



A mutation of the cellulose-synthase-like (*CsIF6*) gene in barley (*Hordeum vulgare* L.) partially affects the β -glucan content in grains



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ABSTRACT

The chemical induced barley mutant *m351* was first selected for its low level of mixed-linkage (1–3,1–4) beta-D-glucan (MLG) in an experimental effort to search for barley lines with varied grain MLG contents. The MLG decrease in *m351* was associated with increased levels of fructans and crude fiber, but maintained the same plant characteristics under field conditions. The mutation was mapped to a genetic locus flanked by two SSR markers, Bmag369 and Bmag564, on chromosome 7H. Molecular cloning of the *CsIF6* gene from the *m351* line revealed the presence of a point mutation, causing a substitution of an alanine for threonine at position 849 in the amino acid sequence of the corresponding protein. The resultant protein retains some functionality and affects other components in the *m351* grain. Those metabolic changes associated with MLG reduction in *m351* is the first case reported of a partially functional *CsIF6* gene in cereal grains. The results contribute to better understanding of the functional effects of the *CsIF6* gene and the mutant has potential implications in grain end-use quality improvement.

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1. Introduction

The MLG content in grains is an important quality trait in cereal species. It has received more attention recently in genetic studies and breeding improvement because of its demonstrated beneficial health effects (AbuMweis et al., 2010; Behall et al., 2004; Brennan and Cleary, 2005; Pick et al., 1998) as well as its negative impact in feed and malting industries (Bedford and Schulze, 1998). Therefore modifications of MLG content in cereal grains in both high and low directions have potential market value. In barley genetic studies, several quantitative trait loci (QTL) for MLG have been located on chromosomes 1H, 2H, 5H, and 7H (Fincher, 2009; Han et al., 1995; Li et al., 2008). Microsynteny analysis between barley and rice genomes helped to identify the specific genes in the QTL loci (Burton et al., 2006; Hazen et al., 2002; Islamovic et al., 2013). The rice genomic DNA sequence between the same DNA markers of *Adh8* and *Bmy2* identified in barley contains several open reading frames of cellulose-synthase-like genes (*Csl*) (Burton et al., 2006). Expression of these rice *Csl* genes in *Arabidopsis* has provided direct evidence for the function of cellulose-like gene group F (*CsIF*) and group H (*CsIH*) in MLG biosynthesis (Burton et al., 2006; Doblin

et al., 2009). Bioinformatics analyses revealed the uniqueness of *CsIF* and *CsIH* families in rice, a Poaceae monocotyledon (Burton et al., 2006). The higher contents of MLG in cereal grains may be closely related to the specific function of *CsIF* and *CsIH* genes.

In addition to the genomic studies, the MLG content also has been demonstrated to be associated with other important seed characteristics and agronomic traits. In limited studies on the relationships between MLG and other grain traits, Hang et al. (2007) detected a negative correlation between MLG and amylose but positive correlation between MLG and protein content in 27 barley lines. The high nitrogen level in growth condition enhances the MLG content while the high irrigation level decreases the MLG content in grains (Güler, 2003). MLG content in barley grains is associated with other phenotypic characteristics via pleiotropic interactions. Examples of the pleiotropic effects involved in MLG are the Risø mutants. Mutant lines of Risø 13, Risø 16, and Risø 29 were reported to have increased MLG levels and reduced grain starch and seed weight (Munck et al., 2004; Tester et al., 1993). Cloning of the corresponding gene in the Risø 13 mutant showed that it encodes the ADP-glucose (ADP-Glc.) transporter (Patron et al., 2004), which is responsible for ADP-Glc transportation from the cytosol to the plastids of the endosperm where starch synthesis takes place. The Risø 16 mutant was caused by a mutation in the gene coding of the small subunit of the cytosolic ADP-Glc pyrophosphorylase (AGPase) which is involved in starch synthesis

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(Johnson et al., 2003). Both Risø 13 and 16 mutants are defective in starch synthesis. The elevated MLG levels in both mutants are likely caused by either a relative increase in the cell wall proportion because of the shrunken endosperm or by alteration of sugar distribution in the cells. Thus both Risø 13 and Risø 16 mutants are good examples of pleiotropic effects of starch biosynthesis genes on the MLG metabolism pathway.

Despite the recent progress in MLG studies, the genes and gene function in the MLG biosynthesis pathway are still poorly understood. We recently studied a barley mutant (*m351*), which was isolated through ethyl methanesulfonate (EMS)-mutagenesis and exhibited a decreased level of MLG. We report here the characterization of the *m351* gene mutation as it involves the partial functionality of the *CsIF6* gene, and describe its effects on the grain composition.

2. Materials and methods

2.1. Plant material

The hulled barley cultivar Harrington was used in EMS mutagenesis by the following procedure: seeds were imbibed in cold water for 4 h and then treated with 0.4% EMS at 0 °C for 2 h. The container was moved from 0 °C to 20 °C for 2 h, and the EMS solution was drained. The treated seeds were rinsed overnight in running water, dried on a paper towel and grown in the field. The *m351* mutant was identified by screening the M2 individual plants for altered MLG content in grains using the method described by Hu and Burton (2008). The mutant was backcrossed to the original parental cv Harrington once. The BC₁F₂ progenies were screened for lines having either the wild type or *m351* mutant phenotypes by measuring the MLG content. Multiple BC₁F₂-derived F₃ families were selected randomly and pooled to better represent each class of mutant or wild type. Those pooled lines of each class provided protection against the inadvertent selection for possible unlinked traits that result from selection of an individual line. The pooled families were advanced for a generation and the seeds harvested were tested for grain chemical composition. By preserving and normalizing genetic variability for unlinked traits in each class, this approach enabled meaningful comparisons in less time than would be required for comparisons of corresponding lines. Plants of the *m351* mutant and wild-type lines were grown in field conditions at Aberdeen, Idaho in 2009, greenhouse condition in 2010, and Aberdeen field in 2011.

The mapping population was created by crossing *m351* as a female to the barley cv Steptoe as the male parent. The F₁ seeds were grown in the greenhouse and F₂ seeds were harvested from individual plants. All F₂ seeds were grown in the field at Aberdeen, Idaho and F₃ seeds from each plant were harvested individually. Eighty-one F₃ families from both wild-type and mutant were selected by determining MLG content and used as the mapping population. For the mapping population, plants were grown in the field at Aberdeen, Idaho, in both 2008 and 2009. Seeds were planted in late April and harvested in late August. RNA was extracted from immature seeds of plants grown in pots under greenhouse conditions.

2.2. DNA, RNA extraction and PCR amplifications

For DNA and RNA extraction, plants were 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. The immature seeds were collected at the 2–3 week stage after pollination.

Total genomic DNA of each line was extracted following the method described by Pallotta et al. (2003) with the exception that the SDS was replaced by CTAB buffer. The DNA concentration was measured by Nanodrop (Model ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, DE) and adjusted to 40 ng/μl for PCR reaction. PCR reactions for the designed markers were performed as described by Röder et al. (1998). Electrophoresis was carried out on 6% polyacrylamide gels (0.4 mm thick) in 0.5 × TBE (90 mM Tris–borate, 2 mM EDTA) at 300 V for 3 h using an ABS gel electrophoresis system for the markers of Bmag369 and Bmag564. The enzyme used in this cloning experiment was IProof high fidelity DNA polymerase (Bio-Rad, Hercules, CA). The Taq DNA polymerase used in the regular PCR reaction was REDTaq (Sigma, St. Louis, MO).

To clone the *CsIF6* coding sequence from *m351* and wild-type plants, two pairs of nested primers (Forward-1: 5'gcggtgattgaggacgacggcc3'; Reverse-1: 5'gactgactgttgatggatgag3'; Forward-2: 5'acggccatggcggccagcgggtggc3'; Reverse-2: 5'tgtccgggcaaatgcatcaa 3') were synthesized based on the suggestions from Dr. G. B. Fincher's lab (Australia). Nested primers were used due to the low expression level of the *CsIF6* gene (Burton et al., 2006).

2.3. RNA extraction and qRT-PCR

Total RNA was extracted from frozen tissue using RNeasy kits from Qiagen (Valencia, CA). RNA samples were tested on a 1% agarose gel for quality evaluation based on the appearance of RNA degradation and RNA concentration was measured in a spectrophotometer. For quantitative RT-PCR testing, all RNA samples were treated with DNase according to the manufacturer's manual (NEB, Ipswich, MA). DNase treated RNA was used for reverse transcription using MLV reverse transcriptase (NEB) with oligo-dT primer. The reverse transcription was performed according to the manufacturer's manual supplied with the enzyme.

2.4. DArT marker analysis

Four DNA samples of the mutant *m351* and the wild-type parental lines, and the mutant and wild-type samples from the backcross progenies, were prepared by mixing tissues from 20 seedlings for each sample and submitted to Diversity Arrays Technology (DArT) (Yarralumla, Australia). Based on DArT marker location identified on the chromosome, known SSR markers near the *m351* linked DArT markers were selected using the GrainGene database (<http://wheat.pw.usda.gov>). For the Bmag369 marker, the following primers were used: forward (5' CACTAGCACCAATGACTG 3') and Reverse (5' ATCGAAAATCTTAGCTTTGG 3') (Ramsay et al., 2000). For the PCR marker Bmag564, the primer pair used was: Forward (5' GTCATCGTCCGTTCTTTG 3') and Reverse (5' ATGTAGCTAGATGTCCACCC 3') (Kikuchi et al., 2003). The primers to cover the mutation base pair in the *CsIF6* gene were *M351*mcF2 (CCCATCATCATCTTCGTC) and *M351*mcR2 (ACTCGCCGTCGA-GAACCT). The primer pair of *M351*mcF2 and *M351*mcR2 was used to amplify the PCR products from the mapping population for allelic genotyping by sequencing in individual lines.

2.5. Quantitative RT-PCR analysis

For quantitative RT-PCR setup, we used 10 μl of EvaGreen 2 × mix (Bio-Rad) in 20 μl reaction volume. The reactions were set up in a 96 well plate with clear strip caps. In addition to the 10 μl EvaGreen mix, the reaction contained 7 μl water, 1 μl diluted cDNA solution, and 1 μl of each of 10 mM forward and reverse primer. The *CsIF6* gene primers used were *CsIF6*qt-F (5'

GAGAAGAAGGACCCCTACG3') and CslF6qt-R (5'TTGAGCCAGTGCCTCCACTC3'), these two primers specifically amplified a 146 bp cDNA sequence of the gene. The internal control used was the actin gene. The primers for actin gene amplification in qRT-PCR reaction were Actin-F (5' GCGAGCACCAACGAGAAGTTC3') and Actin-R (5' GGCAGAGCATTGGATGGATGG3'). The qRT-PCR was conducted in the Mx3005P thermal cycler (Agilent Technologies, Inc., Santa Clara, CA) using the default comparative program with three replicates of each reaction. Annealing temperature was set up at 58 °C, while the remaining parameters of the profile used the default setup. For the data normalization, the qRT-PCR data were first normalized to the internal reference actin gene using $\Delta Ct = Ct(\text{cslf6}) - Ct(\text{actin})$ formula. Then the normalized ΔCt test samples was normalized to the ΔCt of calibrator: $\Delta - \Delta Ct = \Delta Ct(\text{test}) - \Delta Ct(\text{calibrator})$. The relative expression level of the gene was calculated by $2^{-\Delta \Delta Ct}$.

2.6. 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 using a commercial kit of K-Fruc (MegaZyme, Ireland) according to the manual instructions. Glucose was determined as for the MLG measurement with the exception of digestion by lichenase following the published modified protocol (Hu and Burton, 2008).

2.7. Other chemical analyses of barley grains

In a different experiment, all samples were analyzed for total lipid by an Ankom XT10, fat analyzer (Model XT 10, Ankom Technology, Macedon, NY) AOCS Official Procedure Am 5-04; total starch was analyzed by the Megazyme Total Starch Kit (MegaZyme, Ireland); protein was measured by a combustion method (AOAC 2002), using a protein analyzer (Model FT528, Leco Crop. St. Joseph, MI); crude fiber content was measured by AOCS Approved Procedure Ba 6a-05 Ankom Technology method 10 using an Ankom 2000 Fiber Analyzer. Protein content was calculated from a formula of nitrogen weight: $[\text{nitrogen weight (g)} / \text{sample weight (g)}] \times 6.25$.

2.8. Characterization of grains

Average kernel weight of wild type and mutant lines was obtained from three replicates of 100 seeds selected randomly from each sample. Kernel diameter was measured using a digital caliper (Chicago Brand Industrial, Inc., Fremont, CA); a total of 20 kernels were measured for each sample and an average value was obtained. Hardness was measured by a Single Kernel Characterization System (SKCS) instrument, (Model 4100, Perten Instruments, Reno, NV). One hundred grains were used for each sample and were reported as means of hardness index. Threshing broken rate was measured by the weight of broken grains divided by the total weight of grain in 50 g of sample prepared using a head thresher of Almaco (Allen Machine Company, Nevada, Iowa).

2.9. Protein structure and stability assay

Protein structure was analyzed using the InterProScan program (version 4.8. www.ebi.ac.uk). Possible signal peptide was also scanned using the SignalP program (cbs.dtu.dk).

A comparison of the protein stability of CslF6 between *m351* and wild-type lines was obtained using the online software I-Mutant v2.0 (<http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant2.0/I-Mutant2.0.cgi>).

3. Results

3.1. Phenotypic characterization of *m351* mutant

The *m351* mutant showed a decreased level of grain MLG in repeated measurements, ranging from 0.9% to 1.6% both under greenhouse and field conditions, as opposed to 5.0–6.2% in the wild-type plants of the cv Harrington grown in the same conditions. To reduce the effects of other potential unrelated mutations in the *m351* mutant genome background, we back-crossed *m351* to the parental cv Harrington. The resultant mutant lines showed 1.4% MLG versus 5.2% for wild-type plant lines (Table 1), these values were similar to those of the original mutant and wild-type cultivar. No significant changes were observed in amylose, total starch, protein, ash, and free glucose contents (Table 1). Crude fiber and fructan contents were increased in the *m351* mutant grains (Table 1).

No obvious differences in growth rate or seed and plant morphology were observed between the *m351* mutant and the wild-type cultivar (Fig. 1A), indicating that the mutation does not affect the morphology of the plants.

Analysis of physical characters showed that the *m351* mutant has virtually the same kernel weight and kernel diameter as the control seeds (Table 2), however the hardness of the mutant kernels decreased. The broken seed rate was 2.5% in *m351* and 0.6% in the wild-type (Table 2). The broken seed rate affects the end use qualities such as malting because broken seeds do not germinate and influence the amount of extract. The similarity in kernel weight measurement (Table 2) between *m351* and wild-type lines is consistent with their grain appearance (Fig. 1B). Taken together, our data indicate that the *m351* mutant maintained major seed characteristics and chemical compositions of total starch, protein, and amylase as its wild-type. The significant decrease of MLG content was accompanied by slight increases of crude fiber and fructans.

3.2. Mapping and cloning of the *m351* mutation gene

DArT marker analysis using the 4 DNA samples of *m351* barley cv Steptoe, pooled mutant line, and pooled wild-type line derived from backcrossing, showed a cluster of DArT markers linked to the *m351* mutant (Fig. 2). To confirm the mapping location and to search for more user-friendly markers, we selected the simple sequence repeat (SSR) markers near the DArT marker region based

Table 1

Grain chemical compositions of *m351* mutant and the corresponding wild-type plants obtained from grain samples from 2009 Aberdeen field, 2010 greenhouse, and 2011 field.

Components	Wild-type			<i>m351</i>			P value
	2009	2010	2011	2009	2010	2011	
Moisture %	8.4	8.2	9.8	8.2	8.3	10.0	0.4776
Protein %	18.2	17.2	11.2	17.6	17.2	12.4	0.4728
Starch %	59.7	60.5	57.4	60.7	61.0	59.9	0.1237
Oil %	2.2	2.3	2.3	2.3	2.5	2.3	0.1254
MLG %	5.0	5.2	5.3	1.3	1.6	1.3	<0.001 ^b
Amylose %	20.0	22.0	20.2	20.1	20.3	21.2	0.3975
Crude Fiber %	3.3	3.0	4.1	5.5	5.2	4.5	0.0112 ^a
Glucose %	0.09	0.09	–	0.09	0.09	–	1.00
Fructans %	1.0	1.2	1.2	2.0	2.1	2.2	<0.001 ^b
Ash %	2.2	2.3	2.3	2.3	2.0	2.2	0.1743

Values are reported in dry-mass based percentages in each category. Data from three years represented three biological repeats with two technical repeats each. P values were calculated by T-Test under assumption of equal variances in three biological repeats.

^a indicates the significance at 0.05 probability level.

^b indicates the significance at 0.01 probability level.

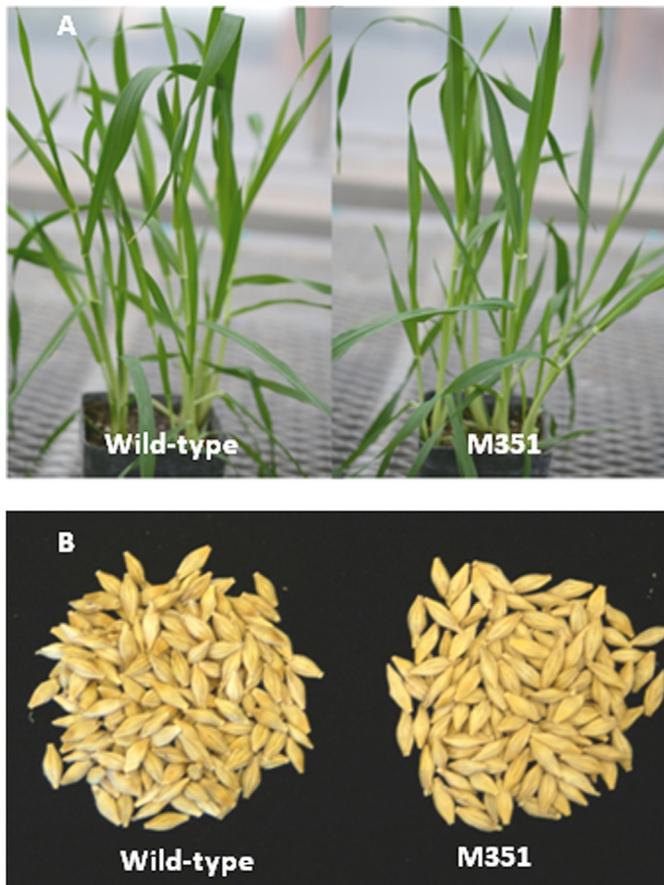


Fig. 1. Plants and Kernel morphology of *m351*. (a) No significant difference in plant morphology was observed between *m351* and its corresponding wild-type line. Plants were grown in the greenhouse at Aberdeen, ID (b). No significant difference in kernel size and shape was observed between *m351* and its corresponding wild-type line. Seeds were harvested from plants grown in the field at Aberdeen, ID.

on the barley consensus map (GrainGene2.0). The selected SSR markers were first screened for polymorphisms between the two parental lines and two markers, Bmag369 and Bmag564 and were found to be polymorphic between the two parental lines (Fig. 2). Both flanking SSR markers were mapped in the segregation population with the *m351* mutant of chromosome 7H. Bmag369 was located 1.2 cM away from the *m351* mutant, while Bmag564 was 14.7 cM away on the other side of the *m351* mutant. All the DNA markers and their mapping positions are summarized in Fig. 2.

Because the *m351* locus was mapped on chromosome 7H in the same region where the gene *CsIF6* is also located (Taketa et al., 2012) and the lower MLG phenotype of *m351* fits well with the logical possibility of a knock-out mutation of the MLG biosynthesis-related gene, we suspected the presence of a mutation on *CsIF6* in the *m351* line. To test the possibility, we decided to clone the full length of the *CsIF6* cDNA from both *m351* and the wild-type plants.

Table 2

Physical characteristics of *m351* and the wild-type grains. Data was obtained from grain samples of 2009 Aberdeen field.

Material	100 Kernel hardness index (HI)	100 Kernel weight (gram)	Threshing broken rate	
Wild-type	61.20	65.80	4.8	0.5%
<i>m351</i> mutant	44.80	52.30	4.7	2.4%

The value was reported as two separate measurements for each category in the table. No statistical analysis was performed on the data.

Sequencing of the cDNA products showed two G to A changes in the *m351* sequence at position 2496 and 2545 compared to wild-type cDNA sequence (Supplement Fig. 1). The nucleotide alteration at 2496 is a silent change without an amino acid change (Fig. 3A; Supplement Fig. 1). The nucleotide change from G to A at position 2545 in the *m351* results in change of the amino acid residue at position 849 from alanine to threonine, (Fig. 3B).

In the *m351* x Steptoe mapping population, a total of 81 families were identified as homozygous for their MLG phenotypes. Flanking DNA markers of Bmag369 and Bmag564 detected 14 recombinants with the MLG phenotype. To characterize the relationship between the MLG phenotype and *CsIF6* gene in those 14 recombinants, we designed a pair of primers, *M351*mcF2 (5'CCCATCATCATCTTCGTC3') and *M351*mcR2 (5'ACTCGCCGTCGAGAACCT3') that amplify a 70 bp fragment flanking the mutated sites. The PCR products from all 14 recombinant genotypes detected by both Bmag369 and Bmag564 markers in the mapping population were sequenced. DNA sequencing results showed that all of the recombinants in the population were agreeable with the MLG phenotype, indicating that there is no recombination between the MLG phenotype and allelic genotype of the *CsIF6* gene. This result provided strong evidence to support our hypothesis that *CsIF6* is the candidate gene in the *m351* mutant. A single amino acid alteration may be critical for the enzymatic function of the protein depending on its location. To evaluate whether the amino acid position 849 in the *CsIF6* protein is critical, we aligned the corresponding region of the *CsIF6*-like proteins from sorghum, oat, wheat, rice, foxtail, *Aegilops tauschii*, *Brachypodium*, *Arabidopsis*, barley (Harrington cultivar) and *m351* mutant line. The amino acid sequence alignment showed that the amino acid at the position corresponding to barley 849 is conserved across all the species (Fig. 3C), indicating that the mutation at 849 in *m351* might be a critical alteration and might affect the enzymatic function of the *CsIF6* protein. The amino acid at position 849 in *m351* is located in one of the predicted trans-membrane domains of the protein (Fig. 3D). The chemical property of alanine is hydrophobic-aliphatic, while threonine is neutral with a polar side chain. Protein stability was analyzed for both mutant and wild-type using Project HOPE software (<http://www.cmbi.ru.nl/hope>). Results indicated that the mutant residue of threonine is bigger in size and less hydrophobic than the alanine present in the mutant. This change could cause the loss of hydrophobic interaction in the core of the protein or with the membrane lipids leading to possible partial functionality of the enzyme. Therefore, the alteration of protein stability might be a potential cause of the *m351* mutant phenotype. The collective evidences of phenotype, mapping location, sequence alternation of the candidate gene, and likely chemical effect of the amino acid alteration in the *CsIF6* protein, lead to the possible conclusion of the *CsIF6* gene mutation in *m351*.

To confirm that the *m351* phenotype is not caused by another gene mutation closely linked to *CsIF6*, we searched other possible genes in *CsIF6* region in the rice syntenic chromosome 6 and 8 (<http://rice.plantbiology.msu.edu>). A *CsIF6* was found in rice chromosome 8 as expected. The whole rice chromosome 8 does not contain any other beta-glucan related gene based on annotations. Search of rice chromosome 6 did not find any beta-glucan related gene either. In addition, we have also searched other possible genes in the *CsIF6* ortholog region in *Brachypodium distarchyon* syntenic chromosome 1 and 3 (http://seacow.helmholtz-muenchen.de/cgi-bin/mips_viewer). The *Brachypodium* chromosome 1 is the syntenic region of barley chromosome 7H where the *CsIF6* is located. Except for the ortholog gene of *CsIF6* gene on *Brachypodium* chromosome 1, there is no other gene possibly related to MLG biosynthesis found on the chromosome identified so far. The fact that there are no other MLG related genes near the *CsIF6* gene in both

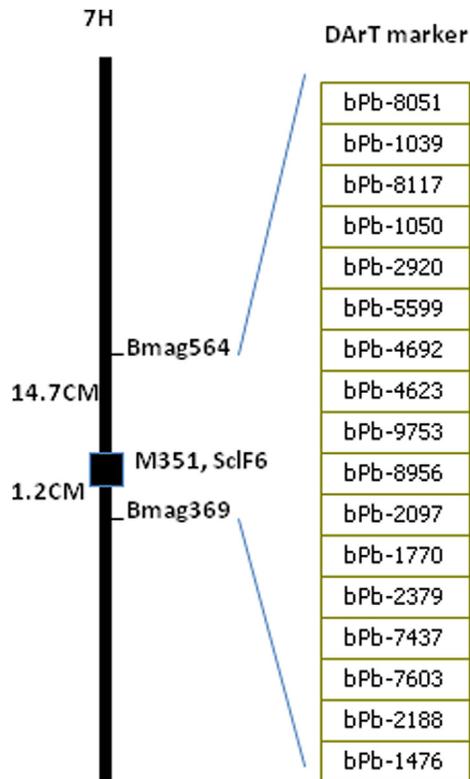


Fig. 2. Genetic mapping results indicated the flanking DNA markers of the *m351* locus. Mapping results are from DArT analysis. *m351*-M and *m351*-Wt. are the pooled DNA samples derived from the F3 population of *m351* × Steptoe. The cluster of DArT markers showing polymorphisms between the mutant and wild-type DNA samples indicate the mapping region of *m351*. Two SSR markers and the *m351*-specific marker were mapped in the 88 F2:3 families of the mapping population. DArT markers were co-segregated with the *m351* gene in pooled DNA samples representing 40 mutant and wild-type plants each. Corresponding DArT markers with the flanking SSR markers were based on the DArT marker map (GrainGene).

rice and *Brachypodium* genomes strongly indicated that it is very unlikely that the *m351* phenotype is caused by another gene near the *CsIF6*.

To rule out the possibility of differential allele expression between mutant and wild-type, we compared the expression levels of *CsIF6* gene in developing seeds using qRT-PCR (Fig. 4). The results showed that there is no significant difference in the gene expression. We cautiously conclude that the low MLG content of the *m351* mutant is not likely due to the reduced expression of the gene in developing seeds.

4. Discussion

In this study, we reported that the *m351* low MLG mutation is very likely due to a missense mutation in the *CsIF6* gene. This is the first case of characterizing the partially functional *CsIF6* gene. Though the *CsIF6* gene in MLG biosynthesis has been defined in transgene expression in *Arabidopsis* (Burton et al. 2006), the *CsIF6* gene mutations, affecting enzymatic functionality, provide additional evidence to evaluate a specific gene structure–function relationship in the MLG metabolism. A recent paper reported that the betaglucanless (*bgl*) barley line presents a point mutation in the *CsIF6* gene (Taketa et al., 2012). The *bgl* mutant had zero betaglucan in the seeds, which indicates the total loss of critical function of this gene (Tonooka et al., 2009). The *m351* mutant is the second case of the *CsIF6* function affected by a point mutation. Because the function of *CsIF6* gene is partially affected in *m351*

while *bgl* is the mutant with a completely abolished function of *CsIF6*, comparison between those two mutants should provide evidence for better understanding of the *CsIF6* gene.

The detailed phenotypic differences between the two mutants are likely outcomes of the allelic functions. The *bgl* mutation results in a smaller plant status, dramatically reduced grain hardness, and sensitivity to cold stress (Taketa et al., 2012; Tonooka et al., 2009). The *m351* mutant, on the other hand, retained 30% of the MLG that may contribute to the normal plant height status (Fig. 1). *Bgl* mutation reduced the hardness index from 74.9 in the wild type to 30.8 in the mutant, a 59% reduction rate, associated with a seed broken rate that increased from 3.8 in the wild type to 35.9 in the mutant, an increase rate of more than 9 times (Tonooka et al., 2009). The *m351* mutant exhibited a reduced grain hardness index from 61.8 in the wild type to 48.8 in the mutant with the reduction rate of 21%, while the grain breakage rate increased four-fold from 0.6% to 2.5% (Table 2). Although the breakage rates were calculated in different processes for the two mutants, the relative grain damage rates compared to their corresponding wild-type genotypes after the processing are still valuable data for evaluating the effects of the mutant gene in two cases. Grain damage rate during the harvesting is an important trait to decide whether the mutant could be used in cultivar development. The lower grain damage rate in *m351* made the mutant more useful in breeding. Retained MLG in *m351* is also likely the reason for a less dramatic effect on grain hardness and broken rate (Table 2). Another difference between *bgl* and *m351* is the glucose content. The glucose content is drastically reduced in the *bgl* mutant (Taketa et al., 2012) while the same amount of glucose was observed between the *m351* mutant and the wild type (Table 1). It is not clear whether the glucose difference in the two mutants is due to the gene function effects or measurement practices, but warrants further research. In addition to the genetic understanding of MLG biosynthesis related to the *CsIF6* gene, phenotypes of the *m351* mutant with a partial functioning *CsIF6* gene may be more useful in a breeding program because possible negative impacts of the mutation are rather mild compared to the dramatic effects of the totally disrupted function in the *bgl* mutant.

Two interesting phenotypes observed in the *bgl* mutant are chlorosis (Tonooka et al., 2009) and chilling sensitiveness (Taketa et al., 2012). Chlorosis is not observed in *m351* at any growing stage of any part of the plants. It is not certain whether the difference is due to the partial versus no function of the *CsIF6* gene in the two mutants or to something else such as a closely-linked chlorosis mutation in the *bgl* mutant. It is noteworthy that a QTL of stress-induced chlorosis is mapped to the same region of chromosome 7H (Li et al., 2008). The data reported in the *bgl* study is not sufficient to rule out the possibility of a closely-linked mutation for the chlorosis phenotype other than possible pleiotropic effects of the *bgl* mutant gene. Chilling stress sensitivity of the *bgl* mutant is likely due to the *CsIF6* gene. It is not as convenient to compare the chilling stress effects between two mutants because *m351* has a spring growth habit. However *m351* showed a slightly weaker germination under a 400 mM salt condition (data not shown). Sensitive responses of the *CsIF6* mutation in both cases is better explained by the same mechanism of a thinner cell wall as mentioned in the *bgl* study (Taketa et al., 2012).

In this report, the characterization of a missense mutation in the *CsIF6* gene is an important contribution to the studies of the MLG biosynthesis genes. The new mutant allele of *CsIF6* in *m351* revealed more molecular evidence for the structure–function relationship of the gene. Availability of the *m351* mutant will help to further explore the gene–structure function affecting MLG and other traits in that biosynthetic pathway. The mild effect of the

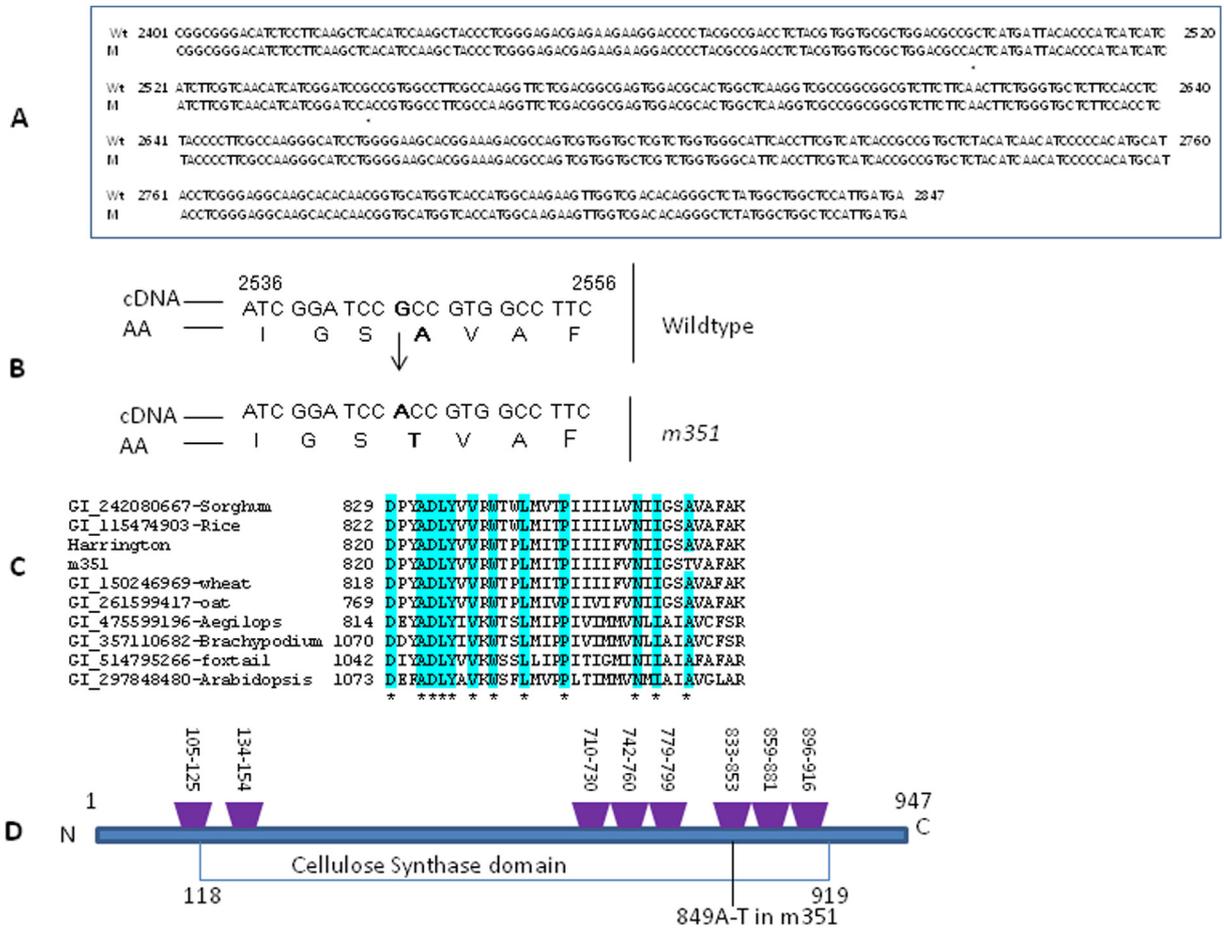


Fig. 3. Illustration of the point mutation of the *CsLF6* gene in *m351* line. A. Alignment of the coding sequence of the *CsLF6* gene between wild-type (Wt) and *m351* (M). Portion of the coding sequence alignment (bp 2401–2847) was shown. The remaining portion of the sequence with no difference between wild-type and *m351* was not shown. There are two G to A changes in the *m351* sequence at position 2496 and 2545. The base pairs change at 2496 is a silent change without resulting in amino acid change. The base pairs change at 2545 resulted in amino acid alteration from alanine to threonine at the amino acid position 849 in the protein. B. The number on the top are the positions of the cDNA sequence of the gene. AA = Amino acid sequence. Sequencing of the full length of the *CsLF6* gene revealed that the point mutation of the 2545 base pairs changed to A from G. Corresponding amino acid changed to Thr from Ala. C. Amino acid sequences of *CsLF6* homoeologous genes from 9 different plant species were aligned in the *m351* mutation region. The amino acids at the corresponding barley 849 positions are very conservative with all alanine where *m351* mutation occurred. D. Protein diagram for *CsLF6* gene. Eight trans-membrane domains were indicated above of the *CsLF6* protein with the start and end amino acid positions for each domain. The program detected another TM domain at the amino acid position of 32–52, but the signal peptide analysis indicated that amino acid position of 1–49 was a signal peptide. Therefore eight predicted TM domains were presented. The cellulose synthase domain was indicated below the protein structure. Mutation in *m351* occurred in the trans-membrane domain.

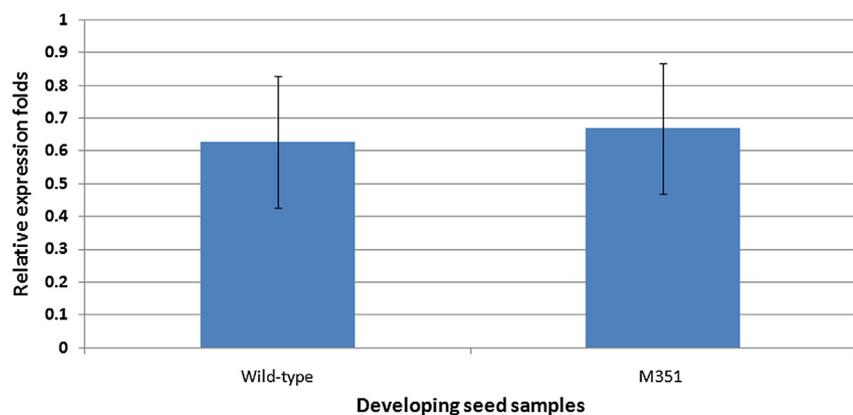


Fig. 4. Measurement of the *CsLF6* gene expression level using qRT-PCR. RNA samples extracted from developing seeds in backcross lines representing the wild-type and mutant plants. The experiments were conducted in two biological repeats and three technical repeats. The values in the figure were the means with standard error.

m351 on other traits makes it useful in MLG improvement for the barley uses where the lower MLG is desirable such as feed, malting, and bio-ethanol production.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jcs.2013.12.009>.

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