for a short period of time, later in development net starch degradation and mobilization occur (Chevalier and Lingle, 1983). In both oilseed rape embryo (da Silva et al., 1997) and tomato pericarp (Schaffer and Petreikov, 1997), the switch to a net degradation of transient starch is accompanied by decreases in starch synthesis enzymes. Precise regulation of *GBSSII* expression might required during the night in leaf

tissue or during pericarp starch degradation and mobilization during the later stages of pericarp development. Conversely, the synthesis and degradation of endosperm starch occur at distinct stages of plant development, with starch degradation taking place during seed germination. If the *GBSSI* gene in endosperm is less sensitive to physiological conditions that affect *GBSSII* expression, the presence of two genes might provide an adaptive advantage.

Starch granules from endosperm and pericarp differ in size and in the ratio of amylose to amylopectin (Nakamura et al., 1998). Pericarp starch granules from waxy and nonwaxy wheat had amylose contents of 19.4% (w/w) and 18.5% (w/w), respectively, whereas endosperm starch granules had an amylose content of 26.3% (w/w) (Nakamura et al., 1998). It seems likely that the tissue-specific expression of *GBSSI* and *II* affects the amylose content of starch granules, since GBSS is a key enzyme in amylose synthesis. However, tissue-specific isozyme composition of other starch synthesis enzymes may also affect the amylose to amylopectin ratio. To further clarify the effect of GBSSII on amylose levels and characteristics, we plan to transform waxy wheat with the *GBSSII* cDNA and characterize the endosperm starch produced by such transformants.

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