Theor Appl Genet (2009) 118:423–431
DOI 10.1007/s00122-008-0908-y

ORIGINAL PAPER

Molecular mapping and characterization of *BLMC*, a locus for profuse wax (bloom) and enhanced cuticular features of Sorghum (*Sorghum bicolor* (L.) Moench.)

Gloria B. Burow · Cleve D. Franks · Veronica Acosta-Martinez · Zhanguo Xin

Received: 2 May 2008 / Accepted: 27 September 2008 / Published online: 5 November 2008

Abstract Sorghum is distinct from other cereal crops due to its ability to produce profuse amount of epicuticular wax (EW or bloom) on its culm and leaves along with less permeable cuticle which are considered to be important traits contributing to abiotic stress tolerance. Here, we report the molecular mapping and characterization of *BLOOM-CUTICLE (BLMC)*, a locus associated with production of profuse wax, using a mutant mapping population developed from a cross between BTx623 (wild type with profuse wax) and KFS2021 (a mutant with greatly reduced wax). The F_2 progenies were genotyped using known and newly developed microsattelite markers to establish a molecular map of *BLMC*. The locus mapped to a 3.6-centimorgans (cM) interval in the terminal end of sorghum chromosome 10 with flanking markers *Xsbarslbk10.47* and *Xcup42*. Targeted mapping delimited *BLMC* to as small as 0.7 cM region and facilitated identification of three cosegregating markers with the trait. The *BLMC* region corresponds to approximately 153,000 bp and candidate genes identified include among others an acyl CoA oxidase (a gene involved in lipid and wax biosynthesis) and seven other putative transcripts. Phenotypic characterization showed that in addition to disrupting the EW production, *BLMC* mutation reduced culm and leaf cuticle, increased plant death rating in the field at anthesis and significantly reduced the C:28 to C:30 free fatty acid fractions of culm and leaf EW. These results clearly support the important role of *BLMC* in the expression of profuse wax and enhanced cuticular features of sorghum. Genetic mapping of *BLMC* opened avenues for identification of genes involved in the cuticle/wax pathway of sorghum and their application for improvement of abiotic stress tolerance.

Communicated by H. T. Nguyen.

G. B. Burow (✉) · C. D. Franks · Z. Xin
Plant Stress and Germplasm Development Unit,
Cropping Systems Research Laboratory, USDA ARS,
3810 4th Street, Lubbock, TX 79415, USA
e-mail: gloria.burow@ars.usda.gov

V. Acosta-Martinez
Wind Erosion and Water Conservation Unit,
Cropping Systems Research Laboratory, USDA ARS,
3810 4th Street, Lubbock, TX 79415, USA

Introduction

Sorghum is one of the most valuable cereal crop in the world and the second main important source of grain which serves as a dedicated substrate for ethanol production in the United States (Rooney 2004). Among cereals, sorghum is distinctive due to its overall abiotic stress tolerance features. A key feature of sorghum that is highly relevant to drought tolerance involves profuse production of epicuticular wax (EW or bloom) on culm and leaves coupled with less permeable cuticle proper (Jenks et al. 1994). Epicuticular wax deposition is highly visible on the abaxial side of the leaf blade, culm, and peduncle of sorghum. EW deposition is expressed at seedling stage and can be readily observed, but is most prominent at the pre-flowering to maturity stage (Jordan et al. 1983; Jenks et al. 1994, 2000). Sorghum produces higher amounts of bloom in culms and leaves than rice and corn both on per area and weight bases (Burow et al. 2008; Beattie and Marcel. 2002; O'Toole and Cruz 1983). This high level of EW deposition is a dominant trait and all sorghum hybrids grown in the US exhibit the feature with some variation (Jordan et al. 1983, 1984).

In sorghum, the role and importance of EW to abiotic stress tolerance has been analyzed in a number of studies

due to ease of its screening. Earlier report suggested that the greater amount of epicuticular wax in sorghum could lead to reduced cuticular transpiration and enhanced stomatal control of water loss (Blum 1975). Jenks et al. (1994) showed that bloomless mutation increased cuticular transpiration with pleiotropic effects and increased the susceptibility to the fungal pathogen *Exserohilum turcicum*. Recently, a physiological and genetic study of the profuse wax trait of sorghum was reported using a mutant population (Burow et al. 2008). In this report, an F_2 population from a cross between a profuse wax producing inbred BTx623 and an irradiated bloomless mutant KFS2021 have been developed. A combination of genetic and physiological approaches was employed to analyze how leaf and culm profuse wax using mutants are correlated with other physiological traits of sorghum. The profuse wax-cuticle trait mutation in this study was found to be under the control of a recessive nuclear gene in agreement with previous reports (Jenks et al. 1994).

Information on the genes and pathways involved in profuse wax production remains to be uncovered in sorghum. Majority of the studies that dealt with the molecular basis of wax synthesis and cuticular function have been conducted with maize and *Arabidopsis* as model species. For maize, molecular analysis of genes involved in cuticular wax synthesis has been deduced using the glossy mutants (Hansen et al. 1997; Xu et al. 1997, 2002; Sturaro et al. 2005). For *Arabidopsis thaliana*, the use of *eceriferum* or *cer* mutants were instrumental in cloning and identification of genes involved in cuticle assembly and wax production (Koorneef et al. 1989; Jenks et al. 1995, 1996). The nature of these genes that have been described were based on loss of function mutations and biochemical analyses of the metabolites. Some examples of the genes that have been identified from Arabidopsis are: *cer1* to *cer4* in the *WS* background and *cer6*, *cer8*, *cer9* and *cer16* in the *Ler* background (Jenks et al. 1995). Other genes that have been identified and cloned that are directly related to cuticular waxes but were not based on *cer* mutants are: *CUT1*, *WAX2*, *fiddlehead*, *LACS2* and *HIC* loci (Millar et al. 1999; Nawrath 2006; Chen et al. 2003). Information on transcriptional activators involved in wax biosynthesis has also been reported from *A. thaliana*. The transcription factor WIN/SHINE from the *AP2* domain containing class of regulatory genes have been studied and used for over expression studies in alfalfa to increase wax production and drought tolerance (Broun et al. 2004; Aharoni et al. 2004; Zhang et al. 2005).

Elucidation of the molecular basis of the wax pathway and cuticular function using *Arabidopsis* has been generally a fruitful undertaking. However, it is essential to complement the information from model plant systems with knowledge from specific crop species like sorghum which has profuse wax and distinct cuticle that has been associated with drought tolerance.

To begin to understand the molecular basis of profuse wax and cuticular features of sorghum, we analyzed the map location of *BLOOM-CUTICLE* (*BLMC*) by genetic mapping and identified candidate genes for the locus. We also characterized the genetic effects of mutation in *BLMC* on cuticular quantity, phenotypic response in the field and biochemical composition of waxes using selected F_3 families. The results from this study will pave the way for cloning sorghum genes which control profuse amount of cuticular wax and enhanced cuticular traits.

Materials and methods

Mapping population

An F_2 population was developed by crossing the mutant line KFS2021 to inbred line BTx623. KFS2021, a bloomless mutant with no visible epicuticular wax, was developed by gamma irradiation of Tx7078 through the research program of the late Dr. Keith F. Schertz (Burow et al. 2008). The locus for the gene from mutant KFS2021 was designated *blmc* (wild type *BLMC* from BTx623) for bloomless trait. This designation is a modification of the nomenclature *bm* which was used earlier for bloomless mutants (Karper 1933; Jenks et al. 1994; Rooney 2000).

The original wild type full sib of mutant KFS 2021 was Tx7078. Tx7078 is an established restorer line characterized by early maturity, pre-flowering drought tolerance and good combining ability. BTx623 is a widely adapted maintainer inbred line which has been used as parent for a number of molecular genetic maps of sorghum (Chittenden et al. 1994; Kong et al. 2000; Menz et al. 2002; Bowers et al. 2003).

F_1 seeds were harvested, grown in two gallon pots in the greenhouse under a temperature regime of 28°C day/25°C night. Plants were maintained under well-watered conditions with automatic drip irrigation and fertilized with Osmocote (composition 18% N, 6% P, 12% K, Scotts Co, Marysville, OH, USA). The F_2 seeds which consist of 2,020 individuals from a single confirmed F_1 plant were harvested. Initially, a total of 220 F_2 individuals were grown and used for genotyping and evaluation of physiological features related to epicuticular wax load (Burow et al. 2008). Only 120 of these F_2 progenies were self-pollinated to produce the F_3 generation in the greenhouse and were included in the mapping population. Later, an additional 100 F_2 progenies were grown, phenotyped, genotyped and self-pollinated for targeted mapping making a total of 220 F_2 progenies used for genetic mapping.

Phenotypic and biochemical analyses

The F_3 families were planted in Lubbock, Texas, for phenotypic and genotypic observation in 2007. Weed control, fertilization and irrigation were applied using standard agronomic practices for sorghum. The bloom/bloomless phenotypes were scored at 30 days after planting (DAP) and at booting stage based on visual observation of the presence/absence of copious amount of EW in culms and leaves. The genotype of each F_3 family was confirmed by observing for segregation of bloom/bloomless trait. Leaf and plant death rating was evaluated at 68 and 80 DAP using a range of 1–8 where 1 = zero to 10% plant death and 8 = 100% plant death (essentially complete drying of all plants).

Detailed phenotypic analysis was conducted using greenhouse grown plants of the parents and five F_3 families of the homozygous bloom/bloomless and heterozygous bloom classes. Each family is represented by nine plants. The experiment was laid in completely randomized design with three replications. Determination of epicuticular wax load in culms and leaves was performed using gravimetric methods described by Ebercon et al. (1977). Epidermal permeability was determined based on chlorophyll efflux from leaves and stems according to protocols by Lolle et al. (1997).

The amount of culm and leaf cuticle (expressed on per area basis) was determined using procedures described by Chen et al. (2003) with modifications. Briefly, 20 discs were soaked for 6 h in 60% (w/v) solution of ZnCl in HCl to separate cuticle membranes. Cuticular samples from 10 to 12 discs separated with a dissecting microscope were pooled, placed in pre-weighed microscope slides, oven dried at 60°C and placed in a desiccator with silica gel for at least 12 h. The average cuticle weights on per area basis for the parents and each F_3 families were determined from three replicates.

The composition of epicuticular waxes was analyzed using a 6890 Hewlett Packard Gas chromatograph Series II (Hewlett Packard, Palo Alto, CA, USA), equipped with flame ionization detector and automatic injector. Briefly, 20 leaf discs carefully collected from leaf and culms were rinsed in 5 ml of chloroform for 30 s. The extracts which contained waxes were dried with a stream of nitrogen gas. The dried residue was weighed and prepared for gas chromatography by derivatization with *N*,*O*-bis(tirmethylsilyl)trifluoracetamide (BSTFA) as described by Jenks et al. (2000).

DNA extraction and genotyping

A high throughput extraction protocol was applied to obtain sufficient amount and good quality genomic DNA from the parents and 220 F_2 progenies comprising the mapping population. Genomic DNA was extracted from 50 mg lyophilized leaf tissue collected from young leaves (collected at around 25–30 DAP) using a combination of methods employing Cetyltrimethylammoniumbromide (CTAB) precipitation, followed by a modified purification with the Magattract Kit (QIAGEN Inc., Valencia, CA, USA). Briefly, freeze dried tissues were ground twice using a Retsch Mixer Mill MM300 grinder with the aid of 5 mm tungsten bead (Maximum Velocity, Peoria, AZ, USA) at 25 Hz for 1 min. Leaf tissue samples were extracted with CTAB buffer (100 mM TrisCl (pH 8.0), 20 mM EDTA, 2% CTAB, 1 M NaCl, 0.1% B-mercapto ethanol added just before use), by incubating at 60°C for 1 h, followed by extraction with chloroform:isoamyl alcohol (24:1). Precipitation of the CTAB-DNA complex was accomplished by diluting [NaCl] of the sample to 0.5 M final concentration with a CTAB dilution buffer (100 mM TrisCl, pH 8.0, 20 mM EDTA, 2% CTAB). Clean-up of DNA with the MagAttract kit was modified by using a resuspension solution containing 0.5 M NaCl and 50% ethanol. The final genomic DNA pellet was resuspended in 100 µl TE, pH 8.0. DNA concentration was quantified using a ND-1000 Spectrophotometer (NanoDrop Technology, Wilmington, DE, USA).

Microsattelite or simple sequence repeat (SSR) mapping was accomplished using a modified forward primer tagged with 20 mer M13 sequence (a GAC GTT GTA AAA CGA CGG CC oligo tag was added at the 5′ end of each forward primer) and a normal reverse primer (without tag) for each marker. The sequences of forward and reverse SSR markers used in this study were obtained from http://sorgblast3.tamu.edu and http://www.gramene.org for *Xtxp* and *Xgap* markers, and that of *Xcup* markers was reported by Schloss et al. (2002). To facilitate detection, fluorescent labeled 20 mer oligo (GAC GTT GTA AAA CGA CGG CC with HEX or FAM or NED tag) was added to the PCR mix. PCR was performed in a volume of 10 µl, containing 1X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.25 units of HotStar *Taq* DNA polymerase (Qiagen Inc., Valencia, CA, USA), 5 pmol of each M13 tagged forward and normal reverse primer, 0.02 pmol of fluorescently labeled 20 mer M13 oligo and 20 ng of template genomic DNA. Amplifications were performed using a PTC-225 (MJ Research, Waltham, MA, USA) thermocycler according to the following conditions: 95.0°C for 15 m (1 cycle); 94.0°C for 1 min, followed by 39 cycles of 94.0°C for 30 s, 53.0°C for 30 s, 72.0°C for 90 s; and a final extension at 72.0°C for 10 min.

Molecular mapping and statistical analyses

A set of 146 SSR markers distributed throughout the genome of sorghum at approximately 20–30 centimorgans (cM) intervals were tested for polymorphism between the

two parents. PCR products were visualized in 3.5% high resolution sieving (SFR) agarose gels or were size separated by capillary electrophoresis using an ABI Prism 3100 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Multiplexing of three differentially labeled PCR products per well was carried out to increase genotyping efficiency. SSRs were analyzed using Genescan 3.1.2 software (Applied Biosystems Inc., Foster City, CA, USA).

To determine tentative association between SSR marker(s) and the *blmc* gene, a total of 20 bloom, and 10 bloomless F_2 progenies were genotyped with polymorphic markers. Targeted mapping of the region of interest was carried out by generating and mapping additional SSR markers mined from assembled whole genome shotgun (WGS) sequence of sorghum available at http://www.phytozome.net. New SSR markers were developed by searching the region of interest for di- to penta nucleotide repeats at every 30,000 bp using SSRIT (Temnykh et al. 2001). Primers were designed for the newly identified SSR markers (which were designated as *Xsbarslbk*) using Primer3 (http://frodo.wi.mit.edu). Genotyping of the parents with *Xsbarslbk* markers was conducted and polymorphic markers were used to genotype the F_2 mapping population. A linkage analysis of 12 SSR markers was conducted to develop the targeted map region of *BLMC*.

Linkage analysis was carried out with Mapmaker 3.0 (Lander et al. 1987) using the F_2 intercross algorithm, a threshold LOD value of 3.0 and a maximum genetic distance of 30 cM. Groups of linked markers were identified using the group command, then the order was further examined using compare command and finally established using ripple command. Genetic distances expressed in cM were calculated using the Kosambi mapping function (Kosambi 1944). Sorghum chromosome designations were based on nomenclature developed by Kim et al. (2005) and illustrated using Map Chart 2.2 (Voorrips 2002).

A physical mapping analysis of the *BLMC* region was performed by applying the information from the finger printed contig assembly of BTx623 bacterial artificial chromosomes (BACs) using PGML BACMan tools available from http://www.plantgenome.uga.edu/bacman and http://www.stardaddy.uga.edu/http://www.stardaddy.uga.edu/ (verified April 30, 2008). BACs were obtained from Clemson University Genomics Institute and were tested using the newly developed SSR markers by PCR.

Results

Phenotypic analysis of *BLMC* mutation

An evaluation of the bloom-bloomless phenotype for each F_3 families allowed the confirmation of the genotype for profuse wax locus. The three phenotypic and genotypic classes for the population were homozygous bloom (*BLMCBLMC*), heterozygous bloom (*BLMCblmc*) and homozygous bloomless (*blmcblmc*). Homozygous bloom and bloomless lines did not show any segregation, while heterozygous families showed segregation for the trait within a family row. These results further confirmed that the bloomless mutation was under the control of single recessive nuclear gene.

Significant reductions in wax load corresponding to approximately 74 and 93% decrease, respectively, for leaves and culms were observed between bloom and bloomless F_3 families (Table 1). An evaluation of the amount of cuticle in each F_3 classes was carried out to determine the phenotypic effect of *BLMC* mutation on cuticular properties of culms and leaves. Bloomless F_3 families showed significantly lower amount of leaf cuticle than bloom sibs. A measurement of cuticular permeability based on chlorophyll efflux from both leaves and culm showed that greater amount of chlorophyll have leached

Table 1 Determination of the phenotypic effect of *BLMC* mutation in F_3 families

Trait	Tissue source	F_3 phenotypic values		
		Homozygous bloom	Heterozygous bloom	Homozygous bloomless
Wax load (mg/dcm^2)	Leaves	2.27 (0.23)	1.81 (0.07)	0.58 (0.07)
	Culm	5.94 (0.25)	5.31 (0.22)	0.43 (0.06)
Cuticle(mg/cm^2)	Leaves	29.25 (2.99)	25.65 (0.02)	8.80 (0.74)
%Chlorophyll efflux at 6 h	Leaves	28.55 (3.29)	30.56 (4.32)	74.06 (1.84)
	Culm	24.48 (4.12)	25.33 (3.70)	79.46 (2.27)
Plant death rating	Whole plant	2.33 (0.11)	2.58 (0.09)	5.41 (0.17)

Data were obtained from greenhouse grown plants and from the field for plant death rating. The experiment was arranged in a completely randomized design with three replications. Values presented are means followed by standard error given inside the parenthesis

out from bloomless F_3 than bloom F_3. In the field, bloomless F_3 families showed significantly higher plant death rating at anthesis than bloom and heterozygous F_3 families (Table 1).

Wax composition and *BLMC* mutation

An analysis of the fatty acid composition of epicuticular wax (chloroform soluble EW) by FAME gas chromatography of parental lines revealed that 95% of epicuticular waxes are free fatty acids. A dramatic reduction in the C:28 to C:30 free fatty acid fractions was found in culm EW of KFS2021 (Fig. 1). Meanwhile in leaves, significant reduction was found in the C:22 to C:30 free fatty acids fractions (Fig. 1). The free fatty acid profile of representative homozygous bloom and bloomless F_3 families showed similar pattern with that of the parents (data not presented). Concomitant with wax analysis, profiling of leaf polar lipids (analyzed by Kansas Lipidomics Research Center, KSU) showed an increase in phosphatidyl glycerol components in the *blmc* mutant as compared to wild type (data not presented).

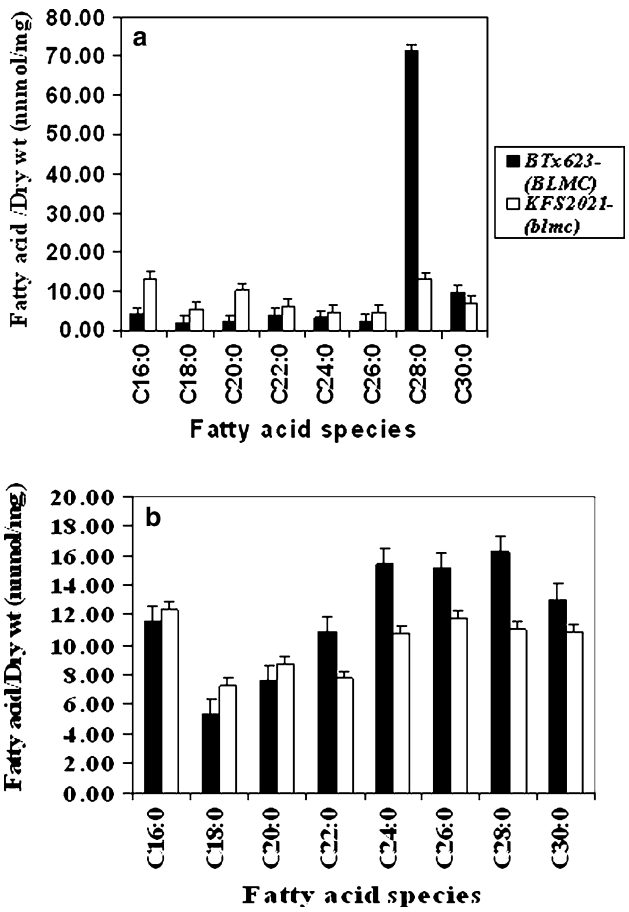

Fig. 1 Wax composition of **a** culm and **b** leaves from parental wild-type and mutant sorghum lines

Molecular mapping of *BLMC* mutation

To assign the locus for profuse wax production in sorghum to a chromosome, a framework map was generated by genotyping 30 selected F_2 offsprings of known phenotype, which include 20 bloom (homozygous and heterozygous individuals), and 10 bloomless progenies from the mapping population. This preliminary mapping step facilitated the placement of the bloom trait to chromosome 10 (Fig. 2). After identifying the chromosome that harbors the locus, genotyping a total of 120 F_2 individuals for all polymorphic markers in chromosome 10 was carried out. Additional SSR markers that were specific for chromosome 10 were

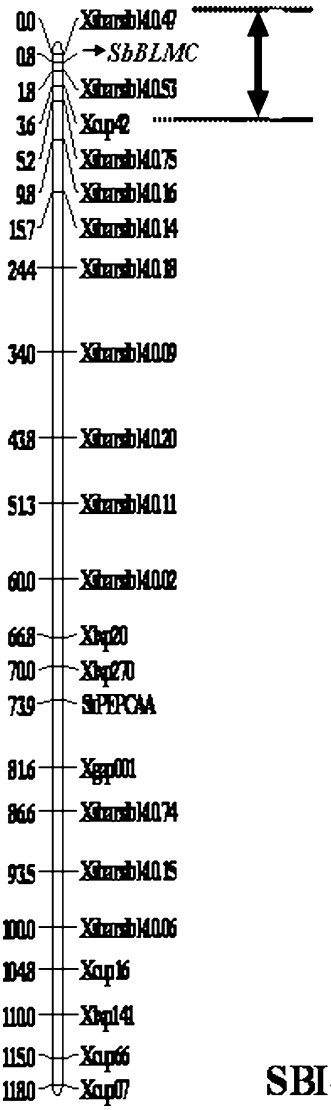

Fig. 2 Molecular map of sorghum chromosome 10 populated with new SSR markers. The numbers on the *left side* represent cumulative distances in cM and the corresponding SSR markers are indicated on the *right side* of the diagram. The gene for profuse wax and enhanced cuticle, *SbBLMC* is indicated and was localized to the terminal end of the chromosome

generated from WGS sequence of sorghum and presented in Table 2. Newly identified polymorphic SSR markers were placed in the map to increase density and identify closer markers. This approach greatly aided in a swifter determination that *BLMC* mapped to terminal end of sorghum chromosome 10 between SSR markers *Xsbarslbk10.47* and *Xcup 42* (Fig. 2). This framework map indicated that *BLMC* resides in a region that spans approximately 3.6 cM.

An analysis of the organization of the terminal region of sorghum chromosome 10 between *Xsbarslbk10.47* and *Xcup42* was conducted using additional SSR markers (Table 2). Targeted mapping of the region using 220 F_2 progenies indicated that *BLMC* could be delimited to a smaller region of 0.7 cM (Fig. 3). The detailed map revealed that *Xsbarslbk 10.48* and *Xsbarslbk 10.49* were localized at about 0.3 and 0.4 cM distances from each side of the locus, respectively. The markers *Xcup49*, *Xsbarslbk10.62* and *Xsbarslbk10.57* appeared to co-segregate with the phenotype (Fig. 3).

An initial study of the physical map corresponding to the terminal end of sorghum chromosome 10 was also performed using BAC clones to establish the physical basis of the *BLMC* region. Three BAC clones from the sorghum BAC library SB_BBc (http://www.genome.clemson.edu) which were previously identified to correspond to the marker *Xcup 49* (Genbank acc# BH245323) were analyzed using flanking SSR markers developed from this study. PCR results showed that the terminal region of sorghum chromosome 10 spanning the *BLMC* region is associated with three overlapping BAC clones 168I12, 97P14 and 36A1 (Fig. 3).

The putative DNA sequence for *BLMC* region was examined by searching the WGS sequence of sorghum. This analysis indicated that the *BLMC* interval corresponded to approximately 153,000 nucleotides. Based on this electronic analysis, a number of open reading frames (ORFs) included in the region could be identified as candidate genes for the *BLMC* locus. The possible candidate genes include glutamine cyclotransferase, an amino acyl-t-

Table 2 Primer sequences of new SSR markers used to build sorghum chromosome 10

Primer ID	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Repeat motif
Xsbarslbk10.02	CGTGAGATGACGAGAAAGCA	CCTTGGCTTACGAGGGAAG	(CT)8
Xsbarslbk10.05	AAAATGTTAGCTATCCCGTAGCA	TCCTACTTACCCCCAATGTCC	(TA)30
Xsbarslbk10.06	TGCACTCACGCGTAAGATTT	ACTTGTGCGCAGGTAGAGGT	(TA)20
Xsbarslbk10.09	TCCCTTCCCAATGGTAAAAA	ACATGCGTGGTCTCTGTTGA	(TATT)14
Xsbarslbk10.11	CCCCCATTTATCCAGCTAAG	CAGCTCAACCTTCCCATATCA	(TA)10
Xsbarslbk10.14	AACAAGCGGGGTCGTTCTA	TGCTCGCTTTTTATGTGTGC	(AT)18
Xsbarslbk10.15	CCTGGTGAAAGGATGTCCAA	AGCTGCACTCAATGCTCCTC	(GA)26
Xabarslbk10.16	GAGGCTGCCTTTGCTTATCA	CGGGCACCAACAACTAACTT	(CGA)19
Xsbarslbk10.18	GGTCTTATGAACTTGCTCAATGAT	AAAAGGGTTTTGGCTGAAAAG	(ATT)35
Xsbarslbk10.20	CGTGAGTAGCCGCAGGTT	ACTTGACGAGCACACACCAA	(CT)18
Xsbarslbk10.21	TCCATCCTCAAGCAGGTCTC	TCCTCAAGCCCACAGGTAAG	(CT)8
Xsbarslbk10.23	TGATCATGGCACTTATGAAATAGA	GTGTCGCTTATTTCGGTATCCT	(AAT)40
Xsbarslbk10.47	CCGGCACTCAAAATCTCTTT	CACATGGTGTCGGAGGATT	(GAA)14
Xsbarslbk10.48[a]	CAAGGCAGTCCCTTCAACAT	CTTGACATCAGCCAAACACG	(AT)33
Xsbarslbk10.49[a]	ACATGGAGCATTTTGCACAC	GTCTTGTCGAAGGTGGGAGA	(CTAT)14
Xsbarslbk10.53	CGGAAGCCCTCATATTGGTA	TGCTTTTCCTTCTGTTCACTCA	(TATC)12
Xsbarslbk10.54[a]	GGCCGGGTGAAAATAGAATA	CAGGGCAGTCTGAGGAAGAG	(TCT)8
Xsbarslbk10.57[a]	ACCACTACCTGGGGTCTGTG	TATGCTCGCTAGCTCACTCG	(CT)15
Xsbarslbk10.58	GACAAATCGTCATGCCACAC	GGTTGACGGTAGAAGGTGCT	(AC)8
Xsbarslbk10.60	CAAGTGGATGGAGTTGCTCA	GGTCTTCAGAGGTCCCCACT	(AT)21
Xsbarslbk10.62[a]	CTGTCCCTGCCTGCTAAAAG	ACCACCAATTAAGGCCACAG	(CA)20(TA)28(TG)8
Xsbarslbk10.63	CACTTGCTGTGTGTGGTTGA	CGCATTTCGCATCTTGTGTA	(GT)10
Xsbarslbk10.64	GCTTACTCGTGTGTCGTTGG	GGGCGTGAGGAAGAATTAAA	(AT)40
Xsbarslbk10.71	AACTCGATCGCAATCTACGG	GGTCGCTCGCTGTTCTTCTA	(GAA)12(GGA)4
Xsbarslbk10.74	AGTTCGAGGCAACAACATCC	GTGGTGTGCCCTAGCTTGAT	(AT)23
Xsbarslbk10.75	TTGGCCACGTATTATTCATCA	GGATCCACAGCTTCCTCAAG	(CT)20

The new SSRs were mined from the whole genome shotgun (WGS) sequence of sorghum available at http://www.phytozome.net/sorghum1.php

[a] SSR markers used for targeted mapping of *BLMC*

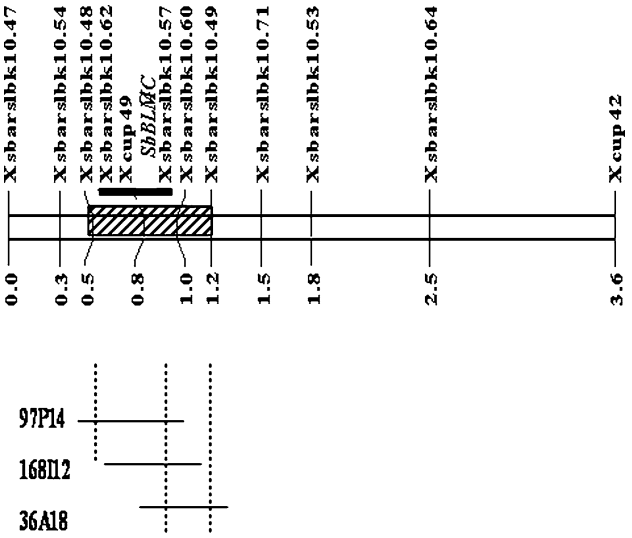

Fig. 3 A detailed map was established for the *BLMC* region using SSR markers mined from the whole genome shotgun sequence of sorghum (http://www.phytozome.net/sorghum1.php). The target interval for *BLMC* is indicated by the *hatched box*. The three BAC clones, 97P14, 168I12 and 36A18 that correspond to the markers for *BLMC* are shown to reveal the physical map of the region

RNA synthetase, a long chain acyl coA oxidase, homogentisate 1, 2 di-oxygenase and four hypothetical proteins with still unknown functions (Table 3).

Discussion

A molecular analysis of the profuse wax gene using the bloomless mutant mapping population was performed in this study. In a previous report (Burow et al. 2008), we have focused on the physiological aspects of the loss of epicuticular wax of the mutation. Here we show that the gene affect the cuticle proper of the culms and leaves of sorghum (Table 1), which seems parallel to the mutant reported by Jenks et al. 1994. However, it is not known yet whether the *blmc* mutant reported here is the same as the *bm2* mutant reported by Jenks et al. 1994 and further complementation tests are needed.

A strong relationship between cuticle and wax load features of sorghum was demonstrated in this work. This is not surprising as these two structures (cuticle and epicuticular wax) make up the outer covering or collectively the epidermal layer of the plant. The effect of the mutation on the cuticle could explain why there is such a dramatic effect on water loss properties of mutant and progenies (Burow et al. 2008). This observation is similar to the findings reported earlier by Jenks et al. (1994), that in sorghum *bm2* mutants both cuticle and EW can be altered by ethyl methane sulfonate (EMS) treatment.

The unique wax composition of sorghum makes it a good source for new epicuticular wax genes which may not exist in other plant species. Gas chromatographic analysis showed that sorghum EW is primarily composed of free fatty acids (Fig. 1). It has been reported that only few plant species has free fatty acids as the dominant EW constituent (Jenks et al. 2000).

The plant cuticle which covers the aerial plant organs, represents the primary barrier between the plant and the environment (Nawrath 2006). It is well recognized that the cuticle plays a crucial role in protecting plants against various abiotic and biotic stress, and may also be involved in a number of developmental processes (Goodwin and Jenks 2005; Nawrath 2006). The cuticle is made up of an outermost layer of epicuticular waxes (referred to as bloom in sorghum) and the cuticle proper is composed of cutin polymers (Nawrath 2006). However, not all cuticles are the same, variations abounds as to their quantity (amount, thickness) and quality (composition and configuration). A number of reports indicate that various cuticle features and their EW configurations perform better in protecting the whole plant from water loss and other stresses (Shepherd and Griffiths 2006).

In the present study, a molecular map for the *BLMC* locus was developed. The over all genetic map obtained from this study using SSRs was consistent with previous

Table 3 Predicted genes in the *BLMC* region of BTx623 based on http://www.phytozome.net whole genome shotgun sequence

Phytozome ID	Gene homology	Genomic span (bp)	Predicted coding sequence (bp)	Total amino acids
Sb10g000310.1	Hypothetical protein	5,879	3,093	1,031
Sb10g000350.1	Hypothetical protein	4,718	2,469	823
Sb10g000360.1	Homogentisate 1,2-dioxygenase	3,023	1,410	470
Sb10g000390.1	Long chain acyl-CoA oxidase	5,530	2,007	669
Sb10g000410.1	Aminoacyl-t-RNA synthetase	11,244	3,105	1,035
Sb10g000420.1	Glutamine cyclotransferase	3,046	1,005	335
Sb10g000430.1	Hypothetical protein	6,471	2,073	691
Sb10g000445.1	Hypothetical protein	5,779	2,421	807

published genetic maps. Placement of SSR markers to linkage group and subsequent chromosomal assignment were in good agreement with the detailed sorghum genetic map by Menz et al. 2002; Bowers et al. 2003 and with the available SSR map of sorghum (Wu and Huang 2006). Previously, Wu and Huang (2006) reported a gap in Sb chromosome 10, resulting in two sublinkage groups. In this study, we were able to fill the gap with new microsattelite markers, linked the two sublinkages 10a and 10b to establish a single chromosome 10 populated by SSR markers with an average interval of 5.4 cM between markers (Fig. 2).

Information on the molecular map of wax genes in sorghum is not available and this is the earliest report to address the subject. This study also applied the newly available genome sequence of sorghum (http://www.phytozome.net) to facilitate chromosome walking and candidate gene identification in sorghum. In this study we localized the *BLMC* locus to chromosome 10, with flanking markers *Xsbarslbk10.47* and *Xcup42* (Fig. 2). Targeted mapping using 220 F_2 resulted in the identification of even closer flanking markers delimiting *BLMC* to a 0.7 cM region (*Xsbarslbk10. 48* and *Xsbarslbk10.49*), and detected three cosegregating markers (*Xcup49*, *Xsbarslbk10.62* and *Xsbarslbk10.57*) with the bloomless phenotype. These markers are now being used as focal point for fine mapping and sequencing of candidate genes.

To complement the molecular map, a physical study of the chromosomal segment that corresponds to the *BLMC* interval was established in this study. The three BACs that were previously associated with *Xcup49* proved to be valuable in revealing the physical basis of the *BLMC* locus. Here, we show that convergence of the molecular and physical maps could now be an easier approach for map-based cloning in sorghum due to the critical mass of genomic resources (including high density maps, finger printed large insert libraries and most recently WGS sequence) that have been developed and made available in the last five years for the species. These genomic resources coupled with the availability of mutants and mutant mapping populations make sorghum an excellent model grass species for gene to phenotype discovery.

An examination of the genome sequence corresponding to the *BLMC* region suggests that it approximately includes 153,000 nucleotides. A group of eight putative ORF candidates can be found in this region and will be used for succeeding candidate gene analysis. A significant and interesting candidate that is being analyzed is the long chain acyl coA oxidase (Sb10g000390.1). It is an interesting candidate because this gene could have direct link to the metabolism of the free fatty acid composition of the sorghum wax. This finding indicates that the sorghum EW-cuticle could provide information on Acyl-CoA elongation associated reactions in the wax metabolic pathway. The reactions involving Acyl-CoA elongation can likely serve as rate limiting steps or as most-highly regulated reaction. This suggests that sorghum could be used as a robust genetic tool for elucidating acyl-CoA elongation and hydrolysis. Acyl-CoA elongation associated reactions are among the most important since these are central and early steps in the wax metabolic pathway and likely serve as rate limiting and most-highly regulated reactions (Jenks et al. 2000).

Genetic mapping of the sorghum *BLMC* locus to chromosome 10 and identification of closely linked markers are important steps toward cloning the genes which play critical roles in wax and cuticle biosynthesis and metabolism. It is possible that these profuse wax genes can be applied as a transgene in other crops to increase wax production and subsequently increase drought tolerance. For sorghum, identification of these genes could lead to new technologies on how to manipulate the wax pathway to make it even more efficient in aiding the crop to better withstand water deficit stress or enhance water use efficiency for growth and grain production.

Acknowledgments The authors would like to thank Dr. Matthew Jenks for helpful discussion and the technical assistance of Halee Hughes, Charles Woodfin, Naomi Kaskela and Lance Layton.

References

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell 16:2463–2480

Beattie G, Marcel L (2002) Effect of alterations in cuticular wax biosynthesis on the physicochemical properties and topography of maize leaf surfaces. Plant Cell Environ 25:1–16

Blum A (1975) Effect of the Bm gene on epicuticular wax and water relations of *Sorghum bicolor* L. (Moench). Israel J Bot 24:50–51

Bowers JE, Abbey C, Anderson S, Chang C, Draye X, Hoppe A, Jessup R, Lemke C, Lennington J, Li Z, Lin YR, Liu S, Luo L, Marler B, Ming R, Mitchell S, Qiang D, Reischmann K, Schulze S, Skinner N, Wang Y, Kresovich S, Schertz KF, Paterson AH (2003) A high density genetic recombination map of sequence-tagged sites for *Sorghum*, as a framework for comparative structural and evolutionary genomics of tropical grains and grasses. Genetics 165:367–386

Burow GB, Franks CD, Xin Z (2008) Genetic and physiological analysis of an irradiated bloomless mutant (epicuticular wax mutant) of sorghum. Crop Sci 48:41–48

Broun P, Pointdexter P, Osborne E, Jiang C, Reichman JL (2004) WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. Proc Natl Acad Sci USA 101:4706–4711

Chen X, Goodwin M, Boroff V, Liu X, Jenks M (2003) Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell 15:1170–1185

Chittenden LM, Schertz KF, Lin YR, Wing RA, Paterson AH (1994) A detailed RFLP map of *Sorghum bicolor* x *S. propinquum*, suit-

able for high density mapping suggests ancestral duplication of Sorghum chromosomes or chromosomal segments. Theor Appl Genet 87:925–933

Ebercon A, Blum A, Jordan W (1977) A rapid colorimetric method for epicuticular wax content of *Sorghum* leaves. Crop Sci 17:179–180

Goodwin SM, Jenks MA (2005) Plant cuticle function as a barrier to water loss. In: Jenks MA, Hasegawa PM (eds) Plant abiotic stress. Blackwell, Oxford

Hansen JD, Pyee J, Xia Y, Wen T-J, Robertson DS, Kolattukudy PE, Nikolau BJ, Schnable PS (1997) The *glossy1* locus of maize and an epidermis-specific cDNA from *Kleinia odora* define a class of receptor-like proteins required for the normal accumulation of cuticular waxes. Plant Phys 113:1091–1100

Jenks MA, Joly R, Peters P, Rich P, Axtell JD, Ashworth EN (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. Plant Phys 105:1239–1245

Jenks MA, Eigenbrode SD, Tuttle HA, Feldmann K (1995) Leaf epicuticular waxes on the *eceriferum* mutants of *Arabidopsis*. Plant Phys 108:369–377

Jenks MA, Rashotte AM, Tuttle HA, Feldmann KA (1996) Mutants in *Arabidopsis thaliana* altered in epicuticular wax and leaf morphology. Plant Phys 110:377–385

Jenks MA, Rich PJ, Rhodes D, Ashworth EN, Axtell JD, Ding CK (2000) Leaf culm cuticular waxes on bloomless and sparse-bloom mutants of *Sorghum bicolor*. Phytochemistry 54:577–584

Jordan W, Monk R, Miller F, Rosenow D, Clark L, Shouse P (1983) Environmental physiology of sorghum. I. Environmental and genetic control of epicuticular wax load. Crop Sci 23:552–558

Jordan W, Shouse P, Blum A, Miller F, Monk R (1984) Environmental physiology of sorghum. II. Epicuticular wax load and cuticular transpiration. Crop Sci 24:1168–1173

Karper R (1933) Inheritance of waxy endosperm in sorghum. J Hered 24:257–262

Kim JS, Klein PE, Klein RR, Price HJ, Mullet JE, Stelly DM (2005) Chromosome identification and nomenclature of *Sorghum bicolor*. Genetics 169:1169–1173

Kong L, Dong J, Hart GE (2000) Characteristics, linkage map positions, and allelic differentiation of *Sorghum bicolor* (L.) Moench DNA simple-sequence repeats (SSRs). Theor Appl Genet 101:438–448

Koorneef M, Hanhart C, Thiel F (1989) A genetic and phenotypic description of *eceriferum* (*cer*) mutants in *Arabidopsis thaliana*. J Hered 80:118–122

Kosambi D (1944) The estimation of map distance from recombination values. Ann Eugen 12:172–175

Lander E, Green P, Abrahamson J, Barlow A, Daly M, Stein L, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lolle S, Berlyn G, Engstrom E, Krolikowski K, Reiter W, Pruitt R (1997) Developmental regulation of cell interactions in the *Arabidopsis fiddlehead*-1 mutant; a role for the epidermal cell wall and cuticle. Dev Biol 189:311–321

Menz MA, Klein RR, Mullet JE, Obert JA, Unruh NC, Klein PE (2002) A high-density genetic map of *Sorghum bicolor* (L.) Moench based on 2926 AFLP, RFLP and SSR markers. Plant Mol Biol 48:483–499

Millar AA, Clemens S, Zachgo S, Giblin M, Taylor D, Kunst L (1999) CUT1, an Arabidopsis gene required for cuitcular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell 11:825–838

Nawrath C (2006) Unraveling the complex network of cuticular structure and function. Curr Opin Plant Biol 9:281–287

O'Toole JC, Cruz RT (1983) Genotypic variation in epicuticular wax of rice. Crop Sci 23:392–394

Rooney WL (2000) Genetics and cytogenetics of sorghum. In: Smith CW, Frederiksen RA (eds) Sorghum: origin, history, technology and production. Wiley, NY, pp 261–305

Rooney WL (2004) Sorghum improvement-integrating traditional and new technology to produce improved genotypes. Adv Agron 83:37–109

Schloss SJ, Mitchell SE, White GM, Kukatla R, Bowers JE, Paterson AH, Kresovich S (2002) Characterization of RFLP probe sequences for gene discovery and SSR development in *Sorghum bicolor* (L.) Moench. Theor Appl Genet 105:912–920

Sturaro M, Hartings H, Schmelzer E, Velasco R, Salamini F, Motto M (2005) Cloning and characterization of a maize gene involved in cuticle membrane and wax production. Plant Physiol 138:478–489

Shepherd T, Griffiths DW (2006) The effects of stress on plant cuticular waxes. New Phytol 171:469–499

Temnykh S, DeClerk G, Lukashova A, Lipovich L, Cartinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.) frequency, length variation, transposon associations, and genetic marker potential. Genome Res 11:1441–1452

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93:77–78

Wu YQ, Huang Y (2006) An SSR genetic map of *Sorghum bicolor* (L.) Moench and its comparison to a published genetic map. Genome 50:84–89

Xu X, Dietrich CR, Delledone M, Xia Y, Wen T-J, Robertson D, Nikolau B, Schnable P (1997) Sequence analysis of the cloned *glossy8* gene of maize suggests that it may code for a *B*-ketoacyl reductase required for the biosynthesis of cuticular waxes. Plant Physiol 115:501–510

Xu X, Dietrich CR, Lessire R, Nikolau B, Schnable P (2002) The endoplasmic reticulum-associated maize GL8 protein is a component of the acyl-coenzyme A elongase involved in the production of cuticular waxes. Plant Physiol 128:924–926

Zhang JY, Broeckling CD, Blancaflor EB, Sledge MK, Summer LW, Wang ZW (2005) Overexpression of WXP1, a putative *Medicago trancatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). Plant J 42:689–707