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ORIGINAL PAPER

Genomic inversion caused by gamma irradiation contributes to downregulation of a *WBC11* homolog in *bloomless* sorghum

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Abstract Epicuticular wax (bloom) plays important roles in protecting the tissues of sorghum (*Sorghum bicolor* (L.) Moench) plants from abiotic stresses. However, reducing wax content provides resistance to greenbug and sheath blight—a useful trait in agricultural crops. We generated a sorghum *bloomless* (*bm*) mutant by gamma irradiation. One *bm* population segregated for individuals with and without epicuticular wax at a frequency of 72:22, suggesting that the *bm* mutation was under the control of a single recessive nuclear gene. Genes differentially expressed in the wild-type and the *bm* mutant were identified by RNA-seq technology. Of the 31 downregulated genes, Sb06g023280 was the most differentially expressed and was similar to *WBC11*, which encodes an ABC transporter responsible for wax secretion in Arabidopsis. An inversion of about 1.4 Mb was present in the region upstream of the Sb06g023280 gene in the *bm* mutant; it is likely that this inversion changed the promoter sequence of the Sb06g023280 gene. Using genomic PCR, we confirmed that six independent F_2 *bm* mutant-phenotype plants carried the same inversion. Therefore, we concluded that the inversion involving the Sb06g023280 gene inhibited wax secretion in the *bloomless* sorghum.

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Gene symbols

Bm	Bloomless
BLMC	Bloom-cuticle
WBC	White-brown complex
CER	Eceriferum

Abbreviations

FATB	Fatty acyl-ACP thioesterase
KCS	β-Ketoacyl-CoA synthase condensing enzyme
ECR	Enoyl-CoA reductase
ABC transporter	ATP binding cassette (ABC) transporter
qRT-PCR	Quantitative reverse transcription-PCR
FPKM	Fragments per kilobase of exon per million fragments

Introduction

The annual sorghum (*Sorghum bicolor* (L.) Moench) crop is the fifth largest in the world. Sorghum has greater tolerance than the other major crops (rice, wheat, barley, and maize) to hot and dry environments; the profuse amount of epicuticular wax (bloom) on the sorghum plant plays an important role in protecting the tissues from abiotic stresses such as drought and ultraviolet light in the semiarid tropics

(Jordan et al. 1983) and from biotic stresses. Reducing epicuticular wax and cuticle deposition increases susceptibility to the fungal pathogen *Exserohilum turcicum* (Jenks et al. 1994). However, *bloomless* sorghum, which has reduced levels of epicuticular wax (Peterson et al. 1982), is not a preferred host of greenbug (*Schizaphis graminum*) (Peiretti et al. 1980; Weibel and Starks 1986) and is resistant to sheath blight (*Rhizoctonia solani* Kuehn) (Kasuga et al. 2001). As greenbug and sheath blight cause serious yield losses, the *bloomless* phenotype is a useful trait for agricultural crops in the temperate zone and in relatively humid areas.

The process of formation of epicuticular wax has been elucidated by using *Arabidopsis thaliana* mutant lines (Samuels et al. 2008). A central stage in wax biosynthesis is the elongation of C16 and C18 acids to very-long-chain fatty acids ($C > 20$). This process is mediated by elongase complexes containing β-ketoacyl-CoA synthase condensing enzyme (KCS), β-ketoacyl-CoA reductase, and enoyl-CoA reductase (ECR) (Samuels et al. 2008). All of the wax biosynthetic enzymes are localized in the endoplasmic reticulum. In Arabidopsis, cuticular lipids are exported from the plasma membrane of the epidermal cells by CER5 (Pighin et al. 2004) and WBC11 (Bird et al. 2007) proteins, which are members of the ABCG/white-brown complex (WBC) subfamily of ATP binding cassette (ABC) transporters. Visible crystals are then formed on the surfaces of the leaf and leaf sheath.

Studies of the functional genomics of sorghum started after completion of the genomic sequence of sorghum BTx623 in 2009 (Paterson et al. 2009). Following the rapid progress of massive parallel sequencing technology, whole RNA sequencing (RNA-seq) has been used for gene expression profiling (Pepke et al. 2009; Wang et al. 2009). Transcriptome analysis of *S. bicolor* in response to osmotic stress and abscisic acid (Dugas et al. 2011) or infection with *Bipolaris sorghicola* (Mizuno et al. 2012) has been reported. A series of programs have been developed for building gene models directly based on the piling-up of short reads: the program Bowtie maps short reads on genomic sequences (Langmead et al. 2009), TopHat concatenates adjacent exons and identifies reads that bridge exon junctions (Trapnell et al. 2009), and Cufflinks constructs gene models on the basis of the exons and bridging sequences predicted by Bowtie and TopHat, and identifies differentially expressed genes (Trapnell et al. 2010). Thus, the use of sequencing-based expression profiling has the potential to overcome the limitations of PCR- or array-based profiling and can be used to identify key genes directly.

Here, we used gamma irradiation to generate a *bloomless* (*bm*) sorghum mutant with normal growth but reduced epicuticular wax content. To directly identify the gene responsible for the phenotype, we applied massive parallel sequencing of RNA and compared expressed genes between the wild-type and the *bm* mutant. The most differentially expressed gene Sb06g023280 had similarity to the gene for an ABC transporter WBC11 for wax secretion in Arabidopsis. Genomic inversion caused exchange of the sequence upstream of the Sb06g023280/WBC11 gene. These results suggest that the genomic inversion caused by gamma irradiation led to downregulation of the Sb06g023280/WBC11 gene and thus inhibited wax secretion in the *bm* sorghum.

Materials and methods

Plant materials

In 2006, about 1,000 seeds of the sweet sorghum cultivar *Italian* (NIAS Genebank Number: JP44728) were irradiated for 20 h with a total dose of 400 Gy of gamma rays from a ^{60}Co source in the Gamma Room, Institute of Radiation Breeding, National Institute of Agrobiological Sciences, Hitachi-Omiya, Ibaraki, Japan. In 2007, we planted 120 M_1 seeds in the field to obtain self-pollinated M_2 seeds by bagging of the inflorescences before heading. In 2008, six seeds from each of the 120 M_2 lines were directly sown in field plots (150 × 20 cm). Two *bm* mutant plants without visible epicuticular wax (bloom) on their areal parts were identified from a single M_2 mutant line, 2I-400-2-11. Self-pollinated M_3 seeds were obtained from all of the M_2 plants, and the two identified *bm* mutant plants, named 2I-400-2-11-2 and 2I-400-2-11-3, were transplanted into the greenhouse following the seed harvest. Hybridizations using the inflorescences of 2I-400-2-11-2 or 2I-400-2-11-3 and an individual with epicuticular wax were made in the greenhouse in the winter. In 2009, ten F_1 seeds were sown in the field and F_2 seeds were obtained by bagging the inflorescences. In 2010, we sowed 96 F_2 seeds from each hybridization in the field in 80 × 20-cm plots at the end of May. F_2 plants with the wild-type phenotype and those with the *bm* phenotype were sampled on 14 August and immediately frozen in liquid nitrogen. The leaf sheaths were subjected to RNA-seq analysis. Moreover, the sorghum *bm* mutant 2I-400-2-11-3 and wild-type plants were grown in the greenhouse for 6 weeks; samples from the plants were then subjected to examination by scanning electron microscopy.

Quantification of epicuticular wax

The sorghum *bm* mutant 2I-400-2-11-3 and wild-type plants were grown in a greenhouse for 2 months. The 4th leaf from the top was cut and the wax was washed from

both surfaces using 1 ml of ethylacetate. The washing solution was collected and dried up, and the dried wax residue was weighed.

mRNA sequencing

Total RNA was extracted from the leaf sheaths of two wild-phenotype plants (wt1, wt4) and three *bm* mutant-phenotype plants (bm14, bm29, and bm68) of sorghum (2I-400-2-11-3) using an RNeasy Plant Kit (Qiagen, Hilden, Germany). RNA quality was calculated using a Bioanalyzer 2100 algorithm (Agilent Technologies, Santa Clara, CA, USA); high-quality RNA (RNA integrity number >8) was used for poly(A) RNA isolation. Oligo(dT) magnetic beads were used to isolate poly(A) RNA from the total RNA samples. Poly(A) RNA was converted to cDNA for massive parallel sequencing in an Illumina Genome Analyzer IIx (Illumina, San Diego, CA, USA), in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina). Details of the cDNA preparation have been given previously (Mizuno et al. 2010). All primary mRNA sequence read data had been submitted to the DNA Data Bank of Japan (DDBJ; PRJDB686). Reads that passed the filter were mapped onto the sorghum reference genome BTx623 (Paterson et al. 2009) by using Bowtie (Langmead et al. 2009) and TopHat (1.1.4) (Trapnell et al. 2009) software, with the default parameters. Uniquely mapped reads were used for further gene model construction and identification of differentially expressed genes by Cufflinks (0.9.3) (Trapnell et al. 2010) software.

Chromosomal walking and genomic sequencing

The unknown region was cloned using a Straight Walk kit (Tsuchiya et al. 2009). Briefly, genomic DNAs were digested with the restriction enzyme *Bgl*II, the DNA fragments were ligated with adaptors, and the target genomic regions were amplified by nested PCR with primers complementary to the genomic sequence and adaptor sequence. The fragments were cloned and sequenced by Sanger method.

Quantitative RT-PCR (qRT-PCR)

RNAs were extracted from the outer layer of the leaf sheath, the leaf, and the inner layer of the leaf sheath in wild-type or *bm* plants at the 3-month stage. Total RNAs of three biological replicates for each sample were extracted using an RNeasy Plant Kit (Qiagen, Hilden, Germany). qRT-PCR primers were designed on the basis of annotation of the BTx623 genome in Phytozome (http://www.phytozome.net/). One microgram of total RNA was reverse-transcribed in a 20-μL reaction mixture from a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed in a 20-μL reaction mixture containing 2×SYBR Master Mix (Kapa, Boston, MA, USA) and 1 μL of cDNA template (1:10 diluted) and gene-specific primers (Online Resource: Supplementary Table 1). qRT-PCR of three technical replicates for each sample was performed with a LightCycler 480 System and its relative quantification software (ver. 1.2) on the basis of the delta-delta-Ct method (Roche). qRT-PCR was performed for 10 s at 95°, 5 s at 55°, and 10 s at 72°. The detection threshold cycle for each reaction was normalized against the expression level of the actin gene (Shih et al. 2006).

Results

Development of the *bloomless* mutants

Seeds of the sweet sorghum cultivar *Italian* were subjected to gamma irradiation to develop mutant plants without visible epicuticular wax (bloom) (Fig. 1a). Two *bm* mutants (2I-400-2-11-2, 2I-400-2-11-3) were screened from a single M_2 mutant line, 2I-400-2-11. We examined 2I-400-2-11-3 under an electron microscope. The leaf sheaths of this *bm* mutant were not covered with epicuticular wax (Fig. 1b). The amount of epicuticular wax chemically extracted was dramatically reduced (Fig. 1c). The F_2 population from the backcross with the wild-type phenotype segregated for individuals with and without epicuticular wax at a frequency of 72:22 ($\chi^2 = 0.1277$; $P = 0.7209$ for a 3:1 segregation ratio, Chi square test), suggesting that the *bm* phenotype was under the control of a single recessive nuclear gene.

Identification of differentially expressed genes

We hypothesized that the gene or genes responsible for the *bm* phenotype were differentially expressed in the *bm* and wild-type plants. To identify differentially expressed genes comprehensively, we applied massive parallel sequencing of RNA (i.e. RNA-seq). RNA from the leaf sheaths of two wild-phenotype plants (wt1, wt4) and three *bm* mutant-phenotype plants (bm14, bm29, and bm68) was sequenced. The sequence fragments were mapped on the sorghum genome (Table 1); 40,681 gene models were then constructed by piling-up the mapped reads (Fig. 2). The FPKM (fragments per kilobase of exon per million fragments) of each gene was calculated to quantify the gene expression (Online Resource: Supplementary Table 2), and differentially expressed genes were identified; 8 genes were upregulated and 31 downregulated in all comparisons of biological replicates (Table 2). Of the 31 downregulated

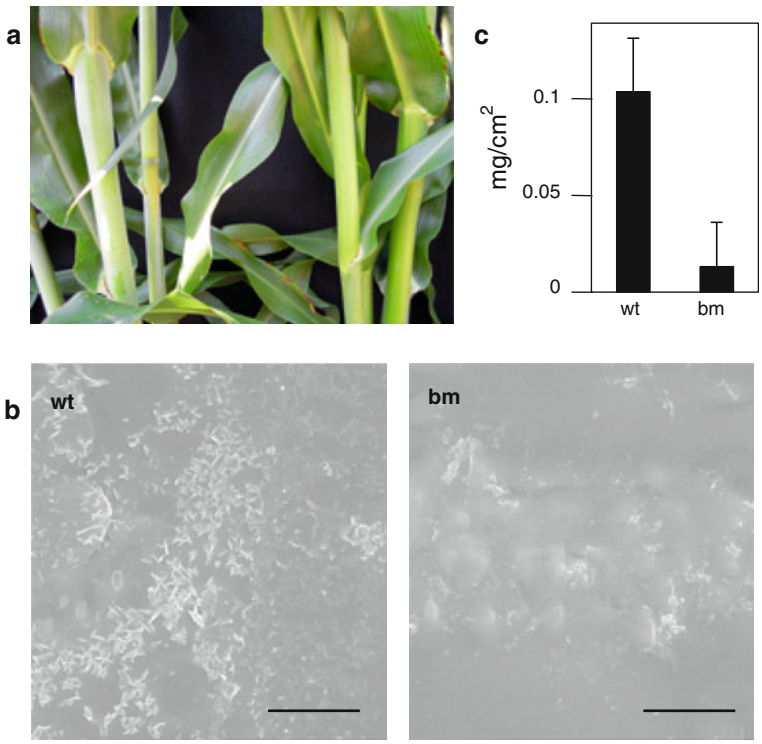

Fig. 1 Reduction of epicuticular wax coating in the *bloomless* mutant of sorghum *Italian*. **a** Epicuticular wax is accumulated in the wild-type (wt: *left*) but not in *bloomless* (bm: *left*). **b** Scanning electron micrographs of abaxial side of leaf sheath. *Bars* 5 μm. **c** Chemical quantification of epicuticular wax

Table 1 Numbers of mapped reads

	wt1	wt4	bm14	bm29	bm68
Adaptor-trimmed reads	18,032,604	18,614,563	18,301,375	19,910,889	21,077,888
Mapped reads	15,233,139	15,543,471	15,241,825	16,902,649	18,283,412

Adaptor-trimmed reads were mapped on the sorghum genomic sequence (Sb1) using the TopHat (1.1.4) program. Gene models were predicted by the Cufflinks (0.9.3) program

genes, Sb06g023280, which is 77.9 % similar to At1g17840/*WBC11*/*COF1*/*DSO* (encoding an ABC transporter responsible for wax secretion in Arabidopsis; (Bird et al. 2007; Panikashvili et al. 2007; Ukitsu et al. 2007), was the most differentially expressed (Table 3).

Genomic sequencing of the Sb06g023280 gene

We hypothesized that downregulation of the Sb06g023280 gene in the *bm* phenotype was caused by a change in the genomic sequence. However, the coding region of the Sb06g023280 gene had neither insertion/deletion nor nucleotide substitution. As our preliminary PCR experiment had suggested that there was a change in the genomic sequence in the promoter region upstream of the Sb06g023280 gene (data not shown), we cloned the region upstream of the Sb06g023280 gene by chromosomal walking. The amplified upstream sequence of the *bm* mutant was different from that of the wild-type. In the *bm* mutant, the sequence upstream of Sb06g023280 was divided into two pieces that were similar to two 1.4-Mb-distant genomic regions in the BTx623 genome (Fig. 3). Part of the upstream sequence was at its usual location (52.4 Mb), but the rest was connected, in inverted orientation, to a region at 53.8 Mb on chromosome 6 (Fig. 3). We also succeeded in cloning the other putative junction region on the *bm* genome by chromosomal walking (Fig. 3). At the junctions, 4 bases at 52.4 Mb and 2 bases at 53.8 Mb were deleted in the *bm* genome (Fig. 3). We confirmed using genomic PCR that six independent F_2 *bm* mutant-phenotype plants carried the same inversion (Online Resource: Supplementary Fig. 1). As the inversion caused an exchange of the promoter sequence of the Sb06g023280 gene, we considered that this difference contributed to the downregulation of this gene. Therefore, we concluded that the Sb06g023280 gene in the *bm* mutant was invertedly located between 52.4 and 53.8 Mb on chromosome 6 (Fig. 3).

Expression of other wax-related genes in the *bm* mutant

We compared the expression of genes putatively encoding proteins responsible for the process of formation of

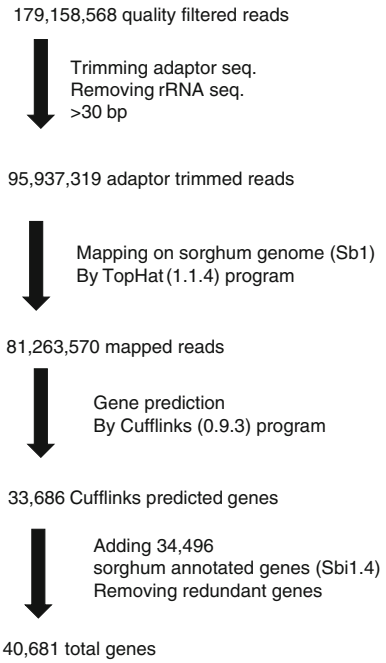

Fig. 2 Strategy for constructing gene models. Reads from RNA-seq were filtered by base quality; adaptor sequences were trimmed and rRNA sequences removed. Adaptor-trimmed reads were mapped on the sorghum genome using the TopHat program. Novel transcripts were predicted by the Cufflinks program on the basis of the piling-up of mapped short reads. By adding the previously annotated sorghum genes (Sbi1.4) and removing redundant genes, we annotated 40,681 gene models

Table 2 Numbers of differentially expressed genes

	UP		DOWN	
	wt1	wt4	wt1	wt4
bm14	925	345	814	388
bm29	792	171	638	300
bm68	168	596	247	686
Common to bm14, bm29, and bm68	33	22	150	107
Common to wt1 and wt4 (vs. bm14, bm29, and bm68)	8		31	

Numbers of differentially expressed genes in each combination of two wild-type (wt) and three *bloomless* (*bm*) mutants are shown. Numbers of commonly differentially expressed genes are also shown

epicuticular wax in the wt and the *bm* mutant. Six other genes with similarity to genes isolated from Arabidopsis mutants were subjected to qRT-PCR analysis. They encoded ABC transporter/CER5 (Pighin et al. 2004) for wax secretion; fatty acyl-ACP thioesterase (FATB) (Bonaventure et al. 2004), β-ketoacyl-CoA synthase (KCS/CER6) (Fiebig et al. 2000), and enoyl-CoA reductase (ECR/CER10) (Zheng et al. 2005) for wax biosynthesis; and CER1 and CER2 (Mcnevin et al. 1993) with unknown function. These six genes either showed no change in expression (in the case of CER2) or were not differentially expressed to as great a degree as the Sb06g023280 gene (Fig. 4). Only Sb06g023280 was substantially downregulated among the seven genes putatively encoding proteins involved in the process of formation of epicuticular wax.

Tissue-specific expression of wax-related genes in wild-type sorghum

We further compared the expression of wax-related genes on the outer layer of the leaf sheath, the leaf, and the inner layer of the leaf sheath in wild-type plants. Genes putatively encoding proteins involved in wax secretion or biosynthesis were barely expressed in the inner layer of the leaf sheath, unlike in the leaf and the outer layer of the leaf sheath (Fig. 5). This is consistent with our observation of wax crystals only on the light-exposed surface of the plant. This indicated that accumulation of wax in wild-type sorghum is controlled by a biosynthesis step before secretion. The finding that the *bm* mutant had downregulation of the Sb06g023280 gene (Fig. 4), which is involved in secretion, but no substantial downregulation of the genes involved in biosynthesis, indicated that exclusive downregulation of the Sb06g023280 gene (Fig. 4) was a feature of the *bm* mutant.

Discussion

Characterization of the *bm* mutant

We generated sorghum *bm* mutants (Fig. 1) by gamma irradiation. As gamma irradiation causes many double-strand breaks (DSBs) in the genome, it would have been difficult to identify the genes responsible for the *bm* phenotype using conventional methods. Sequencing-based expression profiling could be used directly to identify candidate genes responsible for mutants generated by gamma irradiation (Fig. 2; Tables 1, 2). Sb06g023280, which is similar to *WBC11* encoding an ABC transporter responsible for wax secretion in Arabidopsis (Bird et al. 2007; Panikashvili et al. 2007; Ukitsu et al. 2007), was the most differentially expressed (Table 3). We also identified a 1.4-Mb genomic inversion proximal to the promoter region of the Sb06g023280 gene (Fig. 3); this inversion appeared to be responsible for downregulation of the Sb06g023280 gene. The inversion might have been caused by the accidental ligation of two proximal DSBs in the nucleus, as the deletion of four bases at the upstream junction and two at the downstream junction (Fig. 3) suggested that damaged ends had been made blunt prior to ligation. We therefore considered that Sb06g023280 was

Table 3 The 5 most differentially expressed genes having annotations

gene_ID	FPKM_wt	FPKM_bm	Fold change	*P* value	Description
DOWN					
Sb06g023280	92.97	9.35	0.10	2.0.E−09	Similar to OSIGBa0115K01-H0319F09.18 protein
Sb04g033420	85.84	29.70	0.35	8.7.E−04	Similar to fatty aldehyde dehydrogenase 1
Sb02g034370	130.69	51.41	0.39	6.9.E−05	Similar to adenosine 5′-phosphosulfate reductase 2
Sb04g005330	86.51	35.73	0.41	4.1.E−04	Similar to putative uncharacterized protein
Sb03g033760	333.04	159.63	0.48	9.8.E−05	Similar to putative uncharacterized protein
UP					
Sb05g008350	10.48	40.91	3.90	2.7.E−03	Similar to non-TIR-NBS-LRR-type resistance protein
Sb08g005080	30.89	107.53	3.48	4.5.E−04	Weakly similar to WRKY transcription factor 45
Sb03g042500	73.09	250.92	3.43	7.9.E−11	Similar to putative uncharacterized protein
Sb09g024930	129.64	360.94	2.78	1.1.E−10	Similar to putative uncharacterized protein
Sb07g002230	31.19	80.67	2.59	7.7.E−05	Similar to histidine amino acid transporter

Gene ID (gene_id), fragments per kilobase of exon per million fragments mapped in the wild-type (FPKM_wt) or in *bloomless* (FPKM_bm), calculated ratio of FPKM (fold change), *P* value, and description in Phytozome (description) are listed

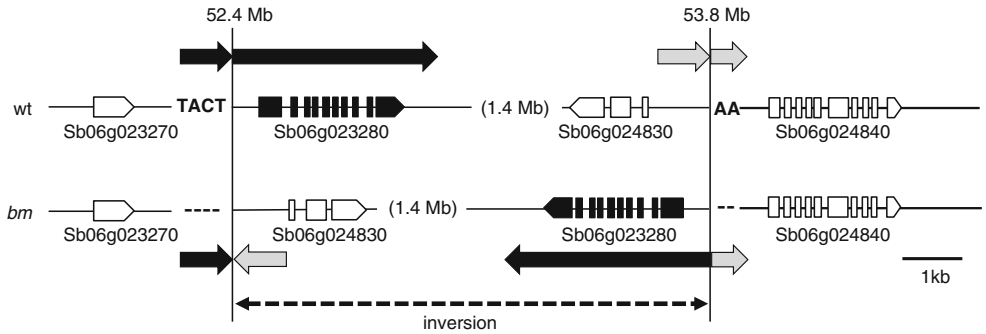

Fig. 3 Genomic inversion in the *bloomless* mutant. An inversion of about 1.4 Mb occurred on chromosome 6 in *bloomless* (*bm*). *Arrows* indicate cloned sequences from the wild-type (wt) and the *bm* genomes. At the junctions, four bases (TACT) or two bases (AA) were deleted. Because of the genomic inversion, the upstream region of the Sb06g023280/WBC11 gene (*black*) was exchanged with the downstream sequence

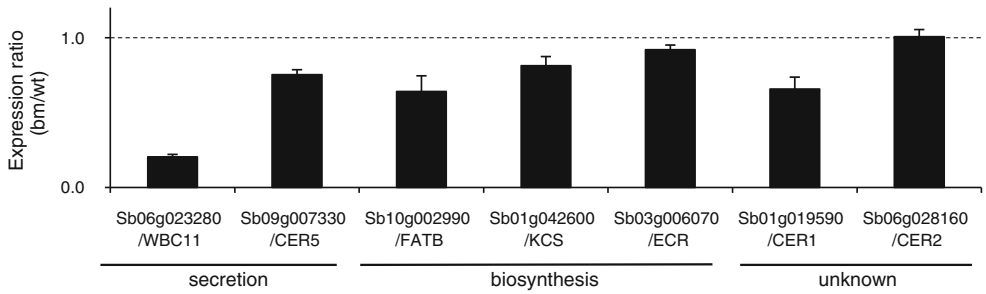

Fig. 4 Sb06g023280/WBC11 gene is downregulated in *bloomless*. Total RNAs were prepared from the leaf sheath. The ratio of expression of the Sb06g023280/WBC11 gene in *bloomless* (*bm*) to that in the wild-type (wt) is shown. Expression levels were quantified by quantitative real-time PCR and normalized against that of the actin gene. Expression ratios of other genes for wax secretion (CER5), for wax biosynthesis [fatty acyl-ACP thioesterase (FATB), β-ketoacyl-CoA synthase (KCS), enoyl-CoA reductase (ECR)], and with unknown functions (CER1 and CER2) are also shown

the candidate gene most likely responsible for the *bm* phenotype.

The genome sequences of six F_2 *bm* mutant-phenotype plants carried the same inversion including the Sb06g023280 gene (Online Resource: Supplementary Figure 1), thus strongly supporting our hypothesis. However, the whole genomic sequence of the *bm* mutant remains to be elucidated. Irradiation, like any other mutagen, can cause mutations in

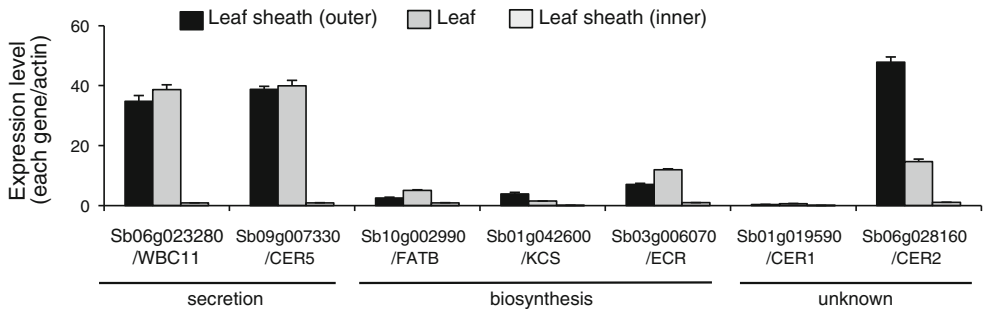

Fig. 5 Genes involved in wax formation are expressed in the outer layer of the leaf sheath and in the leaf, but not in the inner layer of the leaf sheath. RNAs were prepared from the leaf and the outer or inner layer of the leaf sheath of wild-type sorghum. Expression levels were quantified by quantitative real-time PCR. Expression ratios of genes for wax secretion (WBC11, CER5), for wax biosynthesis [fatty acyl-ACP thioesterase (FATB), β-ketoacyl-CoA synthase (KCS), enoyl-CoA reductase (ECR)], and with unknown function (CER1 and CER2) are shown. The expression level of each gene was normalized against that of the actin gene

various places in the genome. It is therefore possible that there are other mutations in the genome that could also have affected the *bm* phenotype and that were not detected by massively parallel sequencing of mRNA. The integration of massive parallel sequencing of RNA and genomic DNA will be a powerful method for identifying other mutated—including not differentially expressed—genes comprehensively in the future.

Wax formation is controlled by both synthesis and secretion steps. In the wild-type, wax formation is regulated mainly by a biosynthesis step in the surface tissues before secretion (Fig. 5). Exclusive downregulation of the Sb06g023280/WBC11 gene, thus impeding only the secretion step, was a feature of the *bm* mutant (Fig. 4). CER5 is another ABC transporter; in Arabidopsis it makes a heterodimer with WBC11/Sb06g023280 to form a functional ABC transporter (Bird et al. 2007). This heterodimer formation may also be required in sorghum, because even though CER5 was expressed in the leaf sheath of *bm* plants (Fig. 4), the secretion step was greatly inhibited. Notably, other genes for wax biosynthesis were slightly downregulated in the *bm* plants (Fig. 4). This might have been due to negative feedback regulation of wax biosynthesis: the inability to secrete wax likely resulted in the accumulation of wax precursors in the cells of the surface of leaf sheath. We therefore consider that the *bm* phenotype resulted mainly from blocking of the secretion step due to downregulation of the Sb06g023280/WBC11 gene.

Other differentially expressed genes were identified in our RNA-seq analysis. Stress-inducible genes (similar to non-TIR-NBS-LRR-type resistance protein gene or WRKY transcription factor 45) were slightly upregulated in the *bm* plants (Table 3). The leaf sheath accumulated red pigment in the *bm* plants (Fig. 1a); this is a typical response to stress. We consider that the upregulated expression of these stress-inducible genes was due to the secondary damage caused by reduction in the wax covering.

Comparison with other mutants with reduced epicuticular wax

Bloom-Cuticle (BLMC), a locus associated with the profuse production of wax, has been identified using a mutant mapping population developed from a cross between BTx623 (a wild-type with profuse amounts of wax) and KFS2021 (a mutant with greatly reduced wax cover) in sorghum (Burow et al. 2009). The *BLMC* region corresponds to approximately 153 kb on chromosome 10; candidate genes include an acyl-CoA oxidase gene encoding a protein involved in lipid biosynthesis and seven other putative transcripts (Burow et al. 2009). None of these candidate genes (Sb10g000310 to -90) was differentially expressed in our study (Online Resource: Supplementary Table 2). Even though we did not genotype the population and perform linkage mapping of the *bm* progeny in *Italian* (2I-400-2-11-3), we knew the location of the *bm* gene on chromosome 6 (Fig. 3) because the whole sorghum genomic sequence is available. Therefore, we consider that the genes responsible for the *bm* in *Italian* (2I-400-2-11-3) and for *BLMC* in KFS2021 were essentially different.

Our sorghum *bm* mutant showed barely any developmental defect (Fig. 1). However, the Arabidopsis *WBC11* mutant shows severely defective growth as well as wax loss (Bird et al. 2007). We consider that this difference is due to the reduced, but not completely blocked level of expression of the Sb06g023280 gene (to 0.10 or 0.21 of the value in the wild-type, depending on the developmental conditions; Table 3; Fig. 4). The complete coding region remained in the sorghum *bm* mutant (Fig. 3), but, in contrast, the Arabidopsis gene has a T-DNA insertion in the coding region and thus encodes a nonfunctional protein (Bird et al. 2007). Therefore, we consider that the reduced level of expression of the Sb06g023280/WBC11 gene contributes to restricted wax secretion in the presence of normal growth. Moreover, as the *bm* phenotype in sorghum

is resistant to greenbug (Peiretti et al. 1980; Weibel and Starks 1986) and sheath blight (Kasuga et al. 2001), the *bm* mutant in this study (2I-400-2-11-3) has the potential to become a useful line of the sorghum cultivar *Italian* in the future.

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