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Polymorphism in the Barley Granule Bound Starch Synthase 1 (*Gbss1*) Gene Associated with Grain Starch Variant Amylose Concentration

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Supporting Information

ABSTRACT: Granule bound starch synthase 1 (GBSS1) accumulation within starch granules and structure of *Gbss1* alleles were determined for nine barley (*Hordeum vulgare* L.) genotypes producing amylose-free (undetectable), near-waxy (1.6−4.5%), normal (25.8%), and increased (38.0−40.8%) amylose grain starches. Compared to normal starch granules, GBSS1 accumulation was severely reduced in three near-waxy, slightly reduced in two waxy, and slightly elevated in three increased amylose starches. *Gbss1* nucleotide sequence analysis for the nine genotypes distinguished them into three *Gbss1* groups with several single-nucleotide polymorphisms. A new unique Q312H substitution within GBSS1 was discovered in near-waxy genotype SB94912 with reduced amylose (1.6%) concentration relative to the other two near-waxy lines, CDC Rattan and CDC Candle (4.5%). The two waxy genotype GBSS1 showed a previously described D287V change for CDC Alamo and a new G513W change for CDC Fibar. Both amino acid alterations are conserved residues within starch synthase domains involved in glucan interaction. The increased amylose genotypes showed several unique nucleotide changes within the second and fourth *Gbss1* introns, but only SB94893 GBSS1 showed a unique amino acid substitution, A250T in exon 6. The *Gbss1* nucleotide differences were used to design genetic markers to monitor *Gbss1* alleles in genotypes with various amylose grain starches.

KEYWORDS: *amylose concentration, granule bound starch synthase gene structure polymorphism, genetic markers*

INTRODUCTION

The composition and structure of starch produced in cereal endosperm are important grain quality traits determining the suitability of grain for different applications. Grain with varied amylose concentration is of particular interest for starch industries providing products for niche markets. As compared to normal starches, amylose-free (waxy) or near-waxy starches provide improved freeze−thaw stability and form nongelling pastes suitable for food thickening and stabilization.[1] High-amylose starches have a high gelling strength and are used for the production of resistant starches and the formation of films and as adhesives in the board and paper industries. Some of the desired starch structures can be attained by chemical treatments;[2] however, these methods are expensive and use chemicals with negative effects on the environment. To develop plants with desired starch composition and structure for various food and industrial applications, it is important to identify genes that favorably affect starch biosynthesis. Genetic markers for these key alleles can then be used in marker-assisted selection to accelerate the development of genotypes with novel starch properties.

Granule-bound starch synthase 1 (GBSS1) is the major enzyme responsible for amylose biosynthesis in plants.[3] Besides producing amylose, the enzyme also has a role in extending amylopectin chains.[4,5] GBSS1 is a member of the large glycosyltransferase family, which catalyzes monosaccharide transfers, usually a nucleotide diphosphosugar or sugar phosphate, to an acceptor molecule that is part of a growing polysaccharide chain.[6] The enzyme classification for GBSS1 was initially EC 2.4.1.21 or EC 2.4.1.11, but the enzyme was reclassified to EC 2.4.1.242 when either UDP-glucose or ADP-glucose was shown to act as substrate.[7]

GBSS1 enzymes in the Poaceae family are well conserved proteins encoded by the *Waxy* locus, which extends >5 kb and carries 12 or 14 exons, of which the first exon is untranslated.[8] Twelve exons are present in barley and wheat *Gbss1*,[9,10] whereas maize, rice, and potato *Gbss1* have two extra exons,[11−13] which are present within the corresponding exons four and six of barley. The barley *Waxy* locus is located on chromosome 7H and encodes a 603 amino acid long polypeptide with a predicted 70 or 75 amino acid long transit peptide.[10,14] Like other GBSS1 produced in plants, mature barley GBSS1 is about 60 kDa and accumulates within starch granules, where it constitutes a major protein.[15]

Amylose-free starch in maize is caused by low *Gbss1* expression due to various insertions of transposable elements within the gene,[16] whereas mutations at the 5′ splice site of Wx^b first intron results in waxy rice.[17,18] In hexaploid wheat, variation in the number and composition of active *Gbss1* alleles results in varying amylose concentration in grain starch from normal (~25%) to amylose-free (waxy) starch.[19,20] Some near-waxy barley lines have a deletion within the *Gbss1* promoter,[21] which drastically reduces *Gbss1* expression.[22] A truncated GBSS1 with no enzymatic activity is produced by waxy barley cultivar Yon M Kei due to a single-nucleotide substitution

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Table 1. Barley *Gbss1*-Specific Primers Used in Study

forward	sequence	position[a]	reverse	sequence	position[a]	amplicon (bp)
Hor_wx 1F	CAAAAAGCGAAGAGGAAGGA	261−280	Hor_*wx* 1R	AGAATCGAACCAACCGAGTG	1090−1071	830
Hor_wx 2F	CACTCGGTTGGTTCGATTCT	1065−1084	Hor_*wx* 2R	CACAGGGTGTGGCTACCTG	1796−1751	704
Hor_wx 3F	CGATCAGTAGCAGTCGTCTCTC	1536−1557	Hor_*wx* 3R	ACGCACCTTCTCCAGGAAC	2266−2248	731
Hor_wx 4F	TACAAGCGCGGAGTGGAC	2207−2224	Hor_*wx* 4R	ACGAGATGTTGTGGATGCAG	2911−2892	705
Hor_wx 5F	TTTTGCTAGGTGGCCTTCTG	2875−2894	Hor_*wx* 5R	TCCGATCACTCAATCATCCA	3593−3574	719
Hor_wx 6F	CTGATAGCTCCCCGTGAGG	3550−3568	Hor_*wx* 6R	CATTGAGCCATGCAGTCTTT	4244−4225	695
Hor_wx 7F	TTGGGATTTTCACAGCAATTT	4204−4224	Hor_*wx* 7R	CACCTCCCACCATCTTTGTT	5028−5008	825

[a]Refers to *Gbss1* sequence of Vogelsanger Gold (accession X07931).

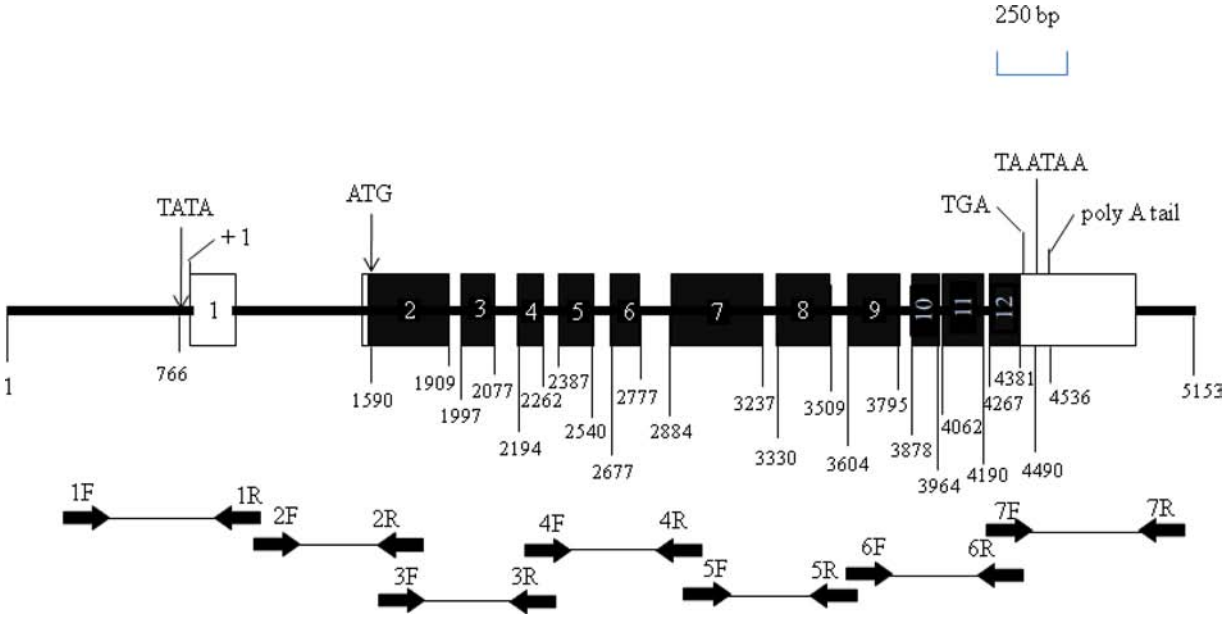

Figure 1. Structure of barley *Gbss1*. The illustration is based on the *Gbss1* sequence of Vogelsanger Gold[10] (accession X07931). Introns and nontranscribed regions are illustrated by a filled horizontal bar. Predicted exons are shown as boxes, where shadowed areas represent translated sequences. The positions of predicted TATA-box, transcriptional start site (+1), start codon (ATG), stop codon (TGA), poly-A signal (TAATAA), and polyA tail are indicated. PCR amplicons generated to assemble *Gbss1* contigs are outlined.

within the coding region.[22] In other waxy barleys which accumulate a full-length GBSS1 within starch granules, a mutation altering a critical amino acid residue at the enzymatic active site has been suggested to underlie the amylose-free starch phenotype as in barley cultivar CDC Alamo.[22]

A higher expression of *Gbss1* leads to increased (>25%) amylose production in some Bangladesh rice cultivars.[23] Other high-amylose starches are generally not due to mutations in *Gbss1*, but rather other starch biosynthetic enzymes. Thus, various mutations affecting starch branching enzyme 2 (*Sbe2*), starch synthase 2 (*Ss2*), or starch synthase 3 (*Ss3*) gene expression or their enzyme activities are known to increase amylose content in wheat,[24,25] barley,[26] rice,[27] and maize[28,29] starches. For barley, high-amylose starch (~35%) produced by Glacier AC38 barley,[30] has been associated with an *amo1* mutation, which is tightly linked to *Ss3a*.[31] Barley *sex6* mutants produce starches with 65−70% amylose, but at the expense of low starch yield and shrunken kernels.[32] Lack of SS2a production with pleiotropic effects on other starch biosynthetic enzymes is responsible for the *sex6* mutant phenotype. However, double-mutant *amo1sex6* produced significantly more starch compared to the *sex6* mutant, without affecting amylose concentration.[31]

To improve our understanding of the effects of different *Gbss1* mutations on amylose production in barley endosperm, characterization of the *Waxy* locus in nine barley genotypes producing waxy, near-waxy, normal, and increased amylose starches revealed several polymorphisms in *Gbss1*. The novel *Gbss1* polymorphisms were used to develop genetic markers for use in marker-assisted selection to develop barley genotypes with desired grain starch amylose concentration.

MATERIALS AND METHODS

Plant Materials. Ten hulless barley (*Hordeum vulgare* L.) genotypes, previously characterized for grain carbohydrate content,[33] were used in the study. All genotypes accumulate a measurable amount of amylose in starch granules, except CDC Alamo and CDC Fibar, which produce amylose-free waxy starch. Starches produced by genotypes CDC Candle, CDC Rattan, and breeding line SB94912 contain low amounts of amylose (4.5, 4.5, and 1.6%, respectively), and are considered near-waxy in this study. CDC McGwire has a normal starch phenotype with 25.8% amylose, whereas breeding lines SH99250, SH99073, and SB94893 produce starches with 38.5, 40.8, and 38.0% amylose, respectively, and are classified as increased amylose lines.

Five plants from each genotype were grown in pots under controlled conditions at 22 ± 1.0 °C and 320 μmol m^{-2} s^{-1} photosynthetic photon flux density. Leaves were harvested at the 5−

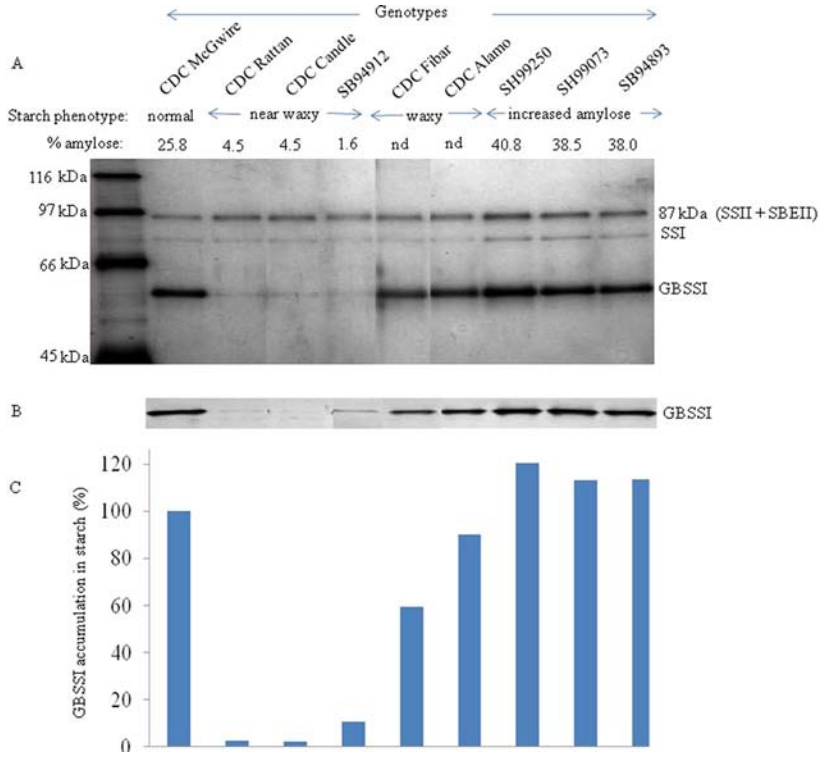

Figure 2. Analysis of GBSS1 accumulation in endosperm starch granules: (A) SDS-PAGE analysis of starch granule-bound proteins of barley genotypes with various amylose concentrations (molecular mass of protein standards is shown to the left, and migration of known starch granule-proteins is shown to the right); (B) immunoreactive signal obtained with GBSS1 antibodies; (C) relative band intensity of immune-reactive bands with normal starch genotype CDC McGwire set to 100%. (Data are based on three independent determinations.)

10 leaves stage, rinsed with distilled water and 70% (v/v) ethanol, and quickly frozen in liquid nitrogen. The plant material was stored at −80 °C and freeze-dried prior to DNA extraction.

Analysis of GBSS1 Polypeptides. Starch was purified from 10 mature barley kernels with embryos removed using a previously described method[34] with minor modifications. The embryo-free seeds were steeped in 2 mL of ice-cold water for 2 h and crushed in a microfuge tube using a small pestle. Produced seed slurry (about 200 μL) was layered onto a 1.5 mL 80% (w/v) cesium chloride solution and centrifuged at 13000*g* for 30 min. The pelleted starch granules were washed twice with 1 mL of wash buffer (55 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol), twice with 1.0 mL of water, and once with 1.0 mL of acetone and air-dried overnight. Starch granule proteins were isolated from a 5 mg aliquot of dry starch granules suspended in 150 mL of sample buffer [62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.0005% (w/v) bromophenol blue], boiled for 5 min, and immediately cooled on ice for 15 min. The protein extract was separated from gelatinized starch by centrifugation at 15000*g* for 20 min at 4 °C and further cleared by an additional centrifugation at 15000*g* for 20 min at 4 °C.

Samples of extracted proteins (5.0 μg) from starch granules were separated by denaturing gel electrophoresis, and polypeptides were visualized by silver staining as described.[34] Polypeptides separated by SDS-PAGE were transferred by vertical electroblotting onto a Hybond nitrocellulose membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA) and the identity of GBSS1 polypeptide was confirmed by protein gel blot analysis using antibodies raised against wheat GBSS1.[20] The wheat antibody reaction with barley GBSS1 was expected as the primary structures of wheat and barley GBSS1 are very similar (94% amino acid identity).

Immunoreactive polypeptides were visualized by chemical staining with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium using a BCIP-NBT kit (Bio-Rad Laboratories, Hercules, CA, USA). Semiquantitative determination of signal intensity from three independent experiments was done by densitometry scanning of protein gel blot analysis/hybridizations using the Gel-Doc Quantity One system (Bio-Rad Laboratories).

PCR Amplification of GBSS1 Gene Fragments. Genomic DNA was extracted from 200 mg of freeze-dried leaf material using a 3% (w/v) cetyl trimethylammonium bromide solution made up in 50 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.5% (w/v) polyvinylpyrrolidone, and 0.2% (v/v) β-mercaptoethanol essentially as described.[35] The DNA quantity was determined by a DU800 UV spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA).

Primers pairs specific to barley *Gbss1* locus (Table 1 and Figure 1) were selected using the Vogelsanger Gold *Gbss1* nucleotide sequence (Genbank accession no. X07932) and Primer3 software.[36] To facilitate assembly of *Gbss1* contigs, the amplicons were designed to overlap each other by at least ≥20 bp (Figure 1). The PCR reactions were performed in a 50 μL volume containing 100 ng of genomic DNA, 1.25 U *Pfu* polymerase (Fermentas, Burlington, ON, Canada), 20 mM Tris-HCl (pH 8.8 at 25 °C), 5 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1 mg/mL BSA, 0.1% (v/v) Triton X-100, 0.5 mM dNTPs, and 1.0 μM each of forward and reverse primers. The amplification conditions consisted of an initial denaturation step at 94 °C for 4.5 min followed by 32 cycles of 94 °C for 30 s, 60 °C for 30−60 s, and 72 °C for 2.0 min and a final incubation at 72 °C for 10 min. Five independent PCR reactions were performed per primer pair and genotype. Amplified DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels and isolated using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Quantification of isolated DNA was done by a DU800 UV spectrophotometer (Beckman Coulter Inc.).

***Gbss 1* Nucleotide Sequence Analysis.** Purified DNA fragments were inserted into pJET1.2 vector using the CloneJET PCR Cloning kit (ThermoFisher Scientific, Waltham, MA, USA). Ligations were transformed into chemically competent *Escherichia coli* DH5α cells and plated on Luria−Bertani solid medium containing 100 mg/mL ampicillin for growth overnight at 37 °C. Transformed colonies

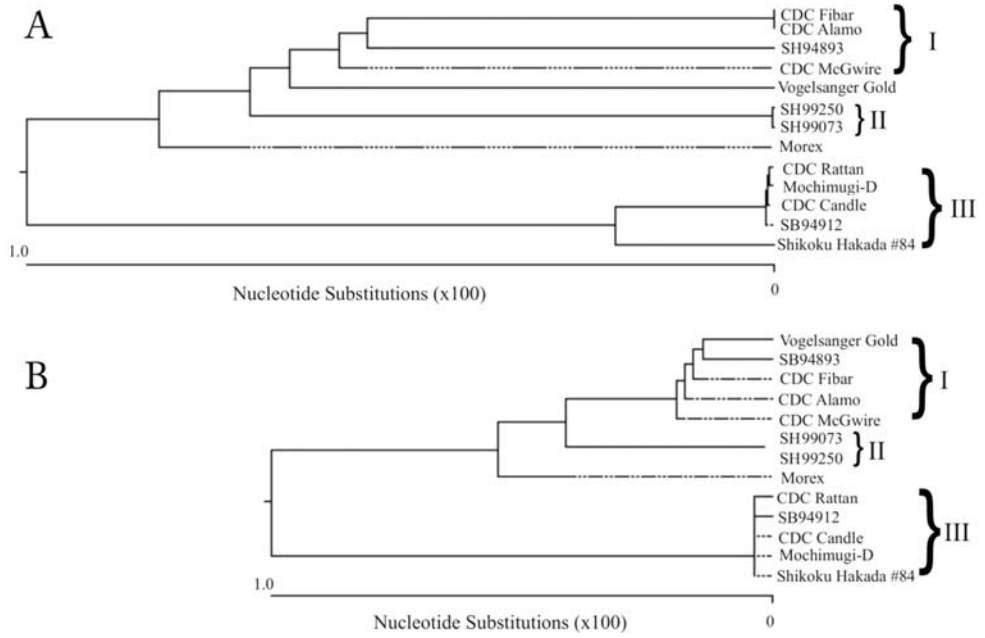

Figure 3. Phylogenetic tree showing relationships between different barley *Gbss1* genes. The trees were constructed using the ClustalW algorithm of DNAStar MegAlign module. Alignment of 5′ UTR sequences is shown in (A) and gene segment from ATG start codon to stop codon in (B). The three clades proposed are indicated.

were tested for desired insert by colony-PCR, and one positive clone per independent PCR reaction was selected. The selected clones were propagated overnight in 100 μL of 2YT medium [1.6% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5%, (w/v) NaCl, 7.5% v/v glycerol] placed in a 96-well microtiter plate at 37 °C. The cultures were frozen at −80 °C and subsequently submitted to DNA Sequencing Service Centre, Plant Biotechnology Institute−National Research Council Canada, Saskatoon, SK, Canada, for plasmid amplification and DNA sequence analysis.

The nucleotide sequences obtained from the seven DNA fragments of each *Gbss1* allele were assembled into a single contig using the SeqBuilder module of the Lasergene software version 7.1 (DNASTAR Inc., Madison, WI, USA). Alignments of contigs to *Gbss1* sequences of barley genotypes Vogelsanger Gold (X07931), Morex (AF47373), Shikoku Hakada #84 (AB088761), and Mouchimugi-D (AB087716) were done by ClustalW using the DNASTAR MegAlign module. To improve the alignments, small adjustments were done manually. Cleavage sites for transit peptides were predicted using the ChloroP 1.1 neural network (http://www.cbs.dtu.dk/services/). Searches for sequence similarities in the NCBI Genbank were done using the blastn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Putative TATA boxes were identified using plant *cis*-acting regulatory DNA elements (PLACE) database (http://www.dna.affrc.go.jp/PLACE).

RESULTS AND DISCUSSION

Analysis of GBSS1 Accumulated in Starch Granules. The nine genotypes selected for the study show various levels of amylose concentration in starch granules[33] (Figure 2). To determine the extent to which the amount of GBSS1 accumulated within starch granules correlated with amylose content, granule-bound proteins were extracted from different starches and analyzed by SDS-PAGE. CDC McGwire, producing starch with normal amylose concentration, showed a prominent granule-bound protein of about 60 kDa (Figure 2A), which was confirmed as GBSS1 by immunoblot analysis using polyclonal antibodies raised against wheat GBSS1 (Figure 2B).

Further analysis of SDS-PAGE and immunoblot revealed drastically reduced GBSS1 levels in starch granules isolated from the three near-waxy genotypes CDC Rattan, CDC Candle, and SB94912 (Figure 2A,B). A densitometric scan of the immunoblot estimated GBSS1 abundance in near-waxy starches to be ≤10% of that of the normal starch produced by CDC McGwire (Figure 2C). Low *Gbss1* expression and production of GBSS1 isoforms that were labile or poorly incorporated into starch granules were possible reasons for the near-waxy starch phenotypes. As compared to the three near-waxy starches, waxy starches of CDC Fibar and CDC Alamo showed a much greater abundance of GBSS1 (Figure 2A,B). For these two genotypes, GBSS1 accumulation was approximately 60 and 90%, respectively, of that of normal starch genotypes (Figure 2C). Starches of the three genotypes with increased amylose concentration (SB99250, SB99073, and SB94893) showed a slightly higher abundance of GBSS1 (10−15%) than normal starch genotypes (Figure 2). However, the intensities of an 87 kDa protein band corresponding to migration of SBEII and SSII polypeptides[37] and 71 kDa SSI[38] were also more intense than in the normal starch sample. One explanation for the higher abundance of certain granule-bound proteins in increased amylose starches could be due to more efficient extraction of granule-bound proteins from amylose-rich starches as compared to amylopectin-rich or normal starch. Alternatively, genes encoding starch biosynthetic enzymes including GBSS1 were more efficiently expressed or encoded with higher affinity for glucan polymers in increased amylose genotypes.

Allele Variants of *Gbss1*. The *Gbss1* sequences generated for all nine barley genotypes corresponded to nucleotides 261−5027 of the Vogelsanger Gold sequence and included 1329 bp upstream of the translational start site (position 1590−1592) to 644 bp downstream of the stop codon (4381−4383). To study the relationship between different *Gbss1* alleles, DNA sequences of the nine contigs for the genotypes with variant starch were compared to corresponding sequences derived from normal starch genotypes Vogelsanger Gold, Morex, Shikoku Hakada #84, and Mochimugi-D.

Table 2. Unique Allele Differences in Waxy, Near-Waxy, and Increased Amylose Genotypes of Barley[a]

			normal starch	near-waxy starch				waxy starch		increased amylose starch		
position[c]	region	poly-morphism	wild types[b]	CDC Rattan	CDC Candle	SB94912	Muchimogi-D	CDC Fibar	CDC Alamo	SH99250	SH99073	SB94893
261	5′	SNP	C	C	C	C	C	**G**	**G**	C	C	C
313	5′	SNP	C	C	C	C	C	C	C	**A**	**A**	C
454	5′	SNP	C	C	C	C	C	C	C	C	C	**T**
559−560	5′	indel	C-A	C-A	C-A	C-A	C-A	C-A	C-A	**CGA**	**CGA**	C-A
584	5′	SNP	A	A	A	A	A	A	A	**T**	**T**	A
623−1026	5′ + exon 1	variation	403, 408, 410, 419 nt	<----------**403 nt deletion**---------->				<----------403 nt---------->		<---------419 nt--------->		410 nt
1195−1198	intron 1	variation	ATGG/CTGG	CTGG	CTGG	CTGG	CTGG	CTGG	CTGG	**CCGGG**	**CCGGG**	CTGG
1206−1207	intron 1	indel	T-C	T-C	T-C	T-C	T-C	T-C	T-C	**TTC**	**TTC**	T-C
1225−1226	intron 1	indel	G-A	G-A	G-A	G-A	G-A	G-A	G-A	**GGA**	**GGA**	G-A
1240	intron 1	SNP	G	G	G	G	G	G	G	**C**	**C**	G
1265	intron 1	SNP	A	A	A	A	A	**C**	**C**	A	A	A
1273−1274	intron 1	indel	G-C	G-C	G-C	G-C	G-C	**GGC**	**GGC**	G-C	G-C	G-C
1291−1292	intron 1	indel	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	G-C	**CCG**
1347−1348	intron 1	indel	TC	TC	TC	TC	TC	**-C**	**-C**	**CT**	**CT**	**-C**
1372	intron 1	indel	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	A-T	**AAT**
1389−1393	intron 1	variation	TTAAT/CCATT	CCATT	CCATT	CCATT	CCATT	TTAAT	TTAAT	TTAAT	TTAAT	**TAATA**
1399−1400	intron 1	variation	AG	AG	AG	AG	AG	AG	AG	AG	AG	**GA**
1429−1430	intron 1	indel	G-A	G-A	**GGA**	G-A	G-A	G-A	G-A	G-A	G-A	G-A
1694	exon 2	SNP	G/C	C	C	C	C	G	G	**A**	**A**	G
2267−2272	intron 4	variation	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	GACCGA	–	GACCGA
2274−2295	intron 4	variation	22 nt	22 nt	22 nt	22 nt	22 nt	22 nt	22 nt	<------**22 unique nt**----->		22 nt
2297−2300	intron 4	variation	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	AGCT	–	AGCT
2303−2307	intron 4	variation	CTTT	CTTT	CTTT	CTTT	CTTT	CTTT	CTTT	**TTCCC**	**TTCTT**	CTTT
2772	exon 6	SNP	G	G	G	G	G	G	G	G	G	**A**
2990	exon 7	SNP	A	A	A	A	A	A	**T**	A	A	A
3051	exon 7	SNP	G	G	G	**T**	G	G	G	G	G	G
3935	exon 10	SNP	G	G	G	G	G	**T**	G	G	G	G
3971	intron 10	SNP	T	**C**	T	T	T	T	T	T	T	T
4536−4546	3′	SSR	$a_{(11)}$; $a_{(12)}$; $a_{(15)}$	**$a_{(14)}$**	**$a_{(14)}$**	$a_{(15)}$	$a_{(15)}$	$a_{(12)}$	$a_{(12)}$	$a_{(12)}$	$A_{(11)}$	$a_{(12)}$

[a]Polymorphism different from *Gbss1* alleles of normal starch genotypes are shown in boldface. [b]*Gbss1* alleles of Vogelsanger Gold, Morex, Shikoku Hakada #84 and CDC McGwire. [c]Based on *Gbss1* sequence of Vogelsanger Gold (accession X07931).

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An alignment of the 5′UTR segments (nucleotides 261−1589) revealed three main clades (Figure 3A). The first clade included sequences of waxy lines CDC Alamo and CDC Fibar, increased amylose genotypes SB94893, and normal starch genotype CDC McGwire. The second clade consisted of increased amylose genotypes SH99250 and SH99073, which were very closely related and showed similar nucleotide sequences. Sequences of near-waxy genotypes (CDC Rattan, CDC Candle, SB94912, and Mochimugi-D) grouped as clade three, which had a 403 nt deletion (Table 2), together with normal starch genotype Shikoku Hakada #84. The *Gbss1* 5′UTR sequences of normal starch genotypes Vogelsanger Gold showed relationships to both clades I and II, whereas the Morex sequence was closest to clade II.

When the sequence from start codon to stop codon (nucleotides 1590−4383) was analyzed, the sequence divergences were reduced (Figure 3B), but essentially the same groupings were seen as for the 5′ untranslated region. One exception was the Vogelsanger Gold sequence, which became included in clade I. The sequence alignments of the two gene fragments showed no clear distinction in *Gbss1* alleles between normal, near-waxy, or increased amylose starch genotypes. This suggested that subtle nucleotide differences for some of the genotypes could underlie the marked variation in GBSS1 accumulation and amylose concentration in grain starch. Therefore, we focused on analyzing only those allelic variations that affect the promoter region and/or starch synthase enzymatic active sites that were not observed in any of the four genotypes with normal grain starch composition.

***Gbss1* Allele Variants in Genotypes with Normal Amylose Concentration in Starch.** Alignment of the 4840 bp contig of normal starch genotype CDC McGwire to sequences of normal *Gbss1* alleles carried by Vogelsanger Gold, Morex, and Shikoku Hakada #84, respectively, was done to identify nucleotide changes that do not significantly alter amylose concentration in starch. This analysis revealed a total of 108 polymorphic sites along the *Gbss1* sequence (Supporting Information, Figure 1). The CDC McGwire allele was most similar to that of Vogelsanger Gold (94.0% identity) and Morex (93.8%) identity, whereas Shikoku Hakada #84 was more divergent (91.8% identity). The Shikoku Hakada #84 sequence differences were mainly due to a 191 bp insertion 263 bp upstream of the ATG site and a 15 bp insertion within the transit peptide coding sequence. These modifications are often found in *Gbss1* genes of Korean and Japanese germplasm with normal starch phenotypes.[14,21]

Alignment of the deduced GBSS1 amino acids for the normal starch lines revealed very high sequence identities (Supporting Information, Figure 2). The 603 amino acid long pre-GBSS1 produced by CDC McGwire was identical to that of Vogelsanger Gold. One amino acid preceding the transit peptide cleavage site differed between CDC McGwire and Morex pre-GBSS1 sequences. Comparison to the Shikoku Hakada #84 genotype revealed several sequence differences, all located close to the amino-terminal end of the preprotein (Supporting Information, Figure 2). The transit peptide cleavage sites predicted by the ChloroP 1.1 neural network (http://www.cbs.dtu.dk/services) were located between the V_{69} and S_{70} residues of CDC McGwire and Vogelsanger Gold. For the Shikoku Hakada #84 and Morex pre-GBSS1, cleavage was postulated between the V_{74} and R_{75} residues and between the V_{69} and R_{70} residues, respectively. Due to the 15 nucleotide long insertion within the coding sequence of the transit peptide, GBSS1 produced by Shikoku Hakada #84 was 5 amino acids longer (74 amino acids) than the other three normal starch genotypes (69 amino acids). These predicted transit peptides were one residue shorter than determined for the amino-terminal end of purified barley GBSS1 from cultivar Satsukinijo.[8] It is possible that the preprotein is cleaved after residue 69, but the first amino acid of the generated protein is proteolytically removed as seen for several plant proteins.[39] Except for the initial amino acid predicted, the four normal starch genotypes produced identical 60 kDa mature GBSS1.

***Gbss1* Allele Differences Associated with Variant Starch Genotypes.** The *Gbss1* contigs generated for the near-waxy lines (CDC Rattan, CDC Candle, and SB94912) were aligned to the four normal *Gbss1* allele sequences to find allele differences unique to the variant starch genotypes (Supporting Information, Figure 1). Also included in the alignment was a *Gbss1* sequence of near-waxy barley genotype Mochimugi-D producing starch with 2.3% amylose.[40] All unique nucleotide differences noted for the genotypes producing variant starches were located to four exons (2, 6, 7, and 10) and three introns (1, 4, and 10) as presented in Table 2.

***Gbss1* Allele Differences Associated with Near-Waxy Starch Genotypes.** The near-waxy genotypes in this study and Mochimugi-D were found to be nearly identical (99.8%). All shared a 403 bp deletion within the promoter region, a 191 bp insertion within intron 1, and a 15 bp insertion in the sequence encoding the transit peptide (Supporting Information, Figure 2). When compared to the Mochimugi-D *Gbss1* sequence, a few additional unique differences were noted for each of the CDC Rattan, CDC Candle, and SB94912 sequences (Table 2). The 191 bp insertion and the additional five codons within the transit peptide were not expected to affect *Gbss1* expression or activity as these insertions are also present in the *Gbss1* of normal starch genotype Shikoku Hakada #84.[22] A more severe effect on *Gbss1* expression was predicted from the 403 bp deletion, as it excluded the TATA box, transcriptional start site, and untranslated exon 1 from the *Gbss1* allele. Further on, the 403 bp deletion is associated with near-waxy starch phenotypes in several six-row Korean and Japanese barley genotypes[14] and is the only *Gbss1* sequence difference between near-waxy Mochimugi-D and normal starch genotype Shikoku Hakada #84.[14] Thus, it was concluded that the 403 bp deletion was the main reason for the near-waxy phenotypes in CDC Rattan, CDC Candle, and SB94912. The sequence alignment also suggested that the origin of the *Gbss1* alleles in Canadian near-waxy genotypes is an Asian germplasm closely related to Mochimugi-D. A likely donor of the near-waxy *Gbss1* allele is the Japanese genotype Murasaki mochi, which can be found in the pedigree of CDC Alamo.[22]

Despite the lack of TATA box signal and surrounding sequences, the near-waxy genotypes were able to produce a small amount of GBSS1. This suggests that an alternate promoter was used for *Gbss1* expression. A search for typical TATA-box signals in region upstream of ATG codon of *Gbss1* carried by near-waxy genotypes did not reveal any sequence matching typical promoter sequences present in the PLACE database (http://www.dna.affrc.go.jp/PLACE/index.html). However, a TATAAG motif matching the TATAΔ-PLM motif identified for a subset of plant promoters[41] was noted for all *Gbss1* alleles (nucleotides 1372−1377 in Vogelsanger Gold sequence). To determine if this potential alternative promoter is utilized in endosperm tissue, a full characterization

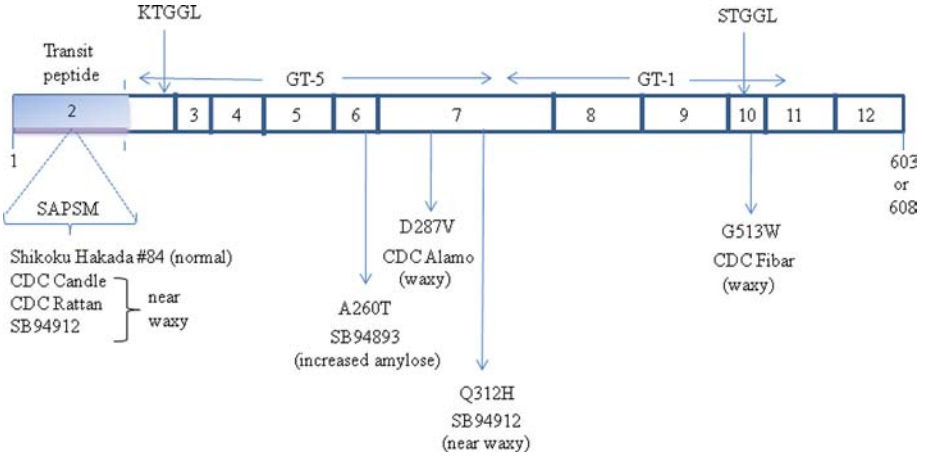

Figure 4. Amino acid variations observed for pre-GBSS1 produced by different barley genotypes. Vertical bar indicates pre-GBSS1 with shadowed area representing transit peptide. Each block of the bar indicates the corresponding exon sequence. Locations of glycosyltransferase domains 5 (GT-5) and 1 (GT-1) are indicated by horizontal arrows. Locations of conserved KTGGL and STGGL motifs with possible role in catalysis and/or substrate binding are indicated. The position of amino acid substitutions extending the transit peptide in genotypes with normal and variant starches is indicated. Amino acid substitutions within mature GBSS1 were all caused by nucleotide divergences from normal *Gbss1* alleles.

of *Gbss1* transcripts produced in normal and starch mutant genotypes needs to be studied.

The nucleotide differences observed within intron 10 and at the 3′ end of CDC Rattan (Table 2) did not affect any sequence that appeared to be critical for *Gbss1* expression. Nor could we find any critical region altered by two SNPs observed for CDC Candle within intron 1 and at the 3′ end, respectively (Table 2). Thus, these unique sequence differences noted for CDC Rattan and CDC Candle were not expected to cause additional effects on *Gbss1* expression than the reduction caused by the 403 bp deletion in the promoter region. For SB94912, the 403 bp deletion and a unique SNP at position 3051 were the only *Gbss1* sequence differences from the normal *Gbss1* haplotype of Shikoku Hakada #84 (Table 2). The unique SNP identified created a *Sph*I restriction site within exon 7 and altered a glutamine residue to histidine in the SB94912 GBSS1 amino acid sequence. The significantly lower amylose concentration in starch of SB94912 (1.6%) as compared to CDC Rattan (4.5%), CDC Candle (4.5%), and Mochimugi-D (2.3%) suggested that the Q312H change had a negative effect on GBSS1 activity. A spontaneous mutation in a Mochimugi-D type *Gbss1* allele or recombination with an allele encoding the H_{312} residue may have created the *Gbss1* haplotype of SB94912.

***Gbss1* Allele Differences Associated with Waxy Genotypes.** The sequence alignment of *Gbss1* contigs and published sequences revealed five polymorphic sites between the *Gbss1* alleles of waxy genotypes CDC Fibar and CDC Alamo and the normal *Gbss1* haplotypes (Table 2). Four of the five sequence divergences were shared between CDC Fibar and CDC Alamo and were all located within a 107 bp region upstream of the TATAΔ-PLM motif (nucleotides 1372−1377) in intron 1. It is possible that one or several of these nucleotide changes affect *Gbss1* transcription, which from a semi-quantitative analysis appears to be higher in CDC Alamo as compared to normal starch barley genotypes.[22] Two unique sequence divergences for *Gbss1* of CDC Alamo and CDC Fibar, respectively, were identified within exons 7 and 10, respectively (Table 2).

An A_{2990} to T substitution in CDC Alamo introduced a *Drd*I restriction site and caused a D287V alteration in GBSS1 amino acid sequence (Supporting Information, Figure 2). CDC Fibar carried a different SNP at position 3935, producing a *Sex*A1 site and causing an amino acid G513W alteration in encoded GBSS1.

***Gbss1* Allele Differences Associated with Increased Amylose Genotypes.** Many unique sequence differences for *Gbss1* were found for the increased amylose genotypes (Table 2), which appeared to accumulate slightly increased amounts of granule-bound proteins within starch granules (Figure 2). Most of the *Gbss1* sequence differences were common to genotypes SH99250 and SH99073 and positioned in the 5′ region and within introns 1 and 4. Genotype SB94893, which had its own set of unique sequence differences, also showed polymorphism within intron 1. The nucleotide sequence differences within intron 1 are of interest as they could have a regulatory function for *Gbss1* expression from the normal promoter and/or the alternate promoter. Whether temporal or spatial expression of *Gbss1* is altered in increased amylose genotypes needs to be determined, before any conclusion can be drawn about the observed allelic differences. Nevertheless, one unique sequence difference from normal *Gbss1* was seen for *Gbss1* in genotype SB94893. A nucleotide variation at position 2772 eliminated an *Eae*I site and caused A250T alteration in GBSS1.

Structural Features of Barley GBSS1. The pre-GBSS1 produced by the normal starch genotype CDC McGwire was 603 amino acids long, and the same number of amino acids was predicted for pre-GBSS1 of increased amylose genotypes and the two waxy genotypes (CDC Alamo and CDC Fibar). A slightly longer pre-GBSS1 (608 amino acids) was produced by the near-waxy genotypes CDC Candle, CDC Rattan, and SB94912 due to a common 15 bp insertion within exon 1. The extra five codons lie within a sequence encoding the transit peptide and also did not affect the site for transit peptide, but did not alter the predicted cleavage site. As the longer transit peptide is produced in barley lines with normal starch composition (e.g., genotype Shikoku Hakada #84), the five extra amino acids within the transit peptide are not likely to affect targeting of GBSS1 to plastids or abundance in starch granules.

Four different amino acid substitutions were identified for mature GBSS1 (Figure 4). Three of the amino acid alterations were positioned within the glycosyltransferase 5 domain of

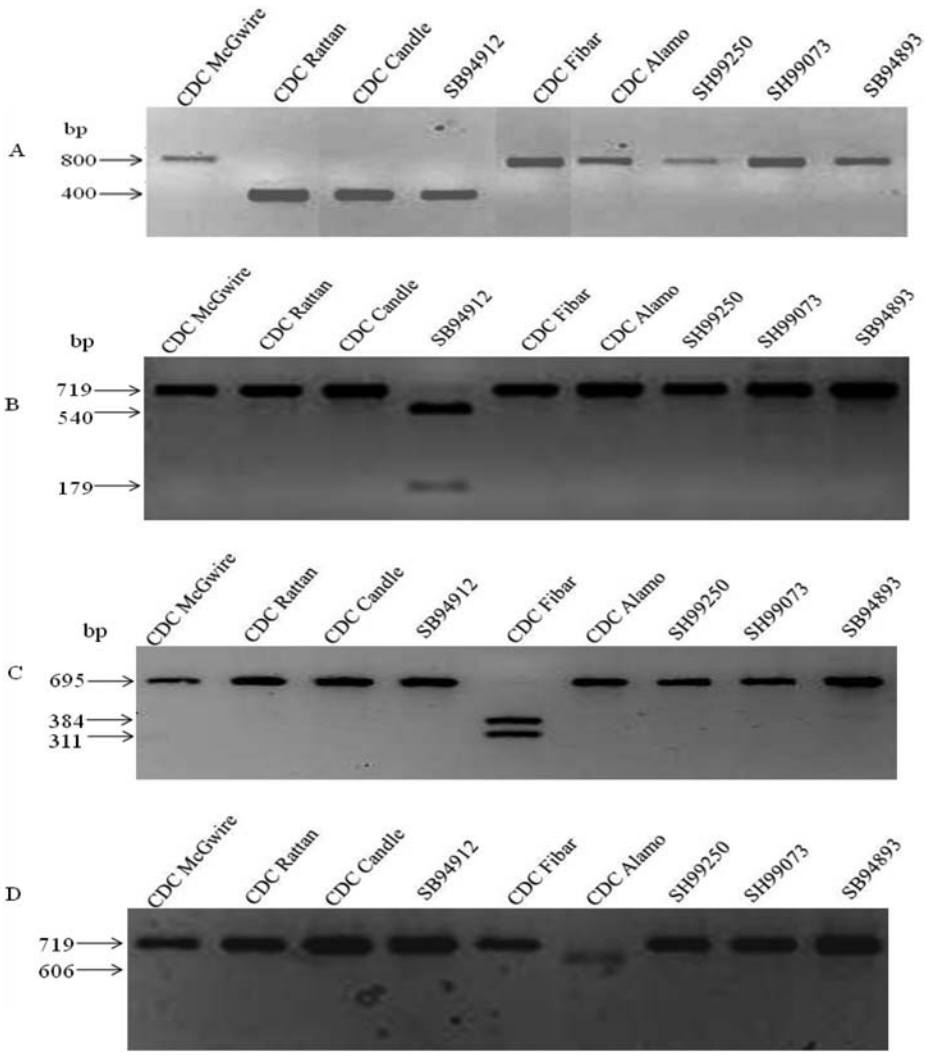

Figure 5. Genetic markers developed for *Gbss1* alleles: PCR assay for promoter deletion in near-waxy *Gbss1* alleles carried by CDC Rattan, CDC Candle, and SB94912 (A); Q312H substitution in GBSS1 of SB94912 (B); G513H substitution in GBSS1 of CDC Fibar (C); and D287V substitution in GBSS1 of CDC Alamo (D).

GBSS1, whereas the fourth substitution was located to the glycosyltransferase 1 domain. The single amino acid substitution A250T in GBSS1 produced by increased amylose genotype SB94893 is positioned immediately in front of α-helix IH5[43] (Supporting Information, Figure 2). Most GBSS1 enzymes of barley, wheat, rice, *Arabidopsis*, *Chlamydomonas* and *Ostreacoccus* carry an alanine residue at this position, whereas isoforms of SSI, SSII, and SSIII of the same species have alanine, serine, threonine, glycine, or proline residues.[42] Thus, the A250T could have a role in GBSS1 enzyme specificity. The α-helix IH5 is followed by a loop denoted 380s, which is highly variable among starch synthases and speculated to be involved in the open-closed transitions upon binding of ADP-glucose.[42] The conserved D_{287} residue within the 380s loop is altered by the CDC Alamo D287V substitution, which may affect the catalytic properties of the enzyme.[42,43] Therefore, the amylose-free starch produced by CDC Alamo is likely due to an inactive GBSS1 as previously suggested.[22] Close to the CDC Alamo D287V substitution, but outside the 360s loop, lies the amino acid substitution Q312H observed for GBSS1 of near-waxy SB94912. The polar uncharged amino acid change to a positively charged residue involves a conserved glutamine residue, supposedly important for binding to substrate or target. Despite the 403 bp deletion in the promoter region and the Q312H change in encoded GBSS1, the SB94912 genotype was able to produce a small amount of amylose. In contrast, the waxy genotype CDC Alamo, which shows near-normal GBSS1 production, did not produce any amylose. This suggests that the D287 residue is much more important for GBSS1 activity than Q312.

The second domain of GBSS1 was affected by a mutation carried by waxy genotype CDC Fibar. For this genotype, the amino acid change G513W is positioned in the C-terminal end that is unique to GBSS1 enzymes.[42] This region of GBSS1 carries a STGGL sequence that is similar to the N-terminal KTGGL motif identified as binding site for ADP glucose in bacterial glycogen synthase.[44] The substituted residue in CDC Fibar was positioned 10 residues downstream of the STGGL motif, and it is the likely cause of GBSS1 inactivity and production of amylose-free starch in the endosperm of CDC Fibar.

The mutation observed in CDC Alamo has been described earlier and exists in several barley genotypes.[22] The other

amino substitutions observed for GBSS1 of SB94912, CDC Fibar, and SB94893 have not been previously described.

Genetic Markers for *Gbss1* Mutant Alleles. Genetic markers for genes of interest are important tools for breeding, as the desired gene combination can be screened early in germplasm development. Thus, markers significantly reduce the time and cost for cultivar development and have become routine in breeding programs. To efficiently monitor *Gbss1* alleles characterized in this study, four genetic markers for positive identification of mutant alleles were developed. The presence of the near-waxy *Gbss1* allele can be screened by PCR amplification using primer pair Hor_wx1F and Hor_wx1R (Table 1). In this assay, genotypes with the 403 bp promoter deletion (CDC Rattan, CDC Candle, and SB94912) produced a 400 bp PCR product, whereas normal or increased amylose starch genotypes produced an 800 bp fragment (Figure 5A). The presence of the Q312H substitution, which potentially reduces amylose concentration in SB94912, can be identified by a cleaved amplified polymorphic sequence (CAPS) marker. PCR amplification with primer pair Hor_wx5F and Hor_wx5R, followed by restriction with *Sph*I, is diagnostic for the SB94912 *Gbss1* allele, which produced a 179 bp fragment in the assay (Figure 5B). The G513W amino acid substitution encoded by waxy starch genotype CDC Fibar can be screened by using primer pair Hor_wx6F and Hor_wx6R and restricting the product with *Sex*AI to give a cleaved product if CDC Fibar *Gbss1* mutation is present (Figure 5C). A CAPS marker for the D287V substitution encoded by *Gbss1* of waxy starch genotype CDC Alamo was obtained by PCR amplification with primer pair Hor_wx5F and Hor_wx5R and restriction with *Drd*I (Figure 5D). Production of a 606 DNA fragment was indicative of *Gbss1* mutation carried by CDC Alamo. No positive diagnostic assay for mutation causing A250T substitution in *Gbss1* of SB94893 was tested. However, the normal *Gbss1* alleles harbor a recognition site for endonuclease *Eae*I at the site of mutation in SB94893 *Gbss1* allele. Thus, PCR amplification using primer pair Hor_wx4F and Hor_wx4R and restriction with *Eae*I would theoretically give a cleaved product for normal *Gbss1* alleles, but not for the SB94893 *Gbss1* allele.

The detailed nucleotide sequence analysis of *Gbss1* from barley genotypes with various grain starch amylose concentrations revealed considerable nucleotide sequence heterogeneity. Three genotypes (CDC Rattan, CDC Candle, and SB94912) showed severely reduced GBSS1 production and amylose concentration due to a 403 bp deletion abolishing the main *Gbss1* promoter, but not affecting a weaker alternative promoter. Similar promoter deletions have been reported for a few other near-waxy barley genotypes.[14,22]

The transit peptides encoded by the various genotypes were of different lengths, where a five amino acid longer transit peptide was proposed for pre-GBSS1 of near-waxy genotypes and the normal starch genotype Shikoku Hakada #84. However, the length of the transit peptide was not expected to have any effect on amylose production. Amino acid substitutions D287V in Alamo and G513W in Fibar were identified as the likely causes of inactive GBSS1 resulting in no amylose in starch granules. A Q312H substitution in GBSS1 of near-waxy genotype SB94912 may, in addition to the promoter deletion, reduce GBSS1 activity and amylose production in endosperm to more reduced concentration as compared to other near-waxy genotypes.

Three genotypes (SB99250, SB99073, and SB94893) with increased amylose concentration seemed to accumulate a slightly increased amount of GBSS1 and other starch biosynthetic enzymes in starch granules, but the reason behind the higher abundance could not be established. The *Gbss1* sequences of increased amylose genotypes displayed many unique sequence differences within introns when compared to normal *Gbss1* alleles. Whether these nucleotide alterations altered temporal or spatial expression of *Gbss1* during endosperm development needs to be determined by additional studies of *Gbss1* expression. It is also possible that mutations in *Gbss1* do not cause increased amylose phenotypes for any of the three genotypes studied. The increased GBSS1 may merely be an effect of a mutation in another starch biosynthetic gene. As pleotropic effects are common for starch mutants,[27,45] they may explain the simultaneous increase of GBSS1, SSI, and 87 kDa polypeptides (SSII and/or SBEII) observed from SDS-PAGE (Figure 2). The altered starch granule profile observed for increased amylose starches in the study showed some resemblance to that of a rice high-amylose mutant, where production of both SSI and GBSS1 is increased due to SSIIIa absence.[46] Increased levels of not only SSI but also SSII and SBEIIb were seen in a proteome analysis of barley high-amylose mutant *amo1*.[47] In contrast, reduced amounts of SSI, SBEIIa, and SBEIIb in starch granules are observed in high-amylose *sex6* barley mutants, which lack production of SSIIa.[32] Thus, variation in protein−protein interactions between different starch biosynthetic enzymes or phosphorylation status may underlie many of the pleotropic effects seen for starch mutants[48,49] and will be important to study in future experiments of increased amylose genotypes. To facilitate studies of *Gbss1* expression, positive assays for four allele variants were developed in this study. These markers may also be useful for monitoring introgression of various *Gbss1* alleles in barley improvement programs.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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