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Cloning and characterization of the granule-bound starch synthase II gene in rice: gene expression is regulated by the nitrogen level, sugar and circadian rhythm

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Abstract A full-length coding domain sequence of a gene analogous to granule-bound starch synthase (GBSS; ADP-glucose-starch glucosyltransferase, EC 2.4.1.21) was cloned and defined as *OsGBSSII* based on a Nitrogen (N)-starvation-induced cDNA library constructed using the rapid subtraction hybridization method. The deduced amino acid sequence of *OsGBSSII* was 62–85% identical to those of GBSS proteins from other plant species. The exon/intron organization of *OsGBSSII* was similar to that of *OsGBSSI*. *OsGBSSII* was mainly expressed in leaves and its protein was exclusively bound to starch granules in rice leaves, which suggests that the amylose in rice leaves is synthesized by *OsGBSSII*. N-starvation-induced expression of *OsGBSSII* could be repressed by supplying nitrate, ammonia or amino acid (glutamic acid or glutamine), glucosamine (an inhibitor of hexokinase) or dark conditions. These results indicate that N-starvation induction was dependent on the photosynthetic product and hexokinase in rice leaves. Sugars induced the accumulation of *OsGBSSII* transcripts in excised leaves through glycolysis-dependent pathways. *OsGBSSII* gene expression is regulated by the circadian rhythm in rice leaves.

Keywords Circadian rhythm · Gene expression · Granule-bound starch synthase · Nitrogen · *Oryza* · Sugar

Abbreviations *AGPase*: ADP-glucose pyrophosphorylase · *BE*: branching enzyme · *C*: Carbon · *GBSS*: granule-bound starch synthase · *Glc*: glucose · *Gln*: glutamine · *Glu*: glutamic acid · *HXK*: hexokinase · *N*: Nitrogen · *RaSH*: Rapid Subtraction Hybridization · *Suc*: sucrose

Introduction

Plant starch almost entirely consists of the glucose (Glc) homopolymers amylopectin and amylose. Amylose is a lightly branched linear molecule with low polymerization, while amylopectin is a much larger molecule with extensive branches resulting from α -1,6 linkages (Smith et al. 1997). Both types of polymer are elongated by starch synthase, which catalyzes the transfer of Glc from ADP-Glc to growing glucan chains via an α -1,4-linkage. Several starch synthases are thought to catalyze amylopectin synthesis, while granule-bound starch synthase I (GBSSI) is solely responsible for the biosynthesis of amylose in storage tissues (Smith et al. 1997). In *waxy* rice, while endosperm starch does not contain amylose, the starch fractions in the embryo, leaf blades, stems, and roots do contain amylose (Blakeney and Matheson 1984). Recent research has identified a new *GBSS*, designated *GBSSII*, in *waxy* wheat and *amf* pea, which is responsible for amylose synthesis in non-storage tissues (Denyer et al. 1997; Nakamura et al. 1998; Vrinten and Nakamura 2000; Edwards et al. 2002).

Scheible et al. (1997) reported that in tobacco, nitrate could be a signal that regulates starch synthesis by inhibiting the expression of the small subunit of ADP-Glc pyrophosphorylase (*AGPase*) and decreasing the concentration of 3-phosphoglyceric acid (3PGA), the allosteric activator of *AGPase*. It has been reported that the genes for starch metabolism, such as *AGPase* in *Arabidopsis* (Sokolov et al. 1998), *GBSSI* in sweet potato (Wang et al. 2001), *AGPase*, *GBSSI*, *SSII* and *SSIII* in potato (Visser et al. 1991; Nakata and Okita 1995; Kossmann et al. 1999), and branching enzyme (*BE*) in cassava (Salehuzzaman et al. 1994) are controlled by sugars. The transitory starch in leaves is synthesized during the day and mobilized at night to supply the carbon requirements of the plant. This circadian fluctuation of starch accumulation in leaves has been reported in many plants (Li et al. 1992; Geiger and Servaites 1994; Mérida et al. 1999; Wang et al. 2001;

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Zabawinski et al. 2001). The genes for starch synthesis, such as *AGPase* and *GBSSI*, have also been reported to be regulated by circadian rhythm (Mériida et al. 1999; Wang et al. 2001; Zabawinski et al. 2001). However, little is known about the integration of the regulation of the genes involved in starch synthesis by circadian, sugar and nitrogen signaling.

In the present study, a cDNA fragment analogous to wheat *GBSSII* was identified from a rice nitrogen (N)-starvation-induced Rapid Subtraction Hybridization (RaSH) cDNA library. The gene was cloned and defined as *OsGBSSII*. The domain structures, exon/intron organization and protein localization were analyzed. The gene expression patterns in rice leaves as influenced by different nitrogen levels, sugars and the circadian rhythm were investigated.

Materials and methods

Plant materials and experimental treatments

Rice (*Oryza sativa* L. cv. Zhe 733, Indica) seedlings were grown in a greenhouse at 28 °C and 80% relative humidity. To construct the N-starvation cDNA library, some of the plants were transferred to N-free rice nutrient solution (Yoshida et al. 1976) at the four-leaf-stage. The seedlings were sampled after being treated for 0.5, 2 and 48 h. The N-starvation-induced RaSH cDNA library was prepared according to the program of Jiang et al. (2000).

To determine the effects of different N sources, the four-leaf-stage seedlings were grown in rice solutions containing 2 mM of KNO₃, NH₄Cl, glutamic acid (Glu) or glutamine (Gln). For the sugar induction test, the four-leaf-stage seedlings were cultured for 2 days under dark conditions to deplete endogenous sugars before treatment with various sugars. After 2 days, the second and third leaves were excised from the plants (about 1 cm in length) and then transferred and incubated in 175 mM of sugar solutions in the dark at 28 °C. The leaf samples were harvested after 12 h of sugar treatment.

OsGBSSII cloning

The cDNA clones isolated from the N-starvation RaSH cDNA library (*DnpII*) were sequenced (MegaBACE 1000; Amersham Pharmacia Biotech). A cDNA fragment of 253 bp showed a high identity to wheat *GBSSII* (AF109395 in NCBI). A BLAST search in the rice genome database and EST database retrieved the genomic sequence of the OSM122258 clone in Monsanto (<http://www.rice-research.org>) and the EST clone of AU030570 in GenBank (<http://www.ncbi.nlm.nih.gov>), which contained the 5'- and 3'- partial sequences of rice *GBSSII*, respectively. One pair of primers, GS1: 5'-tag gtg tca ctg aat ggc tag aac-3' and GS2: 5'-tgg ccc aca tct cta agt aac atc-3', was used for reverse transcription-polymerase chain reaction (RT-PCR) with oligo(dT)-primed cDNA that had been reverse-transcribed from RNA of rice leaves. The cDNA fragment was cloned into pUCm-T vector (Sangon, China) and denoted as *GBSSII*-T. The positive clone with an insert of about 2.0 kb was selected and reproduced in fresh LB medium containing 100 µg/ml ampicillin, and sequenced.

RNA gel blot and RT-PCR analysis

Total cellular RNA (20 µg) prepared from various rice tissues was separated on 1% formaldehyde-agarose gels, and transferred to a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech). The probe used for hybridization was the 253-bp fragment.

Hybridization was carried out as described previously (Jiang et al. 2003a). For RT-PCR, the cDNA was reverse-transcribed from 2 µg of total RNA. The PCR amplification program consisted of an initial 95 °C cycle for 5 min followed by 34 cycles (*OsGBSSII*) or 28 cycles (*OsGBSSI* and *Actin*) of 95 °C, 30 s; 58 °C, 30 s; 72 °C, 2 min, and a final extension at 72 °C for 5 min. The primers were *OsGBSSII*, GS1 (-13 to +11 bp, A-ATG is No. 1) and GS2 (+1987 to +2010 bp); *Actin*, 5'-gga act ggt atg gtc aag gc-3' and 5'-agt ctc atg gat acc cgc ag-3'; and *OsGBSSI*, 5'-tcg aca aca tca tgc ggc tca-3' (+1016 to +1036 bp) and 5'-agc aca ggg cgt tcc gta tct c-3' (+1485 to +1506 bp).

Genomic DNA blot analysis and genomic mapping

The genomic DNA (10 µg) isolated from rice leaves was digested with the restriction enzymes *DraI*, *EcoRI*, *HindIII* and *XbaI*, separated on a 0.8% agarose gel, and blotted onto a nylon membrane (Amersham Pharmacia Biotech). Hybridization was carried out as described previously (Jiang et al. 2003a).

For genomic mapping, the electronic-mapping method was used. A BLAST search in a genome database from GenBank (<http://www.ncbi.nlm.nih.gov>) with the 2,023-bp *OsGBSSII* cDNA sequences retrieved the EST of E51255. When used as a marker, the EST identified loci on the High-Density Rice Genetic Map (<http://rgp.dna.affrc.go.jp>; Wu et al. 2002).

Expression of *OsGBSSII* in *Escherichia coli*

The following primers were used for PCR modification of the N-terminus of *OsGBSSII*: pGS1 [5'-aag gaa ggt acc act gaa tgt cac cca tgg tgc-3' (+262 to +282)] and pGS2 [5'-aaa aaa gag ctc ggg agt ggc cac gtt ttc ctt g-3' (+1803 to +1824)]. These primers were used to introduce *KpnI/SacI* restriction sites at the putative N-terminus of *OsGBSSII* (T₈₈ECHPWC...). The reconstructed sequence of *GBSSII* was subcloned into the *KpnI/SacI* sites of pET-29b (Novagen) and named pGBSSII. Overnight cultures of *E. coli* (BL21) cells transformed with plasmid pGBSSII or with pET-29b were diluted 1:100 (v/v) in fresh LB medium containing 50 µg/ml kanamycin. Soluble cytoplasmic proteins were prepared from IPTG-induced BL21 cells containing pET-29b or pGBSSII according to Harn et al. (1998). A 20-µl aliquot of each extract was assayed for starch synthase activity as described previously (Jiang et al. 2003b). The assay was repeated three times. A unit of activity was defined as the formation of 1 nmol ADP per min at 30 °C.

Preparation of antiserum

The expressed 6 × His-GBSSII in *E. coli* was extracted and purified on an Ni²⁺ nitriloacetic acid metal-affinity column according to the manufacturer's instructions (Qiagen, Hilden, Germany). Samples were mixed with Freund's complete adjuvant, and injected intramuscularly into a rabbit. Crude serum was used in all experiments.

Isolation and analysis of *OsGBSSII* protein from leaves

Protein extracts were separated by 7.5% SDS-PAGE, transferred to polyvinylidenedifluoride (PVDF) membranes (AMRESCO), and visualized using rabbit anti-GBSSII antiserum followed by alkaline phosphatase-conjugated goat anti-rabbit serum (Santa Cruz Biotechnologies, CA, USA). For N-terminal sequencing, starch granule-bound proteins were purified from rice leaves after 2 days of N-starvation treatment and separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by staining with Ponceau S. The amino acid sequences of the polypeptides were determined by Edman degradation analysis at the Medical Center, University of Nebraska.

Preparation of starch granules and SDS-PAGE

Starch granules were extracted by the method described by Nakamura et al. (1998). Starch granule-bound proteins were separated by 7.5% SDS-PAGE. To extract starch granule-bound proteins, 20 mg of purified starch was suspended in 800 µl of sample buffer [0.1 mM Tris-HCl (pH 7.5), 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol] and heated in boiling water. The starch solution was cooled on ice and centrifuged at 15,000 *g* for 10 min, and 20 µl of the supernatant was subjected to SDS-PAGE. To extract the total proteins, leaves were homogenized in eight volumes of sample buffer. The homogenates were heated in boiling water for 7 min, cooled on ice and centrifuged at 15,000 *g* for 10 min, and 20 µl of supernatant was subjected to SDS-PAGE.

Amylose content

Amylose content was measured by the method described by Nakamura et al. (1998), and the λ_{max} of the iodine-starch complexes was determined according to the method of Konishi et al. (1985).

Results

Isolation and characterization of the *OsGBSSII* cDNA clone

A cDNA clone analogous to wheat *GBSSII* was isolated from the N-starvation-induced RaSH cDNA library in rice and named *OsGBSSII*. Northern analysis revealed that *OsGBSSII* was expressed in leaves and up-regulated by N-starvation (Fig. 1a). cDNA containing the full-

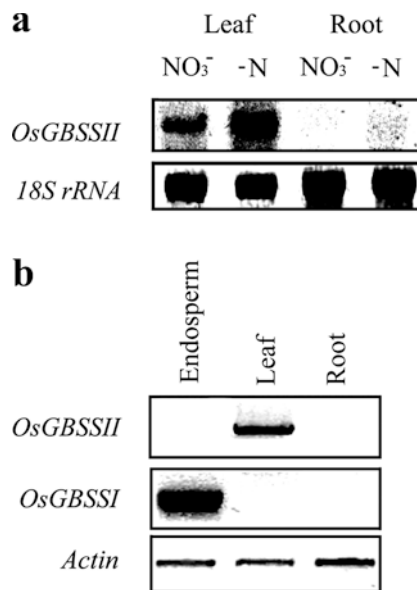


Fig. 1 **a** Northern analysis of *Oryza sativa OsGBSSII*. Total RNAs were prepared from the leaves and roots of Indica rice Zhe733 that had been treated with 2 mM KNO_3 (NO_3^-) or without nitrogen ($-\text{N}$) for 48 h. Lower panel shows the normalization of total RNA levels in each sample by the hybridization of 18S rRNA on the same blot. **b** RT-PCR analysis of *OsGBSSII* and *OsGBSSI* expression in different organs of rice. The amplified fragments of *OsGBSSII* and *OsGBSSI* were 2,032 bp and 490 bp, respectively. A 775-bp *Actin* gene fragment was amplified as a control

length coding domain sequence was obtained by RT-PCR from rice leaves. The spatial expression patterns of *OsGBSSII* were investigated and compared to those of *OsGBSSI* by RT-PCR analysis. The results showed that *OsGBSSII* was mainly expressed in leaves (Fig. 1b), while *OsGBSSI* was expressed in endosperms with much higher transcript levels, which is consistent with previous reports (Hirano et al. 1995). *OsGBSSII* contains an open reading frame of 1,824 bp and predicts a polypeptide of 608 amino acids with a calculated molecular mass of 67.3 kDa and a transit peptide of 77 amino acids (Fig. 2).

Amino acid sequence alignment

The deduced amino acid sequences of *OsGBSSII* were aligned with *OsGBSSI* and nine conserved regions were identified. Eight sequence blocks, which are conserved in plant starch synthases and prokaryotic glycogen synthase (Cao et al. 1999), were present in *OsGBSSII* (Fig. 2). The first homologous region contained the consensus motif KXGG, which is believed to be the

OsGBSSI	MSALTTSQLATSATGFGIADRSAPSSLLRHGFQGLKPRSPAGGDATSLSVTTSSARATPKQ	60
OsGBSSII	M-ARTMGSTPTYCS-YQTNGVGALKQSPHMQFQGSYNYGVRFKRDTLKRDLSVRINKHMAKRI	58
	* * * . . . * * * * * * *	
OsGBSSI	QRSVQGRSRRFPSSVVVYATGAGMNVVFVGEAEMAPWSKTGGLGDLVGLPMAANGHRVM	120
OsGBSSII	ATSTGICTKPRRSHMPVCSAGMTIIFATECHPWCKTGGLGDLVGLPMAANGHRVM	118
	* . . . * * * * * * * * *	
OsGBSSI	VISPRYDQYKDAWDTSVVAEIKVADRYERVRFFHCYKRGVDVRFIDHPSFLEKVGWKTGE	180
OsGBSSII	TIVPRYDQYKDAWDTNVLVEVNIIDRTETVRFFHCYKRGVDVRFIDHPSFLEKVGWKTGP	178
	* * * * * * * * * * *	
OsGBSSI	KIYGPDTGVYKDNQMRFSLLCQAALEAPRIINLNNPYFKGTYGEDVVFVGNWDHWTGPL	240
OsGBSSII	KLYGPTTGDYRDNQLRFCLLCQAALEAPRVNLNNSEYFGPGYGENVVFVANDHWTGVL	238
	* * * * * * * . . . * * * * * * * *	
OsGBSSI	ASYLNKNYPNGIYRNAKVAFCIHNI SYQGRFAFEDYPELNLSERFSFDFIDGYDTPV	300
OsGBSSII	PCYLKSIYQAKGMVYNAKVAFCIHNIAYQGRFAFEDFELLNPSLFSFDFIDGHFKPV	298
	. . . * * . . . * * * * * * * * * *	
OsGBSSI	EGRKINWMKAGILEADRVLTSPYYAEELISGIARGCELDNIMRLTG-ITGIVNGMDVSE	359
OsGBSSII	VGRKINWMKAGITECDLVMTVSPHYVKELASGPKGVLDGILRTKPLETGIVNGMDVYE	358
	* * * * * * * . . . * * * * * * * *	
OsGBSSI	WDPSKDKYITAKYDATTAEAKALNKEALQAEAGLPVDRKIPLIATIGRLEEKGSDVMA	419
OsGBSSII	WNPATDQYISVKYDATTVEARALNKEALQAEAGLPVDSSIPLIVFVGRLEEKGSDIL	418
	* . . . * * * * * * * * *	
OsGBSSI	AAIPELMQEDVQIVLLGTGKKKFEKLLKSMEEKYPGKVRVAVVKNAPLAHLIMAGADVLA	479
OsGBSSII	AAIPEFVEGNVQIVLGTGKKKMEELILLEVYPNTARGLAKFNVPPLAHMMFAGADFI	478
	* * * * * * * * * * * * *	
OsGBSSI	VPSRFEPGGLIQLQGMRYGTPCAGASTGGLVDTVIEGKTGFHMGRLSDVCKVVEPSDVVK	539
OsGBSSII	VPSRFEPGGLIQLQGMRYGVVPICSSTGGLVDTVKEGVTGFHMGFLNVEGCTVDPVDVTA	538
	* * * * * * * . . . * * * * * * * *	
OsGBSSI	VAATLKRAIKVVGTPAYEEMVRNMCNQLSNWGPAPKNWENLLGLGVAGSAPGIEGDEIA	599
OsGBSSII	VASTVKRALQYNTPAFQEMVQNCMAQDLNWKGPAPKNWEEVLLGLGVESQPGIEGEEVA	598
	* * . . . * * * * * * * * * *	
OsGBSSI	PLAKENVAAP	609
OsGBSSII	PLAKENVATP	608
	* * * * * * *	

Fig. 2 Amino acid sequence alignment of *OsGBSSI* and *OsGBSSII*. The sequences were aligned using the CLUSTAL program. Identical and conserved residues are marked with (*) and (:), respectively. Solid lines and numerals 1 to IX indicate conserved regions. Arrows indicate the cleavage sites of transit peptides

ADP-Glc binding site (Furukawa et al. 1993). The ninth conserved region is specific to GBSS-class starch synthase. A phylogenetic comparison of OsGBSSII and other plant GBSS proteins is shown in Fig. 3. The deduced OsGBSSII sequence showed about 85% identity to wheat GBSSII, and 61–73.3% identity to plant GBSSI sequences. The phylogenetic tree divides into four major branches and three monocot GBSSII proteins, OsGBSSII, TaGBSSII and HvGBSSII, fall into class III.

Gene structure and localization

Southern analysis using *EcoRI*, *HindIII* and *XbaI* digests gave single hybridizing bands. Four *DraI* digestion sites within the probe sequences resulted in two digested bands (Fig. 4). The results indicate that the *OsGBSSII* gene is present as a single copy in rice. The *OsGBSSII* gene was identified at a locus on chromosome 7 between anchors C479 and R10193S (49.7 cM) based on the High-Density Rice Genetic Map (<http://rgp.dna.affrc.go.jp>; Wu et al. 2002) (Fig. 5a).

The inferred exon and intron structures of the genes encoding OsGBSSI, OsGBSSII and AtGBSSI are given in Fig. 5b. These three genes showed similar exon

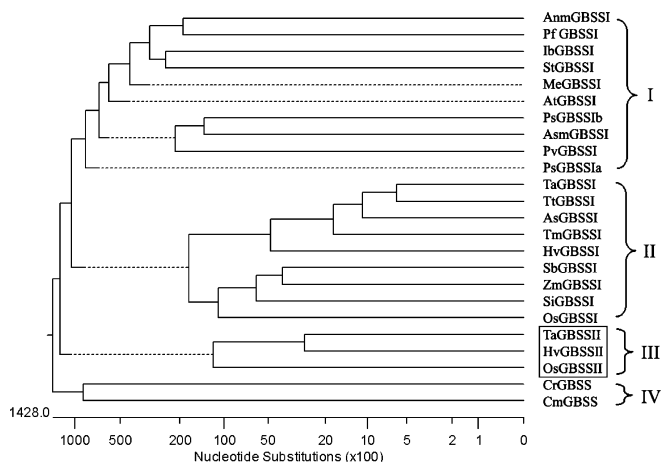


Fig. 3 Phylogenetic relationships of plant GBSS proteins. The tree was generated using the Jotun Hein method in the MegAlign program (DNASTAR). The sequences and their accession numbers in GenBank are: AnmGBSSI (*Antirrhinum majus*, AJ006293), AsGBSSI (*Aegilops speltoides*, AF110374), AsmGBSSI (*Astragalus membranaceus*, AF09792), AtGBSSI (*Arabidopsis thaliana*, AC006424), CmGBSSI (*Chamaebatiaria millefolium*, AF500400), CrGBSSI (*Chlamydomonas reinhardtii*, AF026420), HvGBSSI (*Hordeum vulgare*, AF486517), HvGBSSII (AF486521), IbGBSSI (*Ipomoea batatas*, AB071604), MeGBSSI (*Manihot esculenta*, X74160), OsGBSSI (*Oryza sativa*, X53694), OsGBSSII (AY069940), PfGBSSI (*Perilla frutescens*, AF210699), PsGBSSIa (*Pisum sativum*, AJ345045), PsGBSSIb (X88789), SiGBSSI (*Setaria italica*, AB089141), PvGBSSI (*Phaseolus vulgaris*, AB029546), SbGBSSI (*Sorghum bicolor*, U23945), StGBSSI (*Solanum tuberosum*, X83220), TaGBSSI (*Triticum aestivum*, AB019623), TaGBSSII (AF109395), TmGBSSI (*Triticum monococcum*, AF110373), TiGBSSI (*Triticum turgidum*, AB029062) and ZmGBSSI (*Zea mays*, X03935).

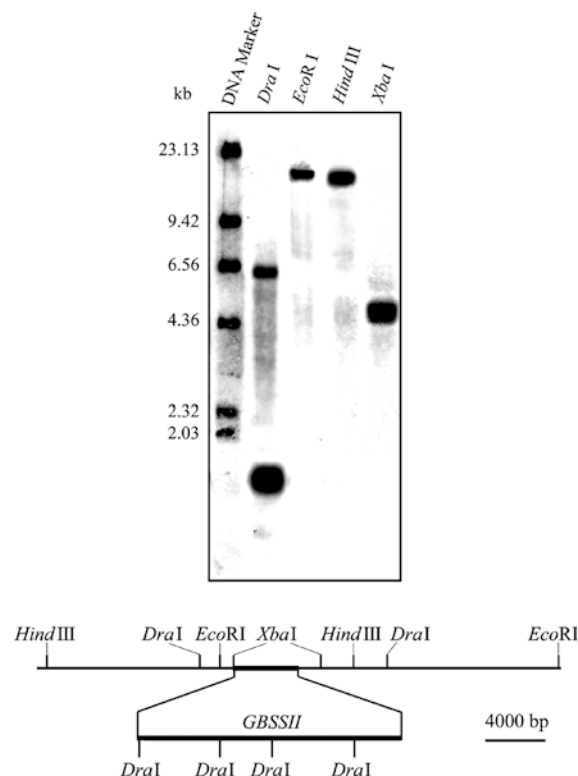


Fig. 4 Southern analysis of *OsGBSSII*. Ten micrograms of genomic DNA was digested with *DraI*, *EcoRI*, *HindIII* and *XbaI*, respectively, and hybridized with the 2,023-bp *OsGBSSII* cDNA clone. *HindIII* λ -DNA size markers are indicated. The map at the bottom shows digestion with *DraI*, *EcoRI*, *HindIII* and *XbaI*, respectively. The region covered by the cDNA probe is indicated by a thick black bar

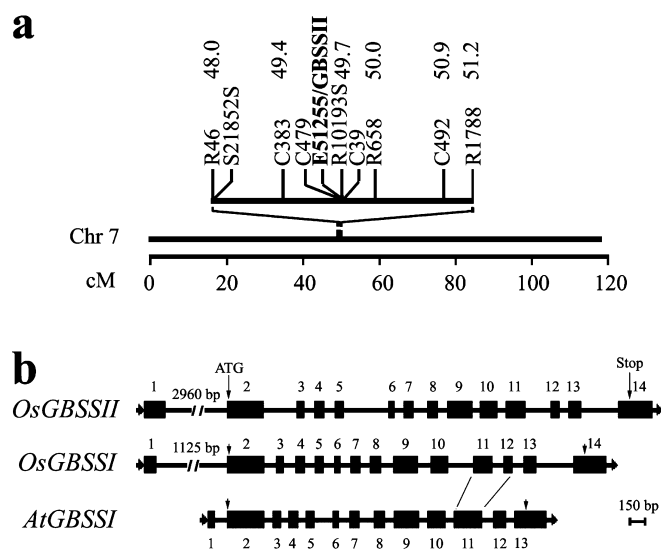


Fig. 5 a Mapping of the rice *GBSSII* gene on chromosome 7 based on RGP-RFLP-2000 Map (<http://rgp.dna.affrc.go.jp/publicdata>). **b** The exon/intron organization of rice *OsGBSSI*, *OsGBSSII* and *Arabidopsis thaliana* *AtGBSSI*. Exons are indicated as black boxes and introns are shown as solid lines. The positions of the start codon ATG and the stop codon are indicated by arrows. The genomic sequences of the *OsGBSSI*, *OsGBSSII* and *AtGBSSI* genes are from GenBank (accession numbers are X53694, AP005325 and AC006424, respectively).

organization, except that the eleventh exon in *AtGBSSI* was separated into two exons in *OsGBSSI* and *OsGBSSII*. *OsGBSSI* and *OsGBSSII* contained larger introns than *AtGBSSI*, especially at the first intron (Fig. 5b).

Expression of *OsGBSSII* in *E. coli*

To determine whether the *OsGBSSII* gene encodes starch synthase, expression of the gene in BL21 was tested. The activity of starch synthase was increased by 4.7-fold above baseline levels of BL21 glycogen synthase activity (Table 1), which confirmed that the *OsGBSSII* gene encodes the starch synthase enzyme.

Localization of OsGBSSII proteins

Polyclonal antibodies raised against OsGBSSII polypeptides were bound to the 60-kDa starch granule-bound proteins from rice leaves and endosperms (Fig. 6). The N-terminus of the 60-kDa protein from rice leaves is S-A-G-M-T-I-I-F-I-A. This sequence shared no significant similarity to the N-terminal sequence of OsGBSSI, but matched that of OsGBSSII. The results indicate that the starch granule-bound 60-kDa protein in leaves is OsGBSSII and is antigenically related to the OsGBSSI protein.

Effects of N-level on *OsGBSSII* gene expression

Western blot analysis indicated that the OsGBSSII proteins were increased and readily detectable after 8 h of N-starvation, and reached a higher level at 48 h (Fig. 7a). The amount of N-starved leaf amylose was also increased at 48 h (Fig. 7b). To investigate whether or not the inhibition of N-starvation-induced OsGBSSII expression is nitrate specific, other N sources were used in solution culture. As quantified using the program ImageQuant (Molecular Dynamics), 2 mM KNO₃, 2 mM NH₄Cl, 2 mM Glu and 2 mM Gln could each reduce the expression of *OsGBSSII* to different degrees (Fig. 7c). To confirm this result, a nitrogen re-supply test was performed. After being grown in N-free nutrient solution for 1 day, the seedlings were transferred to nutrient solutions with different N sources. The results showed again that the expression of *OsGBSSII* was

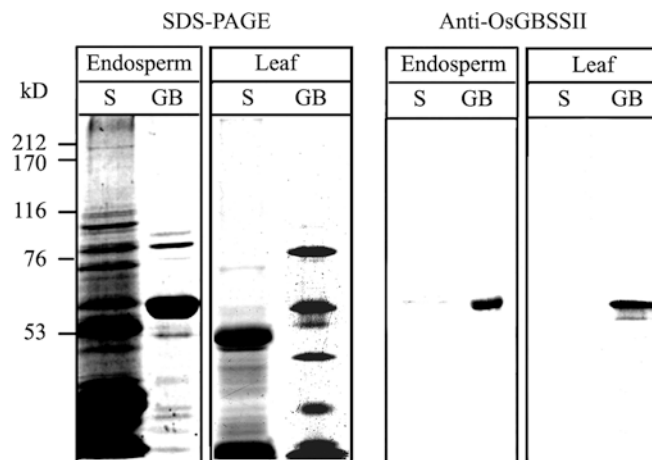


Fig. 6 Localization of OsGBSSII in rice. The proteins were separated by SDS-PAGE and immunoblotted using polyclonal antibodies raised against the OsGBSSII protein. Each lane was loaded with proteins extracted from 2 mg of starch granule (starch granule-bound protein, GB) or 40 µg of soluble proteins (S)

repressed by different N sources in rice leaves (Fig. 7d). This suggests that the expression of *OsGBSSII* is regulated by the intracellular N-level, or the ratio of carbon to nitrogen (C/N) in rice leaves. To test this hypothesis, excised leaves cultured in the dark were sampled. The result showed that the expression of *OsGBSSII* could not be induced by N-starvation (Fig. 7e). Meanwhile, the accumulation of OsGBSSII induced by N-starvation in light could be eliminated by glucosamine, an inhibitor of hexokinase (HXK; Fig. 7e). These results suggest that N-starvation-induced *OsGBSSII* expression in rice leaves is dependent on the photosynthetic product, and HXK may be involved in this regulation pathway.

Effects of sugars on *OsGBSSII* gene expression

Excised leaves were treated with 175 mM of mannitol (Mal), Glc, sucrose (Suc), 3-O-methyl-glucose (3-OMG), and 2-deoxyglucose (2-DOG), respectively, to investigate the effects of sugars on *OsGBSSII* expression. The results of RT-PCR indicated that only sugars produced a significant accumulation of *OsGBSSII* transcripts (Fig. 8a). Glucosamine eliminated the accumulation of *OsGBSSII* transcripts and OsGBSSII proteins induced by Suc (Fig. 8a, b).

Effect of the circadian rhythm on *OsGBSSII* expression

The accumulation of *OsGBSSII* transcript peaked at 4 h of light, and then decreased gradually. When the light period or dark period was prolonged to 24 h, the expression pattern of *OsGBSSII* was similar to that under a normal photoregime (Fig. 9a). To investigate whether sugar was involved in the signal transduction pathway for the circadian expression of *OsGBSSII*, excised leaves were harvested after 12 h of treatment

Table 1 Expression of starch synthase in *Escherichia coli*

Plasmid	Insert	N-terminus	Protein (mg/ml)	Activity ^a (unit/mg protein)
pET-29b (+)	No	–	1.08	12.6
pGBSSII	GBSSII	T ₈₈ ECHPWC	1.42	59.2

^aA unit of activity is defined as the formation of 1 nmol ADP per min at 30 °C using 12.5 mg/ml amylopectin as a primer

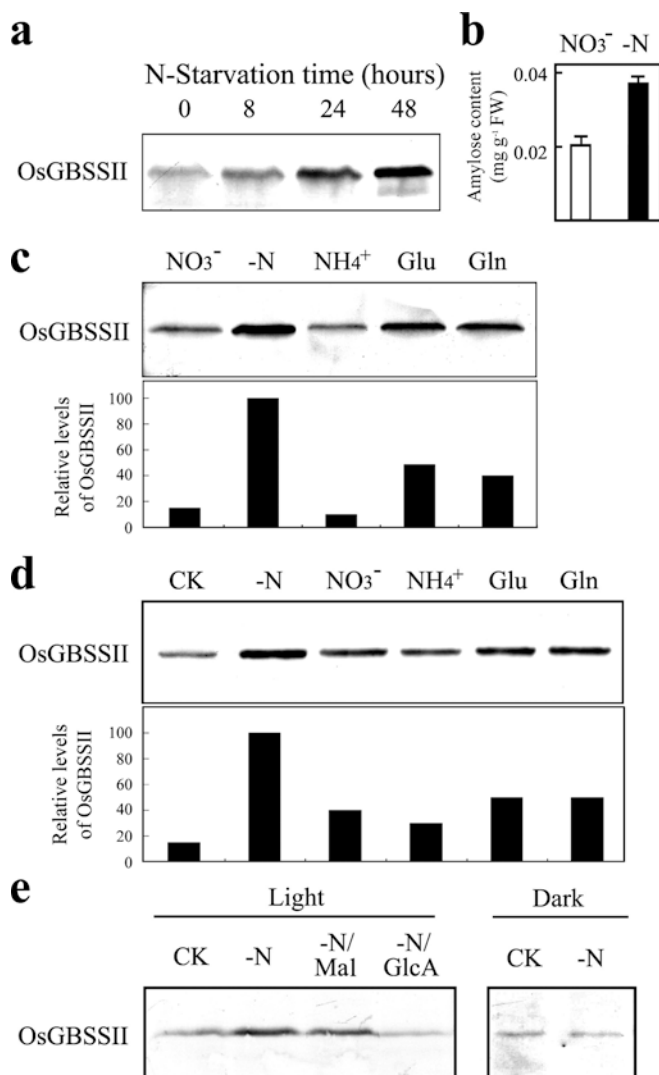


Fig. 7a–e Western analysis to clarify the regulation of *OsGBSSII* expression by the N-level in rice leaves. Proteins (2 μ l each lane) were separated on a 7.5% SDS-PAGE gel and immunoblotted with antiserum against *OsGBSSII*. **a** The seedlings were sampled after being treated for 0, 8, 24 and 48 h with N-free nutrient solution. **b** Amylose content of rice leaves. **c** The seedlings were sampled after being treated for 48 h with nutrient solutions containing 2 mM KNO_3 (NO_3^-), 2 mM NH_4Cl (NH_4^+), 2 mM glutamic acid (*Glu*), 2 mM glutamine (*Gln*), or no nitrogen (-N), respectively. **d** The seedlings were pretreated with N-free nutrient solution for 24 h and then transferred to a nutrient solution with 2 mM KNO_3 , 2 mM NH_4Cl , 2 mM *Glu* or 2 mM *Gln*, respectively, for an additional 24 h. Some seedlings were kept in normal nutrient solution (CK) or N-free nutrient solution (-N) as controls. **Bottom panels in c and d** show the correlation with *OsGBSSII* protein. Relative band areas were determined using the Image-Quant program. Maximum expression was defined as 100%. **e** The excised leaves were incubated in N-free nutrient solution (-N), with 175 mM mannitol (-N/Mal) or 175 mM glucosamine (-N/GlcA), respectively, and maintained under light or dark conditions for 14 h at 28 °C. Some excised leaves were kept in normal nutrient solution as a control (CK)

with 175 mM *Suc* in nutrient solution at various time points (Fig. 9b). As a result, *Suc*-induced *OsGBSSII* transcripts were consistent with the circadian expression pattern (Fig. 9b).

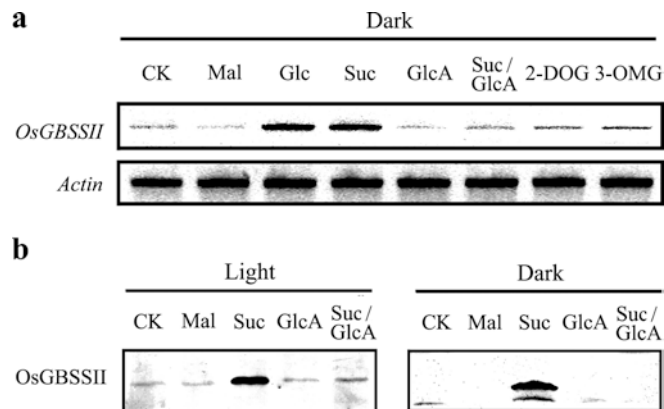


Fig. 8a, b Effects of various sugars and hexokinase inhibitor on *OsGBSSII* expression in rice leaves. **a** RT-PCR analysis of excised leaves supplied with various sugars. The excised leaves were incubated in nutrient solution containing 175 mM mannitol (*Mal*), glucose (*Glc*), sucrose (*Suc*), glucosamine (*GlcA*), sucrose and glucosamine (*Suc/GlcA*), 2-deoxyglucose (2-DOG) or 3-O-methyl-glucose (3-OMG), respectively, and maintained in the dark for 12 h at 28 °C. Some leaf cuttings were incubated in rice nutrient solution as a control (CK). A 775-bp *Actin* gene fragment was amplified as an internal control. **b** Western blot analysis was performed on total protein (20 μ l each lane) of excised leaves supplied with various sugars. Proteins were separated on a 7.5% SDS-PAGE gel and immunoblotted with antiserum against *OsGBSSII*

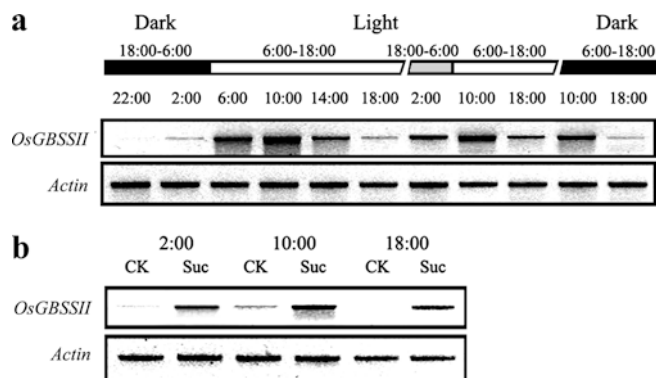


Fig. 9a, b Expression pattern of *OsGBSSII* during a 12 h light/12 h dark photoregime. **a** RT-PCR analysis of *OsGBSSII* expression. The bar at the top of the graph indicates the corresponding day (open box)/night (black box) periods. The period corresponding to night but with the light kept on is indicated by a gray box. The time points when samples were collected for RNA isolation are indicated under the bar. **b** RT-PCR analysis to clarify the effect of sucrose on *OsGBSSII* gene expression during the photoregime. The 775-bp *Actin* gene fragment was amplified as an internal control

Discussion

A cDNA clone of GBSSII was cloned from rice. The *OsGBSSII* protein was exclusively bound to the starch granule of leaves (Fig. 6). The N-terminal amino acid sequence of the 60-kDa starch granule-bound protein from rice leaves matches the deduced *OsGBSSII* mature protein (Fig. 2). The *OsGBSSII* gene has a similar exon

and intron structure to the *OsGBSSI* and *AtGBSSI* genes (Fig. 5b), and *OsGBSSII* protein is highly homologous to plant GBSS (Fig. 3). The expression of enzyme activity was observed in *E. coli* (Table 1). The chromosomal location of *OsGBSSII* is different from that of *OsGBSSI* (Fig. 5a). The deduced *OsGBSSII* protein is closely related to TaGBSSII and HvGBSSII (Fig. 3), and the region of the transit peptide is different between *OsGBSSI* and *OsGBSSII* (Fig. 2), suggesting that *OsGBSSII* protein is transported into chloroplasts in leaves while *OsGBSSI* is transported to amyloplasts in pollen and endosperm. The tissue-specific expression pattern has been observed for other starch-synthesis enzymes in rice and other cereal plants. For instance, rice starch branching enzyme RBE4 (BEIIa) is expressed in vegetative tissues, while RBE3 (BEIIb) is not (Yamanouchi and Nakamura 1992). Similarly, transcripts of the rice starch synthase II-2 (SSIIb) and starch synthase IIIa (SSIIIa) genes were detected mainly in the leaf tissue, while rice starch synthase II-3 (SSIIa) and starch synthase IIIb (SSIIIb) were expressed primarily in endosperm (unpublished data). It has been proposed that in a few cases alternative homologs have been reciprocally silenced in different organs, suggesting subfunctionalization (Force et al. 1999). Our results showed that *OsGBSSII*, *OsSSII-2* and *OsSSIIIa* are expressed in rice leaves and function as transient starch synthases, while *OsGBSSI*, *OsSSII-3* and *OsSSIIIb* function as storage starch synthases in rice.

It has been reported that nitrate could act as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Scheible et al. (1997) reported that nitrate could act as a signal to inhibit the expression and activity of AGPase, and decrease the starch content of leaves. In the present study, the *OsGBSSII* gene was isolated from the N-starvation cDNA library and 2 mM nitrate suppressed the accumulation of *OsGBSSII* transcript and *OsGBSSII* protein in rice leaves. However, the same concentration of ammonium, Glu and Gln also suppressed the expression of *OsGBSSII* in rice leaves to different degrees, respectively, suggesting that the N-level or the C/N ratio may regulate the expression of *OsGBSSII* in rice leaves. The induced expression of *OsGBSSII* by N-starvation in rice leaves under light could be eliminated by glucosamine, which inhibits the enzymatic activity of HXK (Umemura et al. 1998) and dark conditions, which suggests that the N-starvation-induced *OsGBSSII* is dependent on photosynthate and HXK.

Starch-biosynthesis genes, such as *AGPase*, *GBSSI*, *SSII*, *SSIII* and *BE*, have been reported to be up- or down-regulated by sugars (Kossmann et al. 1999; Wang et al. 2001). Three distinct sugar-sensing systems have been suggested in plants: (i) a sugar-transporter sensor pathway, (ii) an HXK sensor pathway and (iii) a glycolysis-dependent pathway (Sheen et al. 1999). Our results showed that both Suc and Glc could up-regulate the expression of *OsGBSSII* in rice leaves under light or dark conditions and that this accumulation of

OsGBSSII could be eliminated by glucosamine. The fact that a penetrating osmoticum, mannitol, has no influence on *OsGBSSII* expression suggests that this stimulation of *OsGBSSII* gene expression by sugars is not the result of osmotic pressure. The ineffectiveness of 3-O-methyl-glucose at triggering induction suggested that sugar phosphorylation might play an important role in signal transduction because it is not a good substrate for HXK (Umemura et al. 1998). Another Glc analogue, 2-deoxyglucose, can be phosphorylated by HXK, but the phosphorylated product 2-deoxyglucose 6-phosphate cannot be metabolized further, and the fact that we were unable to induce the expression of *OsGBSSII* indicated that the induction signals are generated through further metabolism of the sugars (Fig. 8). These results indicate that sugar up-regulates *OsGBSSII* through a glycolysis-dependent pathway in rice leaves.

Li et al. (1992) reported the circadian regulation of starch accumulation in sugar beet leaves. They observed that starch synthesis stopped during the night even when plants were kept under constant illumination, suggesting an endogenous circadian shift in carbon allocation at the end of the day, which diverts newly assimilated carbon to Suc synthesis and increases export at the expense of starch accumulation. There is little information available on the regulation of genes in the starch-synthesis pathway in leaves. Circadian regulation has been reported for the *GBSSI* gene in snapdragon (*Antirrhinum majus*; Mérida et al. 1999) and sweet potato (*Ipomoea batatas*; Wang et al. 2001), and for the small subunit of the AGPase transcript in green alga (*Chlamydomonas reinhardtii*; Zabawinski et al. 2001). Our results reveal that *OsGBSSII* expression in rice leaves is also controlled by a circadian clock. Transcript levels of *OsGBSSII* decreased at the end of the day and increased at the beginning of the day. This response was controlled by an endogenous circadian clock, which could be observed when the light or dark period was extended (Fig. 9). A sequence analysis revealed the presence of *cis* elements (CAANNNNATC) in the promoter region of *OsGBSSII* (data not shown), which have been previously shown to be involved in the circadian control of *Lhc* gene expression in tomato (Piechulla et al. 1998).

This circadian rhythm regulation of starch-biosynthesis genes should greatly facilitate the distribution of carbon from the source to sink tissues. Normally, leaves should accumulate higher levels of photosynthate in the light than in the dark due to active photosynthesis during the day. Thus, greater expression levels of *OsGBSSII* and other starch-synthesis genes during the day could promote the conversion of photosynthate to starch. Under high light or under nitrogen deficiency, more sugars may accumulate because of the faster rate of C-assimilation or the lower usage of carbon skeleton. Sugar then induces the expression of starch-synthesis genes to convert the sugar to starch. This mechanism should prevent the feedback inhibition of photosynthesis or osmotic stress.

In conclusion, our results suggest that two independent pathways may be involved in *OsGBSSII* expression: (i) a sugar-regulating pathway, which is glycolysis-dependent, and (ii) an endogenous circadian rhythm-regulated pathway. N-starvation-induced *OsGBSSII* expression may depend on the sugar-regulating pathway.

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