Theor Appl Genet (2012) 125:1223–1235
DOI 10.1007/s00122-012-1908-5

ORIGINAL PAPER

Genetic and physical fine mapping of the novel brown midrib gene *bm6* in maize (*Zea mays* L.) to a 180 kb region on chromosome 2

Yongsheng Chen · Hongjun Liu · Farhad Ali · M. Paul Scott · Qing Ji · Ursula Karoline Frei · Thomas Lübberstedt

Received: 6 January 2012 / Accepted: 25 May 2012 / Published online: 20 June 2012

Abstract Brown midrib mutants in maize are known to be associated with reduced lignin content and increased cell wall digestibility, which leads to better forage quality and higher efficiency of cellulosic biomass conversion into ethanol. Four well known brown midrib (*bm*) mutants, named *bm1–4*, were identified several decades ago. Additional recessive brown midrib mutants have been identified by allelism tests and designated as *bm5* and *bm6*. In this study, we determined that *bm6* increases cell wall digestibility and decreases plant height. *bm6* was confirmed onto the short arm of chromosome 2 by a small mapping set with 181 plants from a F_2 segregating population, derived from crossing B73 and a *bm6* mutant line. Subsequently, 960 brown midrib individuals were selected from the same but larger F_2 population for genetic and physical mapping. With newly developed markers in the target region, the *bm6* gene was assigned to a 180 kb interval flanked by markers SSR_308337 and SSR_488638. In this region, ten gene models are predicted in the maize B73 sequence. Analysis of these ten genes as well as genes in the syntenic rice region revealed that four of them are promising candidate genes for *bm6*. Our study will facilitate isolation of the underlying gene of *bm6* and advance our understanding of brown midrib gene functions.

Communicated by M. Xu.

Y. Chen · H. Liu · Q. Ji · U. K. Frei · T. Lübberstedt (✉)
Department of Agronomy, Iowa State University,
Ames 50011, USA
e-mail: thomasl@iastate.edu

Y. Chen
Interdepartmental Genetics Program, Iowa State University,
Ames 50011, USA
e-mail: cdjys2007@gmail.com

H. Liu
Key Laboratory of Biology and Genetic Improvement
of Maize in Southwest Region, Maize Research Institute
of Sichuan Agricultural University, Ministry of Agriculture,
Wenjiang, People's Republic of China

F. Ali
Institute of Biotechnology and Genetic Engineering (IBGE),
Khyber Pukhtunkhwa Agricultural University,
Peshawar 25000, Pakistan

M. P. Scott
Corn Insects and Crop Genetics Research Unit,
USDA-ARS, Ames, IA 50011, USA

Introduction

Maize has been cultivated mainly for grain production for thousands of years. It is also used for forage by harvesting the whole above ground maize plant a few weeks before grain maturity. Compared with other forage crops, maize forage has several merits. Its quality is relatively consistent and its yield and energy content are high (Lauer 1995). Moreover, the labor and time required for cultivation and harvesting are lower than for many forage crops, which greatly reduces the cost per unit of dry matter (Lauer 1995). Cell wall digestibility is the key factor determining forage quality (Andrieu et al. 1993; Barrière et al. 2003, 2004b). In Europe, maize is widely grown as forage crop with ~4.6 Mha surface area coverage (Barrière et al. 2004a). In the past decades, breeding efforts in Europe substantially improved in whole plant yield with ~0.1 t/ha increase per year overall. However, cell wall digestibility substantially decreased, which resulted in a reduced feeding value of elite maize hybrids (Barrière et al. 2005).

The USA has the largest maize forage production in the world (Lauer et al. 2001). Despite improvements of 0.13–0.16 t/ha per year of forage yield since 1930, there was no improvement nor reduction for cell wall digestibility (Lauer et al. 2001). Therefore, improvement of cell wall digestibility deserves consideration in future forage breeding.

Currently, interest in lignocellulosic fuel is increasing. Fuel ethanol, especially the lignocellulosic ethanol, is an attractive alternative to petroleum oil, because it burns cleanly, is renewable, and has very large biomass byproduct supplies (Farrell et al. 2006; Rathin et al. 2011). Ethanol is mainly produced from maize grain or sugarcane at present (Vermerris et al. 2007; Demain 2009). Grain-derived ethanol will not be sufficient to satisfy future demand for biofuels. For example, the US government has set a goal to produce 36 billion gallons of biofuel by 2022, of which a big proportion about 58 % (~20.9 billion gallons) will be non-starch based biofuel (Energy Independence and Security Act 2007). The ~75.9 % of the non-starch based biofuel (~20.9 billion gallons) will be cellulosic ethanol. Due to wide geographic adaption and availability, maize stover will likely substantially contribute to cellulosic ethanol production. Currently, cellulosic ethanol is feasible but not economically competitive compared with gasoline and grain-derived ethanol. In order to make cellulosic ethanol economically more competitive, improvement in the quality of byproduct supplies is required along with advancement in bioprocessing and availability of effective enzymes and microorganisms for breakdown of polymeric carbohydrates and fermentation (Wyman 2007).

Cell wall digestibility is highly correlated with the quality of biomass supplies for the purpose of either forage (Barrière et al. 2003, 2004b) or cellulosic ethanol production (Lorenz et al. 2009). Lignin limits access of cellulolytic enzymes or rumen microorganisms to cellulose and hemicelluloses (Moore and Jung 2001). Many studies have shown that lignin content is negatively correlated with either in vitro or in vivo cell wall digestibility, for example, studies conducted by Riboulet et al. (2008), Casler and Jung (2006), and Guo et al. (2001). Lignin structure, for example the ratio of syringyl (S) to guaiacyl (G) units, is also reported to affect cell wall digestibility (Grabber et al. 2004; Fontaine et al. 2003). In studying a 104 silage set, Taboada et al. (2010) reported significant negative correlations between in vivo dry matter digestibility and either G ($r = -0.79$) or S unit ($r = -0.76$), but a positive correlation between in vivo digestibility and the G/S ratio($r = 0.57$). However, both lignin content and ferulate-lignin crossing linking were reported by Grabber et al. (2009) to have more impact on cell wall digestibility than lignin composition.

In maize, there are four well known mutants, known as brown midrib mutants 1, 2, 3, and 4. They are characterized by reduced lignin content and altered composition as well as increased cell wall digestibility (Barrière et al. 2004b). For example, in vivo neutral detergent fiber digestibility of hybrids with homozygous *bm3* alleles is 9 % higher than those with regular *bm3* gene(s) (Barrière et al. 2004a). *bm3* mutations were caused by structural changes of the caffeic acid *O*-methyltransferase (COMT) gene (Vignols et al. 1995; Morrow et al. 1997). The defect of the *COMT* in *bm3* mutant results in a substantial reduction of total lignin content by 25–40 % (Barrière et al. 2004b). Reduced content of sinapyl residues, thus an increased G/S ratio, and elevated level of 5-hydroxyconiferyl alcohol content in lignin were also observed in *bm3* plants (Chabbert et al. 1994a; Provan et al. 1997; Marita et al. 2003). *bm1* was associated with reduced activity of cinnamyl alcohol dehydrogenase 2 (CAD2) gene (Halpin et al. 1998). *CAD* is responsible for converting hydroxycinnamylaldehydes into alcohols in monolignol biosynthesis (Guillaumie et al. 2007b). Thus reduced CAD activity resulted in accumulated aldehyde and reduced monlignol units in the lignin (Barrière et al. 2004b). However, it is still not clear whether *CAD2* is the underlying gene of *bm1* (Guillaumie et al. 2007a). Genes underlying *bm2* and *bm4* have not been isolated yet.

13 additional brown midrib mutants named *bm*A* to *bm*L* are listed in the Maize Genetics Stock Centre (http://maizecoop.cropsci.uiuc.edu/stockcat.php). By tests of allelism among these brown midrib mutants, two new *bm* mutation designated as *bm5* and *bm6* (Ali et al. 2010) were identified. So far, no studies have been conducted on these two new brown midrib mutations. Characterization of these new mutants on cell-wall lignification will advance our understanding of the change in phenolic compound profiles and their impact on cell wall digestibility, and might ultimately provide genetic tools for forage or bioenergy maize breeding. The objectives of this study were to (1) study the effect of *bm6* on cell wall digestibility and plant height, (2) determine the genetic location of *bm6*, and (3) delimit *bm6* to a physical contig to facilitate map-based isolation of *bm6*.

Materials and methods

Mapping population

The mapping population was produced by crossing *bm6* (*bm*J*) and inbred line B73. We chose B73 because it is one parent of the IBM population, and its genome has been sequenced (Schnable et al. 2009). This will facilitate fine mapping and candidate gene prediction. The *bm6* stock

used for crossing with B73 was derived from stock 5803 J *bm*-86-87-8875-6* from maize genetics cooperation stock center (http://maizecoop.cropsci.uiuc.edu/). The stock used for crossing is a segregating family with brown midrib phenotype character which is a spontaneous mutation. A small set of F_2 plants from this population consisting of 181 F_2 (including both regular and brown midrib phenotype) plants were grown in the greenhouse in the winter of 2009 for confirming recessive inheritance and the rough position of *bm6* around bin 2.02 on Chr 2. The information that *bm6* was nearby bin 2.02 was provided by Dr. Sarah Hake and Dr. Erick Vollbrecht (personal communication). As we grew another F_2 set from the same population, we termed that set with 181 F_2 plants as confirming F_2 set hereafter. Leaf tissues and phenotype of all plants in this confirming F_2 set were collected for DNA extraction and for later recessive inheritance and *bm6* rough position confirmation. Leaf tissue of several plants of *bm6* stock were harvested together to represent *bm6*. A large F_2 set with 5,000 seeds was grown at the Agronomy farm of Iowa State University during the summer of 2010 for fine mapping. 25 seeds were sown per row in 200 rows. The final stands were less than 5,000, and we called this set large F_2 set. When the brown midrib phenotype could be observed around the sixth leaf-stage in the field, F_2 plants with brown midrib phenotype were marked in the large F_2 set. One week later, the phenotype was re-evaluated to confirm the result. In the large F_2 set, about 1,000 plants were scored as brown midrib phenotype (we did not count the total F_2 plants), but only 960 brown midrib F_2 (10 plates each with 96 DNA samples being stored) were used for genetic and physical mapping. The leaf tissues of the 960 brown midrib plants were collected for DNA extraction. All the 960 plants were used for physical mapping, and part of them (192) was used for genetic mapping first. To clarify, we called the 960 brown midrib plants physical mapping F_2 set and the 192 F_2 plants genetic mapping F_2 set. The genetic and physical mapping population comprised of plants exclusively showing brown midrib phenotypes, as these are most informative (Ingvardsen et al. 2010).

Investigation of pleiotropic effects of the *bm6* gene

Four 'plots' were randomly chosen from the large F_2 set grown in 2010 summer, each consisting of three adjacent rows. The reason for choosing three rows is to ensure a sufficient number of brown midrib plants in a 'plot', as three quarters were expected as regular plants in F_2 segregating population. The plant height of all the plants in the four 'plots' was measured from soil surface to flag leaf. The recessive effect of the *bm6* gene on plant height was analyzed by linear model $y_{ij} = \mu + g_i + e_{ij}$ with unbalanced data by GLM in SAS (SAS institute) because plant number in each 'plot' was different (35, 36, 41, and 41), where 'i' equals to genotype class ($i = 1$–2, 1 for brown midrib plant, 2 for regular plant), 'j' equals to plant number of each genotype class in each plot ($j = 1$–9, 11, 11, or 15 when $i = 1$ and $j = 1-25$, 26, 26, or 30 when $i = 2$). μ was the overall mean. In order to investigate the digestibility of neutral detergent fiber (DNDF), nine brown midrib (mutant) and nine regular plants were chosen from each of the four 'plots' mentioned above for plant height analysis. The stover of selected mutant plants was harvested together to form a mutant bulk and stover of regular plants together for regular bulks in each 'plot'. Thus, in total four mutant stover bulks and four regular stover bulks were obtained for digestibility analysis. The stover samples were dried and ground to pass through a 1 mm sieve. Quality analysis was performed with near infrared reflectance spectroscopy (NIRS) based on a calibration of 43 samples (Brenner et al. 2012) including one of above eight bulked samples. Wet lab measurement of neutral detergent fiber (%NDF) for the 43 samples was done according to the protocol available on the ANKOM website (http://www.ankom.com). We chose 43 samples because these 43 samples explained most NIR variation of 240 samples including the 8 bulked samples in this and 232 samples from another study (Brenner et al. 2012) in our lab. Finally, neutral detergent fiber (%NDF) and In vitro True Digestibility (%IVTD) (48 h rumen fermentation followed by NDF procedure) were predicted for our 8 samples. DNDF was calculated by $100 \times [1 - (\%\text{INDF}/\%\text{NDF})]$ %, where %INDF was indigestible neutral detergent fiber and calculated by 100 %–%IVTD. A student's t test was used to test the effect of the *bm6* mutation on NDF and DNDF.

DNA extraction

The harvested leaves were freeze dried for 24 h. After drying, two steel beads were added into each sample and samples were ground with the Geno/Grinder 2000 (BT&C, Inc. NJ, USA) at 700 strokes/min for 3 min. DNA extraction was conducted according to the CTAB protocol used at the Plant Genomics Center of Iowa State University (http://schnablelab.plantgenomics.iastate.edu/docs/resources/protocols/pdf/96wellformat.2010.06.23.pdf).

Marker development

After conformation of *bm6* around bin 2.02 in the confirmation F_2 set, nearby anchored SSR and insertion deletion polymorphism (IDP) markers were used for genetic map of *bm6* gene in the genetic mapping F_2 set (which anchored the *bm6* gene between SSR markers umc2245 and umc2363). When public SSR and IDP markers were

exhausted within the genetic interval defined by umc2245 and umd2363, the B73 sequence between the two flanking markers was used for development of new SSR markers. The interval between the two flanking markers spans ~890,000 bp in the B73 genome sequence (http://www.maizesequence.org/blast). The ~890,000 bp sequence was subjected to MIcroSAtellite (MISA), a micro satellite identification tool (Thiel et al. 2003) for SSR motif identification with default parameter settings. After SSR motifs were identified, about 250 bp of sequence at each side of every motif were extracted and the resulting ~500 bp sequences were stored in FASTA format. The extracted sequences were blasted against the Zea repeat database (download from http://plantrepeats.plantbiology.msu.edu/downloads.html) by local alignment search tool (download from http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download). Only the sequences that did not contain any known repeat sequences were used for SSR primer design. Primer design was done in Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm). Default settings in Primer 3 were used, except that GC content was adjusted to 40–60 % and a bracket was used to include the SSR motif, which ensures that the amplified fragment includes the SSR motif. Two cycles of SSR markers were designed. First we tried to find one SSR marker per ~50,000 bp within the 890 kb region. Additional SSR markers were designed for further physical mapping, after the first cycle of SSR markers restricted *bm6* to a smaller region. The newly designed markers were all used to genotype the recombinants (see in the next section) screened out from the physical mapping F_2 set (including 960 F_2 plants).

IDP and SSR marker data collection and analysis

PCR products of SSRs and IDPs were separated on 3 % metaphor or 1 % agarose gels depending on the product size differences between the parental alleles. For co-dominant markers, the PCR band from B73 inbred were scored as "A", while the PCR band from the *bm6* stock was recorded as "a". "AA" and "aa" indicated two alleles at a locus all from B73 inbred and *bm6* stock, respectively. A heterozygous locus was thus recorded as "Aa". "–" was used to represent missing data for co-dominant markers. For dominant markers, only those showing a band in B73 but not in *bm6* stock were used for genotyping. In this case, "A." was used to record the genotype at locus with at least one band from B73 inbred, while "0" was used to record the locus with no band being detected for dominant markers.

The genetic and physical mapping sets comprised of plants exclusively brown midrib phenotypes. Markers showing polymorphisms between two parents will fall into either of the two below mentioned cases: (1) the frequency of 'non-*bm6* allele type' plants which hold "Aa" and "AA" (or "A." in the case of dominant markers) genotype at a marker locus is significantly lower than 0.75, which indicates the marker is linked with the *bm6* gene; (2) the frequency of 'non-*bm6* allele type' plants at a marker is agreement with 0.75 statistically, which indicates that the marker is not linked with the *bm6* gene. At a marker locus linked with the *bm6* gene, the less 'non-*bm6* allele type' plants a marker detects, the closer it is between this marker and *bm6* gene. In the case of a marker co-segregating with the *bm6* gene, this marker will not detect any 'non-*bm6* allele type' plants in the mapping population given the phenotyping is 100 % accurate. Based on the number of 'non-*bm6* allele type' plants in the genetic mapping F_2 set, *bm6* gene can be delimited between two flanking markers (umc2245 and umc2363). These two flanking markers were used to screen recombinant plants from the physical mapping F_2 set. All of the new developed SSR markers designed based on the B73 genome sequence between the two flanking markers were used to genotype the recombinant plants. Recombinant plants here denoted the plants holding at least one B73 allele at the two flanking markers defining the *bm6* gene (umc2245 and umc2363). umc2245 is located upstream umc2363 on the short arm of Chr 2, so we called the plants with B73 alleles at umc2245 in physical mapping F_2 sets as "upstream recombinants" and denoted as U# (# was a number), which helped to define the upstream border of *bm6*. Markers located between umc2245 and *bm6* gene will detect a fraction of the same recombinants identified by umc2245. Similarly, we called the plants with B73 allele at umc2363 in the physical mapping F_2 set as "downstream recombinants" and denoted as D# (# was a number). Markers located between *bm6* gene and umc2363 will only detect a fraction of the recombinants identified by umc2363. These two groups of recombinant plants finally define the physical interval, where *bm6* must be located, based on the physical position of markers in the B73 sequence. In addition, the genetic positions of markers in the target region as well as *bm6* gene were calculated using JoinMap 4 (Van Ooijen 2006). In genetic map construction, brown midrib and regular phenotype were converted into a phenotypic marker designated as *bm6*. Brown midrib phenotype was recorded as "aa" genotype, and regular phenotype was recorded as "A." genotype. In this way, the phonotype caused by *bm6* was converted into a marker.

Candidate gene prediction

After *bm6* was mapped between SSR_308337 and SSR_488638 markers, the start and end position of the physical interval defined by these two markers were obtained by blasting the left and right primer sequences of

SSR_308337 and SSR_488638 on maize sequence, respectively (http://www.maizesquence.org). Subsequently, the view of this physical interval was obtained by entering its start and end position on maize sequence, where the working gene sets in both directions were shown. Additionally, synteny to rice was displayed by the "synteny" function on the same website.

Results

Recessive inheritance of *bm6* phenotype and confirming *bm6* gene nearby bin 2.02

In the confirmation F_2 set, 45 plants showed the brown midrib phenotype, 136 were regular plants with normal midrib. The ratio of brown to green midrib plants is in agreement with a 1:3 segregation ($P = 0.97$), and is in agreement with recessive inheritance of the brown midrib phenotype caused by *bm6*.

Personal communication with Dr. Hake and Dr. Vollbrecht, we got the rough position of *bm6* nearby 2.02, thus we chose two polymorphic SSR markers, umc1165 and umc2363, which are located in bins 2.01 and 2.02, respectively, to genotype the confirmation F_2 set consisting of 181 plants. umc2363 showed co-segregation with the mutation, while 5 recombinants were found between umc1165 and *bm6* in the confirmation F_2 set. The result confirmed that the *bm6* gene is nearby umc2363 on Chr 2.

Effect of *bm6* on plant height and cell wall digestibility

In total, 11, 15, 9, and 11 mutant (brown midrib) F_2 plants, and 25, 26, 26, and 30 regular F_2 plants stood in those four randomly chosen "plots". The least square mean of plant height for regular F_2 plants is 182.1 cm, while the least square mean of plant height for mutant F_2 plants was 171.4 cm, significantly lower than that of regular plants ($P < 0.01$) (Fig. 1a). The student's *t* test revealed that there was no significant difference for average NDF between mutant and regular bulked stover samples ($P = 0.46$), but the average DNDF was higher of mutant bulked stover samples than that of regular bulked samples ($P = 0.07$) (Fig. 1b).

Genetic mapping of *bm6* using publicly available markers

All public SSR markers in bin 2.00, 2.01, and 2.02 were used to test for polymorphisms between B73 and the *bm6* stock. The polymorphic markers showing clear bands were used to genotype the genetic mapping F_2 set (including 192 mutant F_2 plants). As all plants in the mapping population included only brown midrib individuals, the polymorphic markers were indicated to be linked with *bm6* if the detected 'non-*bm6* allele type' plants were significantly lower than 0.75. Mmc0111 only identified 50 'non-*bm6* allele' type plants out of 192 brown midrib individuals (Table 1), far less than expected 144 ($P < 0.01$), which indicates linkage between mmc0111 and *bm6*. umc1552, umc2363, and umc1165 detected only 29, 8, and 8 'non-*bm6* allele type' plants (Table 1). The genetic distance between *bm6* and mmc0111, umc1552, and umc2363 were 14, 8.5, 2.4 cM (Fig. 2). umc2363 and umc1165 co-segregated in this genetic mapping F_2 set. umc2245 detected 9 'non-*bm6* allele type' plants (Table 1), which were different from those identified by mmc0111. Therefore, umc2245 was located on the other side of *bm6* compared with mmc0111. Seven pairs of IDP markers were available between umc2245 and umc1165. Two of them were polymorphic but were dominant. Fortunately, PCR amplified bands appearing in B73, so that they could be used for mapping. IDP4732, IDP7712, and umc2245 detected recombination events on the other side compared with mmc0111 (and umc1552, umc2363, umc1165), and were placed 2.3, 1.8, and 1.8 cM relative to the *bm6* gene (Fig. 2). IDP7712 and umc2245 co-segregated in this genetic mapping F_2 set. Taking together, *bm6* was anchored in a 4.2 cM interval delimited by umc2363 (umc1165) and umc2245 (Fig. 2).

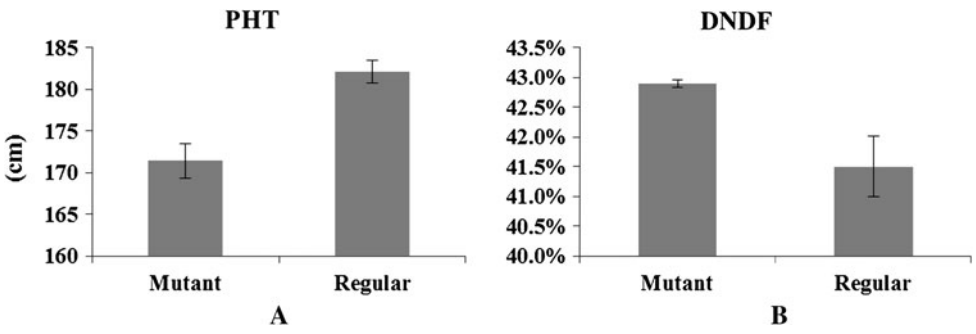

Fig. 1 Pleiotropic effect of *bm6* on plant height and DNDF. **a** The effect of *bm6* gene on PHT (plant height). It reduces plant height by 10.7 cm ($P < 0.01$). **b** The effect of *bm6* gene on DNDF (digestibility of neutral detergent fiber). It increases DNDF by 1.4 % ($P = 0.07$)

Table 1 Mapping of *bm6* by publicly available markers to a region flanked by umc2245 and umc2363

Recombinant individual plants	Markers						
	IDP4732 (35.32)[G]	umc2245 (30.9)	IDP7712 (36.4)	umc1165 (47.4)	umc2363 (54.15)	umc1552 (75.93)	mmc0111 (93.3)
P1-3E	*A.*	aa	0	aa	aa	aa	aa
P1-3D	*A.*	*Aa*	*A.*	aa	aa	aa	aa
P1-6C	*A.*	*Aa*	*A.*	aa	aa	aa	aa
P1-8C	*A.*	*Aa*	*A.*	aa	aa	aa	aa
P2-1H	*A.*	*Aa*	*A.*	aa	aa	aa	aa
P2-3E	*A.*	aa	–	aa	aa	aa	aa
P2-4H	*A.*	Aa	*A.*	aa	aa	aa	aa
P2-6A	*A.*	Aa	*A.*	aa	aa	aa	aa
P2-12C	*A.*	Aa	*A.*	aa	aa	aa	aa
P1-3A	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P1-4D	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P1-12H	0	aa	0	–	–	aa	*Aa*
P2-5C	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P2-5E	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P2-6E	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P2-9F	0	aa	0	*AA*	*AA*	*AA*	*AA*
P2-10H	0	aa	0	*Aa*	*Aa*	*Aa*	*Aa*
P2-12H	0	aa	0	–	*Aa*	*Aa*	*Aa*
P1-2B	0	aa	0	aa	aa	*Aa*	*Aa*
P2-2A	0	aa	0	aa	aa	*Aa*	*Aa*
P1-1H	0	aa	0	aa	aa	a	*Aa*
P2-2C	0	aa	0	aa	aa	a	*Aa*
Nbr of Rec.	9	7	7 (0)	8 (1)	8 (1)	29 (2)	50 (2)

All these markers were used to genotype 192 brown F_2 plants. Not "all non-bm6 allele type" were listed due to space limitation

[G] Numbers in parenthesis indicate the genetic positions on IBM2 2008 Neighbor Frame 2, except umc1552 whose position is inferred from IBM2 2008 Neighbors 2. IDP4732 was expected between umc2235 and umc2363, but was put upstream of umc2245 in our study. Because IDP4732 and IDP7712 were dominant markers, only two kinds of genotypes were detected

Nbr of Rec numbers of recombinants being identified in the genetic mapping F_2 set (with 192 brown F_2 plants). The numbers in the parenthesis were recombinants showing only B73 bands. Each marker detected far less than expected 144 (192×0.75) 'non-*bm6* allele type' plants at $P = 0.01$ level

For co-dominant markers "A" represents bands from of B73, "a" represents bands from of *bm6,* "Aa" represents heterozygotes. "AA" and "aa" indicated two alleles for a plant at a locus were all from B73 and *bm6* stock, respectively. "–"was used to represent missing data for co-dominant markers. For dominant markers, "A." represented a plant holding at least one allele from B73. "0" was used to record the plants with no band being detected at a marker locus

'Non-*bm6* allele type' loci which hold at least one B73 allele ("A") were *italicised*

Development of new SSR markers to saturate the target region

Within the ~890,000 bp region between umc2245 and umc2363, 99 SSR motifs were identified by MISA, and 20 of them were located in repeat sequences. In the first cycle, we tried to find one SSR marker per ~50 kb, but some regions were highly repetitive. Finally, we designed 14 pairs of primers, among which seven detected polymorphisms between both parents (Table 2). After the target region was further reduced in size, the second cycle of primer design provided eight pairs of primers, but only two of them showed polymorphisms. In total, nine polymorphic SSR markers were obtained from 22 pairs of primers tested, including dominant marker SSR-447208.

Physical mapping of the *bm6* gene

umc2363 and umc1165 were used to screen recombinants within the physical mapping F_2 set (including 960 mutant F_2 plants). umc2245 detected 20 'non-*bm6* allele type' plants from 960 plants. These 20 plants were upstream

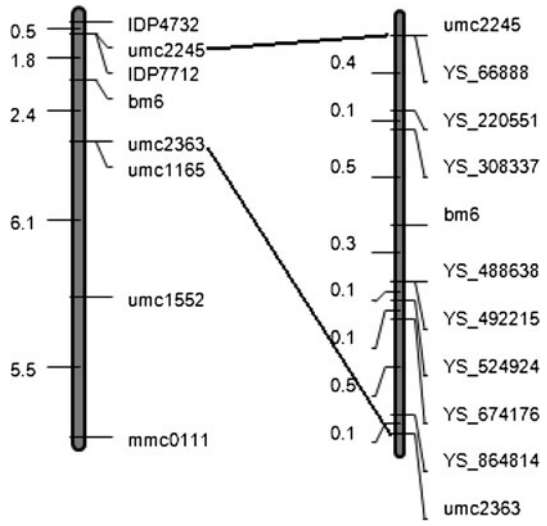

Fig. 2 Genetic map of *bm6*. *Left* genetic map is constructed by public available SSR and IDP markers, where the *bm6* gene was mapped between umc2245 and umc2363. The *right* genetic map was constructed with newly developed markers, where the *bm6* was delineated between YS_308337 and YS_488638

recombinants U1 to U20 (Table 3), indicating the recombination occurring on the upstream of the *bm6* gene. umc2236 detected 19 'non-*bm6* allele type' plants. These 19 plants were downstream recombinants D1 to D19, indicating recombination occurred downstream of the *bm6* gene. All nine newly developed polymorphic markers were used to genotype these 39 recombinants and one control which showed "a//a" at both umc2245 and umc2363 loci.

The dominant marker SSR_447208 was not assigned to the target region, because it detected 31 'non-*bm6* allele type' plants from these 39 recombinants, more than those identified by either umc2245 or umc2363. Although SSR_66888 and SSR_864814 were polymorphic co-dominant markers, they detected only two genotypes (either A//A or a//a) rather than three genotypes in those 39 recombinants. They were still used for mapping as they indicated, whether the B73 allele was present or absent. Finally, eight primers were assigned to the target region flanked by umc2245 and umc2363, as they detected the same or fewer recombinants identified by these two

Table 2 Primers of newly developed SSR markers and their product size in B73 within the *bm6* target region

Primer	Motif	Forward	Reverse	Size (bp)	Poly.
SSR_66888	TGG (7)	TGGCACTGATAGTCGTCCAG	AGCAAAGCCCTGTTATTGGA	101	Y
SSR_102465	AT (6)	GCGAGTGAAAAACACGTGAA	AAAACCGTGTACCCTGCAAT	147	N
SSR-157932	CAA (5)	AAGAACATTGGTGAGCTTGACA	CGAGTGATCGATCTGCATGT	168	N
SSR-220551	TTGCG (5)	CCCTGGAATCTCACACATGA	CCGTCACTTCCACTCCTACC	181	Y
SSR_263186	TG (10)	CGAGGTATTCGGATTCGTGT	AGGCCAGAAGAAACGGAGTT	162	N
SSR_308337	TA (6)	TTCTTGCTTGTCTCTAGCAGCTT	TGTGGCGATGTCCATGATT	179	Y
SSR_347455	CAAG (4)	CGTGCGTGACAGAAGCTG	CATCCGTTAGGTTCCACGAC	178	N
SSR_472348	TTG (9)	GTGCCAAGCAGGACCTAAAA	AAAGACCAAAGCCAAATGGA	153	N
SSR_492215	CCT (7)	CTGCGACGAGGACCACTT	TCTGATCCACGGCCATTATT	158	Y
SSR_524924	CGCT (4)	AAGCCACCTCTCAGCTCTCA	GGATCTTGCTGCTGCATTTC	196	Y
SSR_674176	CGG (5)	GTACCCGTCCATCGTCATCT	CGAAGCCACGAGGAAGTAAC	199	Y
SSR_720602	TG (6)	ATCTGCTGGTTCTGCCAGTT	AAGACCCCCGTACAACACAG	127	N
SSR_798730	CGT (9)	TACCAGTCGGATCGGATTCT	ATAGCCTGCTGCCTAAACGA	176	N
SSR_864814	TA (6)	CTCTTCAACACCATTCTCTAAGACAC	GACGGTCTCCAACAATATGATACA	198	Y
SSR_518876	ATT (5)	ACGACGACAACTTCCGTTTC	TGCAAAGGTGAGACGAACTG	188	N
SSR_232574	AG (9)	GTGGGAAATGAGCGAGAGAG	CCGCCTTTGTTTGTTTTTGT	111	N
SSR_312186	TAT (6)	CTCTGGCTCGGTTGCTTAAC	AGAGGCAAGCAAAACGACAT	191	N
SSR_330561	GAT (5)	ATGGTGTGCCATGATGCTAA	CGATGAATATGCGATGGATG	195	N
SSR_347315	GT (6)	AGGACAGGGCACCACATTAG	GGAAAGATGGCGCTCTGTTA	159	N
SSR_447208	TCG(5)	GAAGACCAGTGGCGTCTAGC	GGCTCGGATGAGTTGGAGTA	231	Y
SSR_488638	GC (6)	AGGCAACTCCTGTGTCTGTGT	CATGATCGCCCACTCCTT	233	Y
SSR_516108	CAT (5)	GAGCCCCGAATCTTTTCTCT	AAGACCTCCGATCCTGCTAAA	145	N

The numbers in the names indicate the relative position in the ~890,000 bp sequence. The first 14 primers are designed from the first cycle, the remaining are from the second cycle

Y detected polymorphisms between two parents, *N* no polymorphisms were detected between two parents

Table 3 Fine mapping of the *bm6* gene to a 180 kb region between ys308837 and ys488638 by newly developed SSR markers

Recombinants	Markers									
	umc2245	ys66888	ys220551	ys308337	ys488638	ys492215	ys524924	ys674176	ys864814	umc2363
Control	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
D1	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D2	aa	aa	aa	aa	aa	aa	aa	aa	aa	*Aa*
D3	aa	aa	aa	aa	aa	aa	aa	0	*AA*	*Aa*
D4	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	AA
D5	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D6	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D7	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D8	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D9	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D10	aa	aa	aa	aa	aa	aa	aa	aa	*AA*	*Aa*
D11	aa	aa	aa	aa	aa	aa	aa	*Aa*	*AA*	*Aa*
D12	aa	aa	aa	aa	aa	aa	aa	*Aa*	*AA*	*Aa*
D13	aa	aa	aa	aa	aa	aa	*Aa*	*Aa*	*AA*	*Aa*
D14	aa	aa	aa	aa	aa	aa	*Aa*	*Aa*	*AA*	*Aa*
D15	aa	aa	aa	aa	aa	aa	*Aa*	*Aa*	*AA*	*Aa*
D16	aa	aa	aa	aa	*Aa*	*Aa*	*Aa*	*Aa*	*AA*	*Aa*
D17	aa	aa	aa	aa	*Aa*	*Aa*	*Aa*	*Aa*	*AA*	*Aa*
D18	aa	aa	aa	aa	*Aa*	*Aa*	*Aa*	*Aa*	*AA*	*Aa*
D19	aa	aa	aa	aa	*Aa*	*Aa*	*Aa*	*Aa*	*AA*	*Aa*
U1	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U2	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U3	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U4	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U5	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U6	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U7	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U8	*Aa*	*AA*	aa	aa	aa	aa	aa	aa	aa	aa
U9	*Aa*	*AA*	*Aa*	aa	aa	aa	aa	aa	aa	aa
U10	*Aa*	*AA*	*Aa*	aa	aa	aa	aa	aa	aa	aa
U11	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U12	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U13	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U14	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U15	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U16	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U17	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U18	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U19	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa
U20	*Aa*	*AA*	*Aa*	*Aa*	aa	aa	aa	aa	aa	aa

"A" represents bands from of B73, "a" represents bands from of *bm6* stock, "Aa" represents heterozygotes. "AA" and "aa" indicated two alleles for a plant at a locus were all from B73 and *bm6* stock, respectively, "–" represents missing data

'Non-*bm6* allele type' loci which hold at least one B73 allele ("A") were *italicised*

flanking markers (umc2245 and umc2363). B73 segments expanded to SSR_308337 based on the genotypes of U11–U20, and B73 segments expanded to SSR_488638 based on the downstream recombinants of D1–D19 (Table 3). Thus, the closest markers flanking *bm6* from our study were SSR_308337 and SSR_488638, which defined about

Table 4 Candidate genes for *bm6* in maize and synteny to rice

Zea mays genes ID	Location	Gene annotation	*Oryza sativa* homologues ID	Location	Gene annotation
GRMZM2G046968	2:3396664–3402959	Putative uncharacterized protein	LOC_Os04g56750 (LOC_Os04g56750)	4:33663503–33668885	Zinc finger C-x8-C-x5-C-x3-H type family protein, expressed
GRMZM2G047018	2:3393037–3396062	PHD finger-like domain-containing protein 5A, Putative uncharacterized protein	No homologues		
GRMZM2G047448	2:3387696–3389171	WUS1 protein	LOC_Os04g56780 (LOC_Os04g56780)	4:33675265–33676315	Homeobox domain containing protein, expressed
GRMZM2G046852	2:3433019–3433460	Novel	No homologues		
GRMZM2G062420	2:3541475–3547749	GTP cyclohydrolase I 1	LOC_Os04g56710 (LOC_Os04g56710)	4:33629046–33633632	GTP cyclohydrolase I 1, putative, expressed
GRMZM2G062396	2:3556786–3558729	Putative uncharacterized protein	LOC_Os04g56700 (LOC_Os04g56700)	4:33624235–33627190	Naringenin, 2-oxoglutarate 3-dioxygenase, putative, expressed
GRMZM2G047321	2:3389845–3393264	Novel	LOC_Os04g56770 (LOC_Os04g56770)	4:33671620–33673950	Conserved hypothetical protein
GRMZM2G348909	2:3409613–3414955	Putative uncharacterized protein	LOC_Os04g56740 (LOC_Os04g56740)	4:33654902–33660829	IQ calmodulin-binding motif family protein, putative, expressed
GRMZM2G511859	2:3420867–3421201	Novel	No homologues		
GRMZM2G342107	2:3434908–3435997	Novel	LOC_Os05g48850 (LOC_Os05g48850)	5:27940521–27942357	No apical meristem protein

180 kb in length referring to B73 genome sequence. The relative genetic position of *bm6* to both flanking markers was about 0.5 cM to SSR_308337 and 0.3 cM to SSR_488638 based on the information from 960 brown midrib F_2 plants, respectively (Fig. 2).

In the physical mapping process, another type of "recombinant" was detected. Six plants were heterozygous at umc2245 (two plants) or heterozygous at both umc2245 and umc2363 loci (four plants). However, the B73 fragment spans from umc2245 to SSR_674176 for all these six plants. This result was in conflict with the above mentioned mapping results. If these are brown midrib plants, there should be no B73 segments in the region between SSR_308337 and SSR_488638. We suspect that these "recombinants" resulted from phenotyping and/or sampling errors. This would reflect a phenotyping/sampling error rate of $6/(960 \times 4) = 0.00156$, which is low given that phenotyping was done on single plants. Alternatively, there were double recombination events occurred between SSR_308337 and SSR_488638 within these six plants, but they were not captured as no markers were located within the 180 kb region. Anyway, these six plants were considered as suspicious plants and were excluded from the physical mapping process.

Candidate genes

In the target region between markers SSR_308337 and SSR_488638, ten gene models were predicted in maize B73 sequence (version 2.0, http://www.maizesequence.org), including a putative GTP cyclohydrolase I 1 gene (GRMZM 2G062420), a putative WUS1 gene (GRMZM2G047448), and eight putative uncharacterized or novel genes (Table 4). Among the eight putative uncharacterized or novel genes, one contains a PHD finger-like domain (GRMZM2G 047018), indicating it might be a transcription factor. The remaining seven genes do not have any indicated function. However, a comparison of the target region with rice revealed good synteny with a region on Chr 4 of rice. Seven out of the ten genes in the target region have homologous

genes in rice. Most showed synteny with Chr 4 except for GRMZM2G342107, with a rice gene hit on Chr 5. Six out of the identified seven homologous genes in rice have gene function annotation, which was used to infer the function of their orthologous counterparts in maize. Based on the information from rice, the genes GRMZM2G046968 might be a zinc finger family gene. The rice homologous counterpart of GRMZM2G342107 was annotated as no apical meristem (NAM) protein-like encoding gene. GRMZ2G 062396 is homologous to the naringenin 2-oxoglutarate 3-dioxygenasease encoding gene (*F3H)*. The rice homologous counterpart of GRMZM2G348909 is an IQ calmodulin-binding motif family gene, which might interact with cell wall related kinase WAK2 (Rohila et al. 2006).

Discussion

Brown midrib mutants that are characterized by brown-reddish coloration of the vascular tissue in the leaf blade and sheath are reported to have altered cell wall composition (Chabbert et al. 1994a, b; Marita et al. 2003; Barrière et al. 2004a, 2004b). They are also reported to have favorable properties for forage (Barrière and Argillier 1993) and cellulosic ethanol production due to reduced lignin content (Lorenz et al. 2009), especially *bm3*. Only four mutants known as *bm1–4* were characterized in maize until recently. More recently, additional *bm* mutants in maize were described. Haney et al. (2008) first reported *bm*F* as *bm5*. By allelism testing among all available brown midrib mutants from the maize stock centre including *bm1–4*, *bm*F* was confirmed as *bm5*, and *bm*E* and *bm*G* were allelic to *bm*F* (Ali et al. 2010). In addition *bm*J* was another mutation locus and designated as *bm6* (Ali et al. 2010). The well known *bm1–4* mutants are mapped on bins 5.04, 1.11, 4.05, and 9.07 (Vermerris 2009). This study mapped *bm6* to Chr 2, which confirmed *bm6* as a novel brown midrib gene. Furthermore, the physical mapping of *bm6* into a 180 kb region will enable final isolation of the underlying gene.

bm1–4 in maize and *bmr6*, *bmr12*, *bmr18* in sorghum are characterized by reduced lignin content and altered lignin composition, but they often have negative effects on agronomic traits although genetic background and environment might confound the effects of brown midrib genes (Pedersen et al. 2005). Homozygous status of *bm3* in maize significantly reduces plant grain and/or forage yield (Miller et al. 1983; Cox and Cherney 2001), and plant height (Lee and Brewbaker 1984). Homozygous status of *bmr6* and *bmr18* in sorghum might also suppress plant height compared with the normal isogenic lines (Casler et al. 2003; Oliver et al. 2005a, b). F_2 plants homozygous for the *bm6* mutation were significantly shorter than regular F_2 plants in our study. Average DNDF of mutant bulks was 1.4 % higher than average of regular bulks. However, the difference of average DNDF between mutant and regular bulks was just suggestive significant ($P = 0.07$), which might be due to the small sample size. In our study we had only 4 mutant bulks and 4 regular bulks, respectively. In addition, the genetic background was expected to be quite different across the bulks as each bulk contained only nine F_2 plants. Digestibility is complex traits and is controlled by many QTL, therefore the non-uniform genetic background might confound the effect of *bm6* gene on DNDF. Thus, the suggestive significance at $P = 0.07$ encourages studying the effect of *bm6* on DNDF and other cell-wall properties by isogenic lines. Our mapping result provided tightly linked markers, which will speed up development of near isogenic lines by marker-assisted selection. Comparing near isogenic lines with and without *bm6* will help to evaluate the effect of *bm6* on cell wall digestibility traits and agronomic related traits, such as plant height, flowering time, lodging, grain, and forage yield. As near isogenic lines cannot distinguish between genic pleiotropy and tight genic linkage, knocking out the candidate gene by RNAi and transposon tagging will help to discriminate pleiotropy and linkage at the gene level (Chen and Lübberstedt 2010).

The mechanism underlying the brown coloration in the vascular tissue is still not clear. However, Satter et al. (2010) proposed that color change in the lignin-rich tissue is a good indication of disturbance of monolignol, also probably flavones biosynthesis in C4 grasses. In our study, the *bm6* gene was mapped into a 180 kb region in bin 2.01. Nearby this region, some monolignol biosynthesis genes are located, for example *CAD6/SAD* (Ac2155 994.3_FG038) in bin 2.02, *PAL2a* (AC213314.3_FG039) and *PAL2b* (AC213314.3_FG037) in bin 2.03 (Barrière et al. 2009). In our target region, ten gene models are predicted in B73 sequence. Among these ten genes, four are promising candidate genes. GRMZ2G062396 is homologous to the naringenin 2-oxoglutarate 3-dioxygenase gene, which is also called flavanone 3-hydroxylase (*F3H*) gene. GRMZ2G06396 contains the oxoglutarate/Fe-dep oxygenase domain and could oxidize phenols or other products in secondary metabolism. Flavonoids as well as lignin are synthesized from phenylpropanoid pathway. *p*-Coumaryl CoA which is the product of the 4-coumarate:CoA ligase (4CL) enzyme in the phenylpropanoid pathway is used for either lignin monolignol or flavonoid biosynthesis. *F3H* is co-expressed with other flavonoid enzymes involved in anthocyanin biosynthesis and responsible for plant colorations (Han et al. 2010). In *Arabidopsis*, genes in the anthocyanin biosynthesis pathway are co-regulated by MYB11 and MYB12 (Stracke et al. 2007). Interestingly, the lignin biosynthesis genes are also co-regulated by MYB transcriptional factors (Zhao

and Dixon 2011). Additionally, lignin biosynthesis is carbon consuming and cross-talking with other physiological processes (Zhao and Dixon 2011). For example, the reduction of the flux of carbon into lignin resulted in accumulation of flavonoid due to a mutation in the *HCT* gene (Besseau et al. 2007). Thus, it is reasonable to speculate that a mutation in *F3H* involved in flavonoid biosynthesis will affect lignin biosynthesis due to either synchronous regulation of flavonoid and lignin biosynthesis or a trade-off of resources between the two biosynthesis branches. Naringenin, substrate for *F3H* in the flavonoid biosynthesis pathway, inhibits the activity of 4CL in vitro in several species such as petunia (Ranjeva et al. 1976), loblolly pine (Voo et al. 1995), maize, rice, and *Echinochloa oryzicol* (Deng et al. 2004). Addition of naringenin to rice produced browning leaf tips and decreased coniferyl alcohol as well as the lignin content by about 10 % (Deng et al. 2004). In vitro application of naringenin could also inhibit the growth of maize, rice, *Echinochloa oryzicol* (Deng et al. 2004). Mutation in *F3H* might lead to accumulation of naringenin, which will suppress plant growth. In our mapping population, we chose 153 F_2 plants in the plant height analysis. The average plant height of mutant plants (46 plants) was significantly lower than that of regular plants (107 plants).

Lignin biosynthesis is highly coordinated by transcription factors (reviewed by Zhong and Ye 2009; Zhao and Dixon 2011). Most of the monolignol biosynthesis genes, except *F5H*, are regulated by transcription factors MYB58/63/85 through AC elements or more degenerate AC elements (Zhou et al. 2009; Zhao et al. 2010). Investigating the differential gene expression by *bm1–4* isogenic lines, Guillaumie et al. (2007a, 2007b) identified other transcription factors and regulatory genes besides MYB factors showing differential expression between regular and mutant isogenic lines. Among them were three zinc finger like protein coding genes. Thus GRMZM2G046968 gene is another candidate for *bm6,* since its syntenic counterpart in rice is a Zinc finger gene.

Gene GRMZM2G348909 is homologous with a rice IQ calmodulin-binding motif family gene. In rice, a calmodulin-binding motif protein was reported to interact with wall-associated kinase (WAK)-2 like protein (Rohila et al. 2006). WAKs are receptor like protein kinase, which are tightly linked with cell wall (He et al. 1996), and are involved in regulation of cell elongation (Lally et al. 2001) and expansion (Wagner and Kohorn 2001). This regulation requires a calcium-dependent binding protein to form the signaling complex (Decreux and Messiaen 2005). In *Arabidopsis*, the expression of WAK1 was more often seen in vascular cells with high levels of expression in older leaves than younger ones (Wagner and Kohorn 2001). Secondary cell wall of vascular tissue is normally with high level of lignifications. Taking together, the putative IQ calmodulin-binding motif gene in our target region might be involved in lignin regulation by interacting with *WAK* proteins.

Another interesting gene in our target region is GRMZM2G047488 which is homologous to the *WUSCHEL1*(*WUS1*) gene in *Arabidopsis*. There are two copies of *WUS* genes in maize (NCBI), which contain homeobox domains. In *Arabidopsis*, there are seven KNOX homeobox genes. BP (BREVIPEDICELLUS) is one of the seven KNOX homeobox genes and it determines not only internode patterning, but also regulates lignin biosynthesis (Mele et al. 2003). *bp* mutants resulted in increased lignin, while overexpression of *BP* resulted in decreased lignin (Mele et al. 2003). However, no similarity between *WUS1* and *BP* was found. Although above mentioned four genes could be candidate genes of *bm6*, we cannot rule out the possibility of other genes with unknown function as the candidates of *bm6* currently. Further study of these ten genes in our target region will help us to isolate the *bm6* gene.

Acknowledgments We would like to thank Elizabeth Bovenmyer, Janine Comstock, and Alice Wang for help in field. We appreciate the generosity of Dr. Sarah Hake (Adjunct Professor and Center Director; Plant Gene Expression Center USDA-ARS) and Dr. Erick Vollbrecht (professor in Department of Genetics, Development and Cell biology at Iowa State University) to provide position information of *bm6* gene. Yongsheng Chen was supported by the Interdepartmental Genetics Graduate Program as well as RF Baker Center for Plant Breeding at Iowa State University. Hongjun Liu is a visiting student at ISU, supported by China Scholarship Council.

References

Ali F, Scott P, Bakht J, Chen Y, Lübberstedt T (2010) Identification of novel brown-midrib genes in maize by tests of allelism. Plant Breed 129:724–726

Andrieu J, Demarquilly C, Dardenne P, Lila M, Barrière Y, Maupetit P, Rivière F, Femenias N (1993) Composition and nutritive value of whole maize plant fed fresh to sheep. I. Factors of variation. Ann Zootech 42:221–249

Barrière Y, Argillier O (1993) Brown-midrib genes of maize—a review. Agronomie 13:865–876

Barrière Y, Guillet C, Goffner D, Pichon M (2003) Genetic variation and breeding strategies for improved cell wall digestibility in annual forage crops. A review. Anim Res 52:193–228

Barrière Y, Emile JC, Traineau R, Surault F, Briand M, Gallais A (2004a) Genetic variation for organic matter and cell wall digestibility in silage maize. Lessons from a 34-year long experiment with sheep in digestibility crates. Maydica 49:115–126

Barrière Y, Ralph J, Méchin V, Guillaumie S, Grabber JH, Argillier O, Chabbert B, Lapierre C (2004b) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants. C R Biol 327:847–860

Barrière Y, Alber D, Dolstra O, Lapierre C, Motto M, Ordas A, Van Waes J, Vlasminkel L, Welcker C, Monod JP (2005) Past and prospects of forage maize breeding in Europe. I. The grass cell wall as a basis of genetic variation and future improvements in feeding value. Maydica 50:259–274

Barrière Y, Méchin V, Lafarguette F, Manicacci D, Guillon F, Wang H, Lauressergues D, Pichon M, Bosio M, Tatout C (2009) Toward the discovery of maize cell wall genes involved in silage quality and capacity of biofuel production. Maydica 54:161–198

Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2007) Flavonoid accumulation in *Arabidopsis* repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell 19:148–162

Brenner EA, Blanco M, Gardnber C, Lubberstedt T (2012) Genotypic and phenotypic characterization of isogenic doubled haploid exotic introgression lines in maize. Mol Breed. doi: 10.1007/s11032-011-9684-5

Casler MD, Jung HG (2006) Relationships of fibre, lignin, and phenolics to in vitro fibre digestibility in three perennial grasses. Anim Feed Sci Technol 125:151–161

Casler MD, Pedersen JF, Undersander DJ (2003) Forage yield and economic losses associated with the brown-midrib trait in sudangrass. Crop Sci 43:782–789

Chabbert B, Tollier MT, Monties B, Barrière Y, Argillier O (1994a) Biological variability in lignification of maize: expression of the brown midrib bm3 in three maize cultivars. J Sci Food Agric 64:349–355

Chabbert B, Tollier MT, Monties B, Barrière Y, Argillier O (1994b) Biological variability in biological variability in lignification of maize: expression of the brown midrib bm2 mutation. J Sci Food Agric 64:455–460

Chen Y, Lübberstedt T (2010) Molecular basis of trait correlations. Trends Plant Sci 15:454–461

Cox WJ, Cherney DJR (2001) Influence of brown midrib, leafy, and transgenic hybrids on corn forage production. Agron J 93:790–796

Decreux A, Messiaen J (2005) Wall-associated kinase WAK1 interacts with cell wall pectins in a calcium-induced conformation. Plant Cell Physiol 46:268–278

Demain AL (2009) Biosolutions to the energy problem. J Ind Microbiol Biotechnol 36:319–332

Deng F, Aoki M, Yogo Y (2004) Effect of naringenin on the growth and lignin biosynthesis of gramineous plants. Weed Biol Manag 4:49–55

Energy Independence and Security Act of 2007, P.L. 110–140

Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311:506–508

Fontaine AS, Bout S, Barrière Y, Vermerris W (2003) Variation in cell wall composition among forage maize (*Zea mays* L.) inbred lines and its impact on digestibility: analysis of neutral detergent fiber composition by pyrolysis–gas chromatography–mass spectrometry. J Agric Food Chem 51:8080–8087

Grabber JH, Ralph J, Lapierre C, Barrière Y (2004) Genetic and molecular basis of grass cell-wall degradability. I. Lignin–cell wall matrix interactions. C R Biol 327:455–465

Grabber JH, Mertens DR, Kim H, Funk C, Lu FC, Ralph J (2009) Cell wall fermentation kinetics are impacted more by lignin content and ferulate crosslinking than by lignin composition. J Sci Food Agric 89:122–129

Guillaumie S, Pichon M, Martinant JP, Bosio M, Goffner D, Barrière Y (2007a) Differential expression of phenylpropanoid and related genes in brown-midrib bm1, bm2, bm3, and bm4 young near-isogenic maize plants. Planta 226:235–250

Guillaumie S, San-Clemente H, Deswarte C, Martinez Y, Lapierre C, Murigneux A, Barrière Y, Pichon M, Goffner D (2007b) MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol 143:339–363

Guo D, Chen F, Wheeler J, Winder J, Selman S, Peterson M, Dixon RA (2001) Improvement of in-rumen digestibility of alfalfa forage by genetic manipulation of lignin *O*-methyltransferases. Transgenic Res 10:457–464

Halpin C, Holt K, Chojecki J, Oliver D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA (1998) Brown-midrib maize (bm1)-a mutation affecting the cinnamyl alcohol dehydrogenase gene. Plant J 14:545–553

Han Y, Vimolmangkang S, Soria-Guerra RE, Rosales-Mendoza S, Zheng D, Lygin AV, Korban SS (2010) Ectopic expression of apple F3′H genes contributes to anthocyanin accumulation in the *Arabidopsis* tt7 mutant grown under nitrogen stress. Plant Physiol 153:806–820

Haney LJ, Hake S, Scott PM (2008) Allelism testing of Maize Coop Stock Center lines containing unknown brown midrib alleles. Maize Genet Coop Newslett 82:4–5

He ZH, Fujiki M, Kohorn BD (1996) A cell wall-associated, receptor-like protein kinase. J Biol Chem 271:19789–19793

Ingvardsen CR, Xing Y, Frei UK, Lübberstedt T (2010) Genetic and physical fine mapping of Scmv2, a potyvirus resistance gene in maize. Theor Appl Genet 120:1621–1634

Lally D, Ingmire P, Tong HY, He ZH (2001) Antisense expression of a cell wall-associated protein kinase, WAK4, inhibits cell elongation and alters morphology. Plant Cell 13:1317–1331

Lauer J (1995) Corn germplasms for silage uses. Field Crops 28:4–5

Lauer JG, Coors JG, Flannery PJ (2001) Forage yield and quality of corn cultivars developed in different eras. Crop Sci 41:1449–1455

Lee MH, Brewbaker LL (1984) Effects of brown midrib on yields and yield components of maize. Crop Sci 24:105–108

Lorenz AJ, Anex RP, Isci A, Coors JG, de Leon N, Weimer PJ (2009) Forage quality and composition measurements as predictors of ethanol yield from maize (*Zea mays* L.) stover. Biotechnol Biofuels 2:5

Marita JM, Vermerris W, Ralph J, Hatfield RD (2003) Variations in the cell wall composition of maize brown midrib mutants. J Agric Food Chem 51:1313–1321

Mele G, Ori N, Sato Y, Hake S (2003) The knotted1-like homeobox gene BREVIPEDICELLUS regulates cell differentiation by modulating metabolic pathways. Genes Dev 17:2088–2093

Miller JE, Geadelmann JL, Marten GC (1983) Effect of the brown midrib-allele on maize silage quality and yield. Crop Sci 23:493–496

Moore KJ, Jung HG (2001) Lignin and fiber digestion. J Range Manag 54:420–430

Morrow SL, Mascia P, Self KA, Altschuler M (1997) Molecular characterization of a brown midrib3 deletion mutant in maize. Mol Breed 3:351–357

Oliver AL, Pedersen JF, Grant RJ, Klopfenstein TJ (2005a) Comparative effects of the sorghum bmr-6 and bmr-12 genes: I. Forage sorghum yield and quality. Crop Sci 45:2234–2239

Oliver AL, Pedersen JF, Grant RJ, Klopfenstein TJ, Jose HD (2005b) Comparative effects of the sorghum bmr-6 and bmr-12 genes: II. Grain yield, stover quality in grain sorghum. Crop Sci 45:2240–2245

Pedersen JF, Vogel KP, Funnell DL (2005) Impact of reduced lignin on plant fitness. Crop Sci 45:812–819

Provan GJ, Scobbie L, Chesson A (1997) Characterization of lignin from CAD and OMT deficient Bm mutants of maize. J Sci Food Agric 73:133–142

Ranjeva R, Boudet AM, Faggion R (1976) Phenolic metabolism in petunia tissues. IV. Properties of *p*-coumarate coenzyme A ligase isoenzymes. Biochimie 58:1255–1262

Rathin D, Maher MA, Jones C, Brinker RW (2011) Ethanol—the primary renewable liquid fuel. J Chem Technol Biotechnol 86:473–480

Riboulet C, Lefevre B, Denoue D, Barrière Y (2008) Genetic variation in maize cell wall for lignin content, lignin structure,

p-hydroxycinnamic acid content, and digestibility in set of 19 lines at silage harvest maturity. Maydica 53:11–19

Rohila JS, Chen M, Chen S, Chen J, Cerny R, Dardick C, Canlas P, Xu X, Gribskov M, Kanrar S, Zhu JK, Ronald P, Fromm ME (2006) Protein-protein interactions of tandem affinity purification-tagged protein kinases in rice. Plant J 46:1–13

Satter SE, Funnel-Harris DL, Pedersen JF (2010) Brown midrib mutations and their importance to utilization of maize, sorghum, and pearl millet lignocellulosic tissues. Plant Sci 178:229–238

Schnable PS, Ware D, Fulton RS, Stein J, Wei F, Pasternak S et al (2009) The B73 maize genome: complexity, diversity, and dynamics. Science 326:1112–1115

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J 50:660–677

Taboada A, Novo-Uzal E, Flores G, Loureda M, Ros Barceló A, Masa A, Pomar F (2010) Digestibility of silages in relation to their hydroxycinnamic acid content and lignin composition. J Sci Food Agric 90:1155–1162

Thiel T, Michalek W, Varshney RK, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor Appl Genet 106:411–422

Van Ooijen JW (2006) JoinMap 4, software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V, Wageningen

Vermerris W (2009) Cell wall biosynthetic genes of maize and their potential for bioenergy production. Handbook of maize genetics and genomics. Springer Science and Business Media, New york, pp 741–769

Vermerris W, Saballos A, Ejeta G, Mosier NS, Ladisch MR, Carpita NC (2007) Molecular breeding to enhance ethanol production from corn and sorghum stover. Crop Sci 47:S142–S153

Vignols F, Rigau J, Torres MA, Capellades M, Puigdomènech P (1995) The brown midrib3 (bm3) mutation in maize occurs in the gene encoding caffeic acid *O*-methyltransferase. Plant Cell 7:407–416

Voo KS, Whetten RW, O'Malley DM, Sederoff RR (1995) 4-Coumarate: coenzyme a ligase from loblolly pine xylem. Isolation, characterization, and complementary DNA cloning. Plant Physiol 108:85–97

Wagner TA, Kohorn BD (2001) Wall-associated kinases are expressed throughout plant development and are required for cell expansion. Plant Cell 13:303–318

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153–157

Zhao Q, Dixon RA (2011) Transcriptional networks for lignin biosynthesis: more complex than we thought? Trends Plant Sci 16:227–233

Zhao Q, Wang H, Yin Y, Xu Y, Chen F, Dixon RA (2010) Syringyl lignin biosynthesis is directly regulated by a secondary cell wall master switch. Proc Natl Acad Sci USA 107:14496–14501

Zhong R, Ye ZH (2009) Transcriptional regulation of lignin biosynthesis. Plant Signal Behav 4:1028–1034

Zhou J, Lee C, Zhong R, Ye ZH (2009) MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis*. Plant Cell 21:248–266