Molecular and Chemical Characterization of a New *Waxy* Allele in Barley (*Hordeum vulgare* L.)

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ABSTRACT

Barley *m38* mutant was selected for its high level of mixed-linkage (1,3),(1,4)-β-D-glucan (MLG) in the grain. This elevated level of MLG was found to be associated with decreased amylose accumulation as well as other chemical composition alterations. Molecular characterization results revealed *m38* as a new allele of the *Waxy* gene, encoding an endosperm-specific granule-bound starch synthase I (GBSSI). Additional mapping data from amylose phenotype and GBSSI gene specific markers supported the conclusion of the GBSSI mutation in *m38*. The *m38* locus contains a nucleotide alteration that would result in the substitution of glycine at position 263 with serine in the putative adenosine-5′-diphosphate-glucose binding domain. This amino acid substitution alters loop structures on the exterior surface of the folded protein and may affect its enzyme activity. Characterizations of *m38* in this report provide for a new allele of the *Waxy* gene and additional evidence of pleiotropic effects on other chemical components including increased MLG, fructans, and fats and decreased amylose and protein.

Mixed-linkage (1,3),(1,4)-β-D-glucan (MLG) exists as a major noncellulosic polysaccharide in cereal crops including barley, oats, rye, and wheat (Smith and Harris 1999). MLG has become an important target trait for barley and oat quality improvement because of its well-known benefits to human health. In genetic studies of MLG, quantitative trait loci (QTL) have been identified for their contributions to the grain MLG content in barley (Han et al 1995; Igartua et al 2002; Molina-Cano et al 2007; Li et al 2008; Fincher 2009). Specific cellulose-synthase-like (*Csl*) genes *CslF6* and *CslH1* demonstrated their function in MLG synthesis in transgenic studies and are located on 7H and 2H, respectively (Burton et al 2006; Doblin et al 2009). The zero MLG in grain from a knock-out mutation of *CslF6* in barley further confirmed the crucial function of the gene in MLG biosynthesis (Tonooka et al 2009). Thus, the QTLs for MLG are defining genes that could be either directly (as with *CslF* and *CslH* genes) or indirectly involved by pleiotropy in MLG synthesis and regulation.

In addition to the *Csl* genes mentioned, genetic studies have also revealed roles of other genes in the MLG content accumulation in barley grains. Mutant lines Risø 13, Risø 16, and Risø 29 were reported to have increased MLG levels and reduced grain starch and seed weight (Tester et al 1993; Munck et al 2004). The gene in Risø 13 was shown to encode the adenosine-5′-diphosphate-glucose (ADP-Glc) transporter (Patron et al 2004), which is responsible for ADP-Glc transportation from the cytosol to the plastids of endosperm, where starch synthesis takes place. The Risø 16 mutant resulted from a mutation in the gene coding the small subunit of the cytosolic ADP-Glc pyrophosphorylase (Johnson et al 2003), a key enzyme regulating the rate of starch biosynthesis. Both Risø 13 and Risø 16 mutants are actually defective in starch synthesis. The elevated MLG levels in both mutants may be caused either by the relative increase in the cell wall percentage as a result of less starch in the shrunken endosperm cell or by a pleiotropic effect of the mutant genes. The pleiotropic regulation mechanism can be used to improve a targeted phenotype such as MLG because genotypes containing as high as 11–20% have been bred for food barley (Fujita et al 1999; Munck et al 2004).

Granule-bound starch synthase I (GBSSI) has been related to starch and MLG in grains. The reduced starch in the waxy endosperm was usually accompanied by a small increase in sugars and MLG (Ullrich et al 1986; Newman and Newman 1992; MacGregor and Fincher 1993; Xue et al 1997; Hang et al 2007). However, evidence also showed that a high-amylose mutation can increase MLG content (Mann et al 2005). Another example is the high-amylose mutation of the *amo1* gene (Glacier AC38), which has 6.3% MLG compared with 5.2% in the parent (Oscarsson et al 1996). Thus, most of the waxys are slightly elevated in MLG, including the *m38* mutant in this report.

All the waxy-endosperm barley lines are caused by the disruption of the *GBSSI* gene. Three mutant alleles of the *GBSSI* gene are identified in waxy barley lines. Those three alleles include the promoter region deletion, a point mutation at the 580 position of the coding sequence leading to premature protein product, and a nonsynonymous mutation at the 860 position of the coding sequence (Domon et al 2002; Patron et al 2002). In this study, we report the characterizations of the *m38* gene mutation, its effects on grain composition, and evidence indicating that *m38* is a new mutant allele of the *GBSSI* gene.

MATERIALS AND METHODS

Plant Materials. The *m38* mutant was isolated from sodium ethyl methanesulfonate (EMS) M2 seeds of barley cultivar Harrington by screening for high MLG contents in the grain. The mutant was backcrossed once to the parental line of Harrington. The 78 BC1F3 families harvested from the field in 2008 were tested for MLG. Eight lines with similar MLG content of *m38* were pooled to represent the mutant line, and eight lines with similar MLG content were pooled to represent the wild-type line. Those lines were used for characterizations and comparison of grain components between *m38* and its wild type.

Plants of *m38* and the wild-type lines were grown under field conditions for grain characteristics in 2009 at Aberdeen, Idaho. Both mutant and wild-type lines were planted in three replicates of 1.5 × 3.0 m plots. The plots were under irrigation and fertilized as with the normal field management of farmland in the region. The mutant and wild-type lines were also planted in greenhouse conditions in 2010 at Aberdeen, Idaho. Plants were grown in the greenhouse with two replicates of one pot with two plants.

*The *e*-Xtra logo stands for "electronic extra" and indicates that a supplemental figure appears online.

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A mapping population was created by crossing *m38* to Steptoe. Harrington and Steptoe are the barley cultivars with normal amylose content. The F1 seeds were planted in a greenhouse, and F2 seeds were harvested by plant. All F2 seeds were planted in the Aberdeen field in 2009, and F3 seeds from each plant were harvested individually. Eighty-seven homozygous F3 families for both wild-type and mutant lines based on the consistent parental type of MLG contents for *m38* × Steptoe were selected as mapping populations.

MLG, Amylose, and Sugar Analyses. The contents of MLG and amylose were measured as described previously (Hu and Burton 2008; Hu et al 2010). Fructans were measured with a commercial kit (K-FRUC, Megazyme, Bray, Ireland). The measurement was performed according to the instructions in the manual accompanying the kit. Glucose was determined as for the MLG measurement with the exception of digestion by lichenase (Hu and Burton 2008). All measurements were performed with a Synergy HT autoreader (BioTek Instruments, Winooski, VT, U.S.A.).

Other Chemical Analyses of Barley Grains. In a different experiment, all samples were analyzed for total lipid by an Ankom XT10 fat analyzer (Ankom Technology, Macedon, NY, U.S.A.) following AOCS official method Am 5-04. Total starch was analyzed with a Megazyme total starch kit. Protein was measured by a combustion method (AOAC 2002), with a protein analyzer (FT528, Leco, St. Joseph, MI, U.S.A.). Crude fiber content was measured by AOCS approved procedure Ba 6a-05 (Ankom Technology method 10) with an Ankom 2000 fiber analyzer. Protein content was calculated considering 1 g of nitrogen produced from 6.25 g of protein. All other uncharacterized components were classified as others.

Hardness was measured with a single-kernel characterization system (4100, Perten Instruments, Reno, NV, U.S.A.). One hundred grains were selected for each sample and were reported as means and standard deviations of hardness index.

Molecular Analyses. For DNA and RNA extraction, seeds of plant materials were germinated and grown in the greenhouse. Root and seedling tissues were collected at the 3–4 leaf stage. Tissue samples were immediately submerged in liquid nitrogen after harvesting and later transferred into a –80°C freezer for storage. For developing seed tissue samples, entire inflorescences were collected at the 2–3 week stage after pollination.

DNA Extraction and Polymerase Chain Reaction (PCR) Amplifications. Total genomic DNA of each line was extracted from freeze-dried tissue samples following the method described by Pallotta et al (2003) with the exception that SDS was replaced by cetyltrimethyl ammonium bromide buffer in the protocol. The DNA concentration was measured with a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, U.S.A.) and adjusted to 40 ng/μL for PCR. PCR for the designed markers was performed as 25 μL volume containing 1 μL of DNA, 1 μL of each primer at 10μM, 2.5 μL of 10× buffer, 1 unit of Taq DNA polymerase (Sigma, St. Louis, MO, U.S.A.), 2 μL of deoxynucleotide triphosphates at 1.25mM for each, and water to 25 μL total volume. The enzyme used in cloning was iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA, U.S.A.).

RNA Extraction and cDNA Synthesis. Total RNA was extracted from frozen tissue with RNeasy kits from Qiagen (Valencia, CA, U.S.A.). RNA samples were tested on 1% agarose gel for quality evaluation based on appearance of RNA degradation, and RNA concentration was measured in a spectrophotometer. The reverse transcription was performed according to the manufacturer's manual supplied with the enzyme. The reverse transcription reaction solution was diluted with water to provide a 1:10 ratio for cDNA amplification.

DNA clones were sequenced by the Idaho State University Core Facility Center at Pocatello, Idaho. Sequence alignments were performed with the online software ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2). Conformation images of the Waxy protein were generated with Modeller software (Chen et al 2006).

Statistical Analysis. The *t*-test analysis was performed for comparisons between *m38* and wild-type grains for each chemical composition with the statistical analysis function in Excel. The average value of each component analysis was reported. The *P* value for the *t* test was also reported.

RESULTS

Phenotypic Characterization of the *m38* Mutant. Line *m38* was selected as a high-MLG mutant from a barley cultivar Harrington population mutagenized with EMS. The mutant plants appeared normal under field growth conditions (Fig. 1A). However, the chemical characterization of grains showed an elevated MLG level of *m38* in repeated measurements, ranging from 7.5 to 9.2% depending on growth conditions, as opposed to 5.0 to 6.2% in the wild-type Harrington plants under the same growth conditions. To eliminate the effects from the genome background, mutant and wild-type lines of *m38* were developed from a backcross with Harrington. Grain quality characterization showed that the

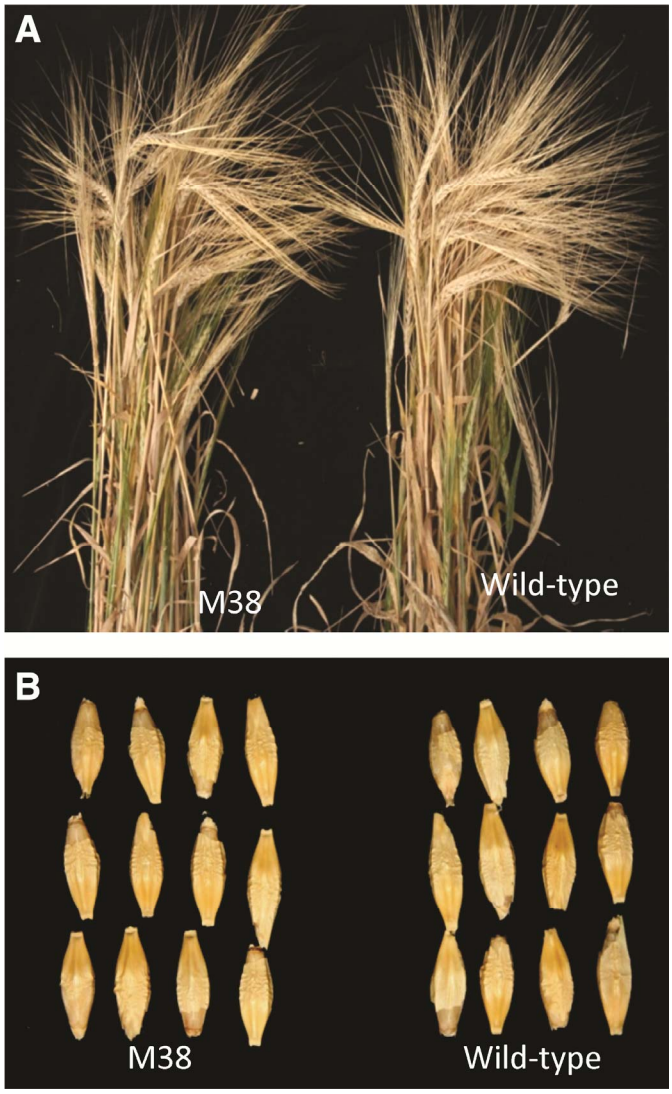

Fig. 1. Kernel and plant morphology of *m38* and the wild-type line. The plants and grains were collected from the 2009 Aberdeen field. **A,** Both *m38* and the corresponding wild-type plants did not show any clear difference in the field growth condition. **B,** No significant differences in kernel size and shape were observed between *m38* and its corresponding wild-type near-isogenic line.

m38 mutant contains 8.1% MLG, whereas the wild-type plants have only 5.2% (Table I). One of the major changes in grain quality was a drastic reduction in amylose content, dropping from 20.1% in the control to 8.4% in the mutant (Table I). Other grain quality compositions including starch, ash, and soluble glucose remained statistically the same in *m38* plants (Table I). We noticed that *m38* had a decrease of 2.3% in protein content, which was close to the 2.9% increase in MLG in the mutant (Table I). This observation may represent an example of negative correlation between MLG and protein content. It should be noted that the negative relationship between MLG and protein was a preliminary observation; more studies are needed to draw a final conclusion. Other chemical changes in *m38* grains include increases in fructans and crude fiber (Table I). All characterizations were performed with grains harvested in the 2009 field and 2010 greenhouse.

Analyses of physical properties showed that the *m38* mutant had virtually the same kernel hardness, diameter, test weight, and plumpness compared with the control plants, although statistical analysis was not performed (Table II). The similarities between *m38* and its corresponding wild-type line were consistent with their grain appearance (Fig. 1A). Taken together, our data indicated that the *m38* mutant maintained most seed characters of its corresponding wild-type line, except for the contents of MLG, amylose, fructans, crude fiber, and protein. The similarity in starch content and test weight suggested that the elevated level of MLG in *m38* was not caused simply by the reduced volume in the shrunken endosperm. Our data on soluble sugar contents indicated no change in glucose, but the increasing fructans indicated a shift of component biosynthetic directions (Table I). Accumulation of fructans and MLG with reduction in protein content in *m38* further showed the complex interactions among the multiple metabolic pathways beyond starch composition.

TABLE I
Grain Chemical Compositions of *m38* Mutant and the Corresponding Wild-Type Plants Obtained from Grain Samples[a]

Components	Wild-Type	*m38*	Standard Error	Test Replicates/ Sample	*P* Value
Test 1					
Moisture %db	8.94	8.69	0.43	6	0.7120
Protein %db	17.4	15.1	1.04	6	0.0250
Starch %db	58.9	57.7	2.29	6	0.3070
Oil %db	2.28	2.61	0.23	6	0.0940
Crude fiber %db	4.09	4.98	0.08	6	0.0200
Ash %db	2.22	2.28	0.23	6	0.9060
Others %db	6.2	8.6	…	6	…
Test 2					
Fructans %db	1.1	2.0	0.09	4	<0.0001
MLG %db	5.2	8.1	0.29	4	<0.0005
Amylose %starch	20.1	8.4	0.75	4	<0.0001
Soluble glucose %db	0.09	0.09	0.01	4	0.5000

[a] Grain samples for all analyses in the table were harvested from plants grown in the field in Aberdeen, ID, in 2009 and in a greenhouse in 2010. Extra samples harvested from the Aberdeen field in 2010 were analyzed for the components in test 1. Values were reported in dry mass based (db) percentages except amylose in each category. Data are reported as the average measurement value, standard error, number of biological replicates for each sample, and the *P* values calculated by *t* test under the assumption of equal variances in two biological replicates. MLG = mixed-linkage (1,3),(1,4)-β-D-glucan.

TABLE II
Physical Characteristics of *m38* and the Wild-Type Grains[a]

Material	Hardness Index[b]	100 Kernel Weight (g)	Kernel Diameter (mm)
Wild-type	61.55	5.30	2.50
m38 mutant	63.05	5.15	3.10
Standard error	2.62	0.18	0.50
P value	0.6788	0.2519	0.8195

[a] Data were obtained from 2009 field-grown grain samples. The value was reported as mean of two separate measurements for each category in the table. Standard error and the *P* values are from the Student *t* test.
[b] Measured with a single-kernel characterization system (mean, *n* = 100).

Genotypic Mapping of the *m38* Mutation into the *Waxy* Locus. To map the mutation, we created a mapping population by crossing *m38* with Steptoe. The homozygous lines were determined according to the MLG phenotypes of F2 plants that were similar to either parental line in each population and the confirmation of the MLG phenotypes in the corresponding F3 families.

Because the reduced level of grain amylose in *m38* was similar to that reported previously in waxy grains of naturally existing barley cultivars and mutant lines, we assumed that the *m38* locus might encode an allele of the *Waxy* gene. To test this hypothesis, we synthesized a pair of primers corresponding to the known barley *Waxy* gene (Patron et al 2002), which could detect a polymorphic region in the 5′-untranslated region (UTR) of the *Waxy* gene (Fig. 2A). The polymorphism between *m38* and Steptoe was the 191 base pair (bp) difference in PCR products with the *Waxy* gene primer pairs (Fig. 2B). The Waxy-F1 (5′-CACTCGGTTGGTTCG ACCTC-3′) and Waxy-R1 (5′-CATGGTTTTGTCCGGTTTTC-3′) primer pair is the specific marker in the 5′ UTR region of the *GBSSI* gene. The polymorphism of 191 bp between *m38* and Steptoe is not responsible for the waxy endosperm phenotype because it is a deletion in the first intron of the *GBSSI* gene,

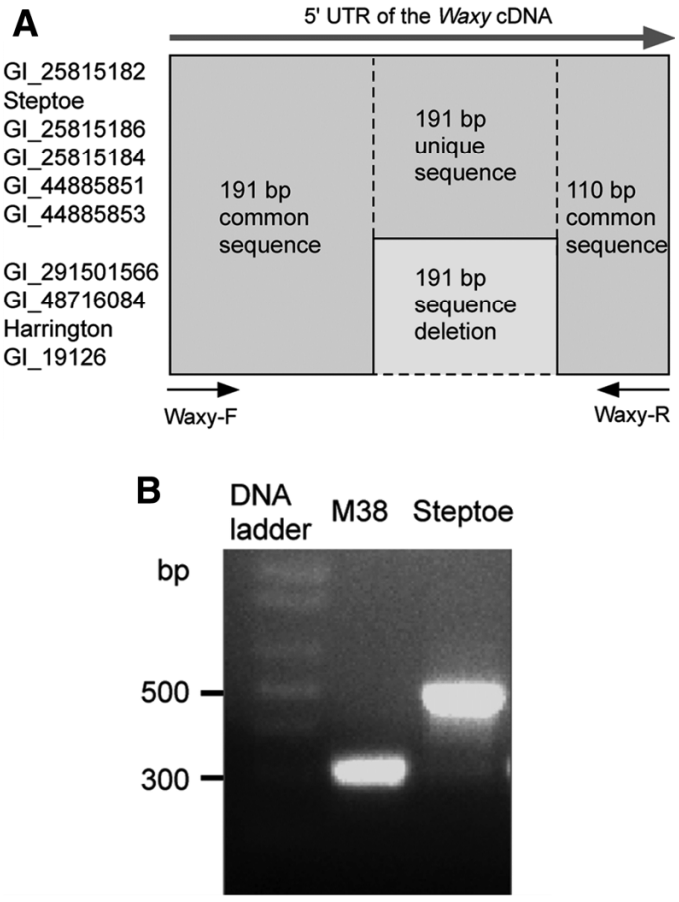

Fig. 2. Detection of polymorphisms at the 5′ untranslated region (UTR) of the *Waxy* cDNA by using gene-specific primers. **A,** The top six sequences contain a 191 bp DNA sequence that is missing in the bottom four lines. The positions of *Waxy*-specific primers, Waxy-F and Waxy-R, are indicated. The detailed sequence information is available in the specific accessions in the database. **B,** Barley cultivar Steptoe and *m38* are polymorphic in the *Waxy* gene, differing in the length of 191 bp between the polymerase chain reaction products. DNA marker, 100 bp ladder from Bio-Rad.

which could be used as a DNA marker (Domon et al 2002). The 191 bp deletion region in Harrington is different from the 413 bp deletion of the GBSSI promoter region described by Patron et al (2002). There is no overlap between the two deletion regions. Genetic mapping was done with the primer pairs in the *m38* × Steptoe population. Results showed that all 46 families with the mutant MLG phenotype carried the *m38* allele, whereas all 41 families with the wild-type MLG phenotype carried the Steptoe allele. The perfect linkage between MLG phenotype and genetic alleles of the *GBSSI* gene strongly supported the hypothesis that the *m38* mutation is the *GBSSI* allele.

To prove our hypothesis further, we designed primers of Waxy-F (5′-CTCTCACTGCAGGTAGCCAC-3′) and Waxy-R (5′-GGCTAT CTCCCATACGAGA-3′) to amplify the whole gene sequences based on the *GBSSI* gene sequence in the database. High-fidelity DNA polymerase was used to amplify the cDNA products by using RNA samples isolated from the *m38* mutant and the wild-type plant. Sequencing of the cDNA products showed that there were nucleotide changes in *m38* compared with the wild-type plant (*JX676772*). The nucleotide change G^{787} to A resulted in an alteration of the amino acid residue at position 263 from glycine to serine, Gly^{263} to Ser. The full coding sequence alignment is shown in Supplemental Figure 1. We designed a pair of primers, M38-F4 and M38-R2, to amplify the 267 bp genomic fragment corresponding to the cDNA sequence between the 713 and 873 bp positions (*JX676772*) flanking the mutated sites of G^{787} to A in *m38*. Using these primers, we performed PCR reactions on all 87 homozygous families from the *m38* × Steptoe population. Sequencing of the PCR products showed the *m38* allele to be perfectly aligned with the endosperm waxy phenotype. The linkage of *m38* allele with the phenotype, plus the amino acid change of Gly^{263} to Ser in the GBSSI protein in *m38* (Fig. 3A and B), led us to conclude that the *m38* mutant is allelic to the *Waxy* gene. Similar amylose content of *m38* to the barley lines without a functional *Waxy* gene such as CDC Alamo and Azhul (Hu et al 2010) indicated that the *Waxy* gene in *m38* is also very low or nonfunctional. Mapping of *m38* was not a typical genetic mapping experiment that used markers against a segregating population and generated a linkage map. We exploited the uniqueness of the waxy phenotype candidate gene of *GBSSI* and directly worked on the linkage of the gene-specific markers and waxy phenotypes in the populations. Because the *Waxy* gene was mapped on chromosome 7H (Ramsay et al 2000), we decided that the *m38* mutation was mapped on chromosome 7H.

To exclude the possibility of another starch synthase gene mutation nearby, we searched the 7H synteny rice chromosome 6 and found only one starch synthase gene on the entire rice chromosome. The locus is designated as *LOC_Os06g04200* (http://rice.plantbiology.msu.edu). Examination of the os06g04200 region did not locate any possible genes that potentially affect amylase synthesis in cells. In addition, when we searched the *Brachypodium distachyon* database (www.plantgdb.org/BdGDB/cgi-bin/blastGDB.pl) with the *GBSSI* gene sequence in barley, the only ortholog gene found was *Bradi1g50090.1*. The ortholog gene identified in *Brachypodium* is located on the chromosome 1 that is the barley 7H synteny chromosome in *B. distachyon*. There are no other genes in the *Brandi1g50090.1* locus region that could possibly affect amylase biosynthesis. All the GBSSI synteny region investigation of both rice and *Brachypodium* genomes supported our conclusion that *GBSSI* gene mutation in *m38* is the cause of the phenotypic changes.

Amino Acid Mutated in *m38* Is a Critical Position Cross Species. To further enhance the conclusion that the amino acid change in GBSSI is responsible for the mutant phenotype in *m38*, we analyzed the conservative status of the changed amino acid among the different species. Alignment of GBSSI amino acid sequences of different plant species included *Arabidopsis* and potato, *Chlamy*, and *Escherichia coli* (Fig. 3C). Results showed that the glycine amino acid is conserved at the position among all the species. Highly conserved glycine at the position in the *GBSSI* gene indicated that it is a critical position and that any change at the position could seriously affect the enzymatic function.

The waxy endosperm of the *m38* mutant strongly indicated the mutation of the *GBSSI* gene. Mapping results pointed to the waxy gene region on chromosome 7H. A GBSSI-specific marker showed perfect linkage to the waxy phenotype of *m38*. Sequencing of the *GBSSI* gene from *m38* showed two nucleotide changes, one of them resulting in an amino acid change from glycine to serine. Alignment of GBSSI amino acid sequences from different species demonstrated that the amino acid position mutated in *m38* is very conservative, indicating the possibility of enzymatic activity alteration if that amino acid changes. Investigation of rice and *Brachypodium* synteny chromosomal regions of *GBSSI* of barley 7H showed no other possible gene near *GBSSI* and excluded the possibility that another gene mutation near the *GBSSI* locus caused the phenotype changes in *m38*. Together, the evidence strongly supports the conclusion that

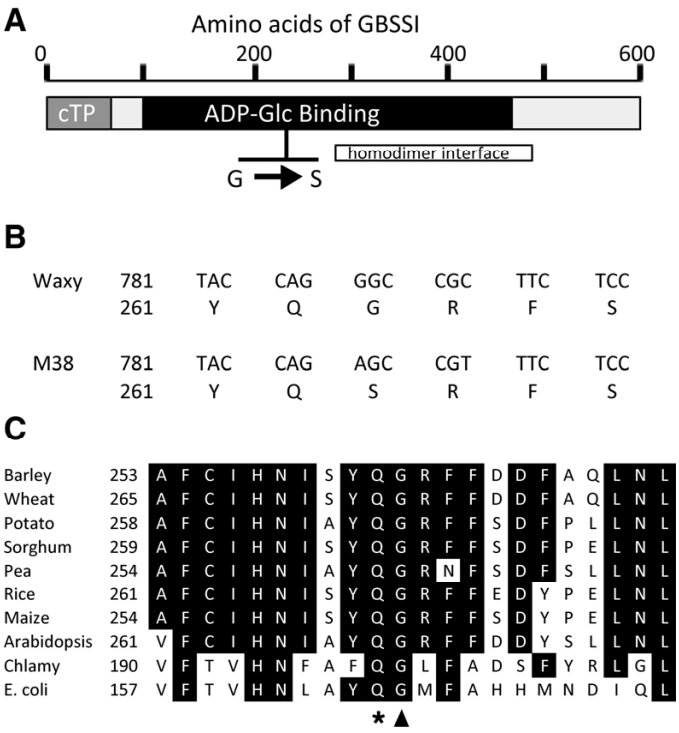

Fig. 3. Functional domains of GBSSI and the mutations in *m38*. **A,** GBSSI encodes a polypeptide of 603 amino acids. The N-terminal 69 residues, identified using ChloroP software (Emanuelsson et al 1999) serve as a chloroplast targeting peptide (cTP) that is removed during translocation into the chloroplast, or amyloplast and plastid in nonphotosynthesis tissues. The adenosine-5′-diphosphate-glucose (ADP-Glc) binding domain present between amino acid residues 80 and 490 and the homodimer interface found between residues 320 and 530 were identified with the BLASTP program. **B,** There are two nucleotide changes at the *m38* locus, compared with the wild-type *Waxy* gene encoding GBSSI. The first nucleotide change of G to A at position 787 of the *Waxy* coding region results in the substitution of glycine with serine at position 263 of the deduced *Waxy* peptide. **C,** The *m38* mutation is present in a highly conserved domain of GBSSI. The G^{263} to S of *m38* is indicated by an arrowhead, whereas a previously identified mutation (Q^{268} to H) in sorghum (Sattler et al 2009) is denoted by a star. Both Gly (G) and Gln (Q) are absolutely conserved in starch synthases from *Escherichia coli* to higher plants. GenBank accession numbers of starch synthases used for the alignment are barley, CAA30756; wheat, AAB26860; potato, Q00775; sorghum, Q43134; pea, CAA61268; rice, CAA37732; maize, CAA27574; *Arabidopsis*, AAN31102 (At1g32900); *Chlamydomonas*, EDP08594; and *E. coli*, P0A6V0.

mutation in the *GBSSI* gene in *m38* is the cause of the mutant phenotype.

Possible Protein Conformation Change in *m38*. To understand how the point amino acid change in *m38* may affect its possible enzymatic function, we simulated the three-dimensional folding of the deduced *m38* peptide with bioinformatics software (Chen et al 2006). We observed three alterations in secondary structure, each creating a new short stretch of β-strand in *m38* (Fig. 4A and B). Alteration positions 2 and 3 (Fig. 3B) were located in the proposed catalytic pocket and may affect binding with ADP-Glc and enzyme catalysis. Alteration position 1 (Fig. 4B) was on the protein surface, which may affect interaction with other potential regulatory proteins. Protein dimerization did not appear to be changed in *m38*, because the dimerization interface remained unchanged (Fig. 4C). We concluded that the changed MLG phenotype in the *m38* mutant was likely caused by the point mutation in the *Waxy* gene.

DISCUSSION

The phenotype of waxy endosperm, which contains mostly amylopectin starch with a low level of or no amylose, has been observed in the grains of many plants, either in naturally existing cultivars or in mutant lines isolated from a mutagenized population (Bhatty and Rossnagel 1997; Graybosch 1998). Aligning the starch synthase sequences from different species showed that both Gly (G) and Gln (Q) at the amino acid position of 263 were absolutely conserved in starch synthases from *E. coli* to higher plants. The previously identified waxy mutation in sorghum was the substitution at the critical position (Q^{268} to H) (Sattler et al 2009). The analysis of the sequence alignments supported the conclusion that substitution of glycine at position 263 with serine in the putative ADP-Glc binding domain resulted in the *m38* mutation.

Low-amylose mutations in barley are all related to the amylose biosynthesis gene *GBSSI*. Patron et al (2002) studied low-amylose mutations (waxy endosperm) in barley and classified the mutants into three alleles of the mutant *GBSSI* gene. The first one is the 413 bp deletion in the promoter region that causes low expression of the gene leading to low-amylose endosperm. Barley lines with the mutant allele include waxy Betzes, waxy Hector, waxy Oderbrucker, and SB85750. The second allele is the T to A point mutation at the 860 position of the coding sequence leading to the corresponding amino acid alternation from Val to Asp. The barley lines carrying the mutant allele include CDC Alamo and Azhul. This allele contributes to very low amylase (Asare et al 2012). The third allele is the point mutation of C to T change at the 580 position of the coding sequence. Barley lines carrying this allele include Yon M Kei and SH97 (Domon et al 2002). The fourth mutant allele of the *GBSSI* gene is *m38*. Identification of the new allele in *m38* should provide more options to use the mutant allele in waxy endosperm barley development and to study the allelic interactions of the waxy endosperm in barley.

The pleiotropic effects of endosperm metabolic pathways in barley grains are known, because previous reports revealed similar phenomena. The well-known examples in barley endosperm are the lysine mutations (Munck et al 2004; Rudi et al 2006). Low-MLG mutants of *lys3* have low MLG, high lysine, reduced starch, and high fat contents, whereas *lys5* mutations have high MLG, reduced starch, and increased fat contents (Munck et al 2004). In the *GBSSI* gene mutation, Ullrich et al (1986) showed the MLG increase in the waxy background by using isogenic lines in their experiment. The similar linkage relationship between *Waxy* gene activity and MLG contents was also observed in other studies in barley (Swanston et al 1995; Izydorczyk et al 2000). Xue et al (1997) reported wide effects of the waxy endosperm, including increased MLG, fat, and free sugars and decreased

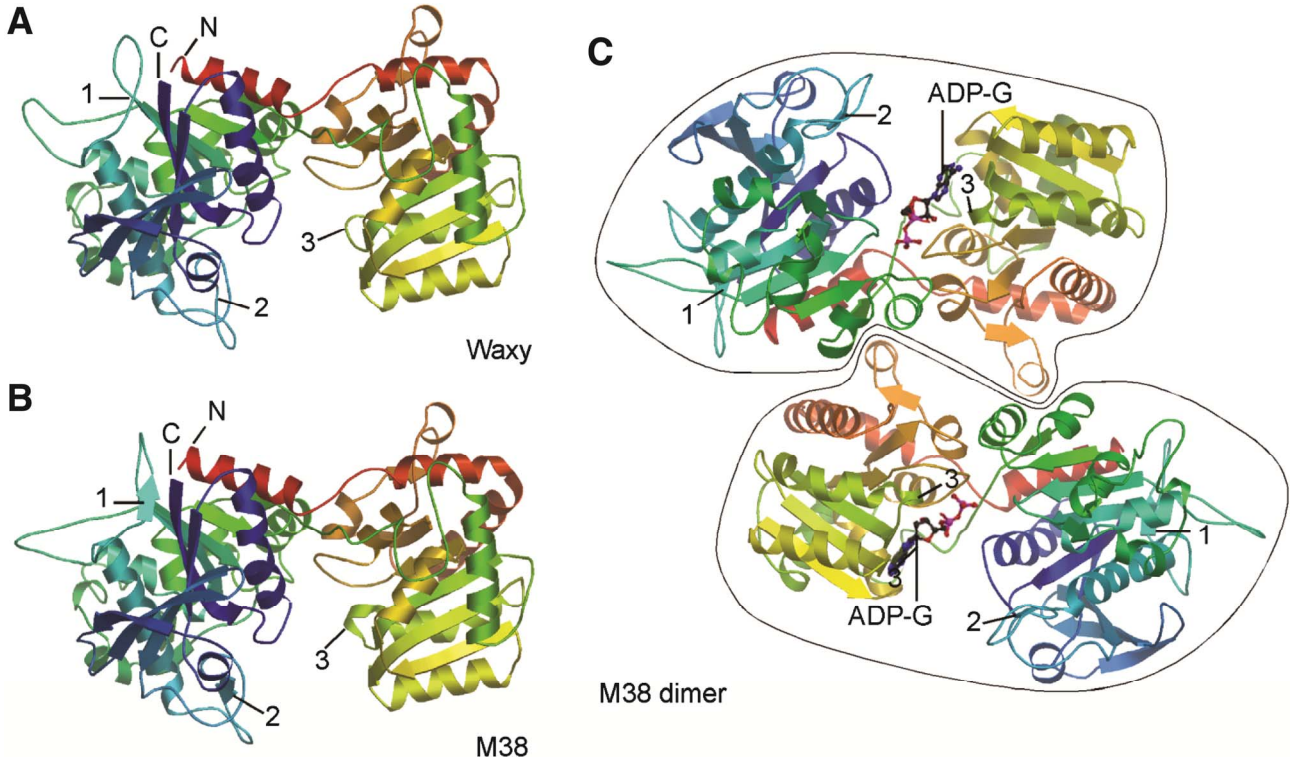

Fig. 4. Homology modeling of the wild-type *Waxy* and *m38* mutant peptides. Conformation images of the *Waxy* protein (**A**) and *m38* mutant protein (**B**) generated with Modeller software (Chen et al 2006). The N- and C-termini of the proteins are indicated by N and C, respectively. Numbers indicate three major visible alterations in secondary structure found between the two protein conformation models. All three alterations involve additional short β-strands found only in the *m38* mutant protein. Model of dimerized *m38* mutant proteins (**C**) containing adenosine-5′-diphosphate-glucose (ADP-G). Alterations 2 and 3 are close to the catalytic center and may have an impact on ADP-G binding and enzyme catalysis. Alteration 1 is located on the surface of the dimer and affects interaction with other regulatory proteins.

starch in an experiment that used six isotypes of barley lines, but they reported no change in protein content. Yasui and Ashida (2011) recently reported that the waxy endosperm in wheat also showed similar pleiotropic effects on other grain quality traits such as increases of MLG, fat, and fructans, but the starch content remained the same. It is interesting to note that the *m38* mutation did not change the starch and decreased protein content. The remaining starch in *m38* resembles the function of the waxy allele in wheat rather than of the barley alleles reported. Although the exact mechanism is unknown, phenotypic characterizations of waxy endosperms indicate that different mutant alleles could have different impacts on other traits. The known example of different phenotypes caused by different alleles in barley is the *lys3* gene. The *lys3c* retained the MLG content, whereas other *lys3* alleles showed reduced MLG (Munck et al 2004). The unchanged starch and decreased protein characteristics of *m38* are apparently different from known alleles of the *Waxy* gene and are therefore worth further study into the physiological and genetic regulations of starch and MLG in barley grains.

CONCLUSIONS

In this study, we isolated and characterized a barley mutant of *m38* with increased MLG content from the population derived from an EMS-treated hulled cultivar of Harrington. Additional characterizations of grains indicated that the mutant decreased protein and amylose and increased crude fiber and fructans at significant levels, whereas other major components remained the same. Other traits including plant heights and physical properties of grains were unchanged. Molecular characterizations of the mutant indicated that the mutant is actually a new allele of the *Waxy* gene. The sequence change and structural prediction of protein demonstrated that the mutant phenotypes are very likely caused by the mutant allele of the *Waxy* gene in *m38*.

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