RESEARCH ARTICLE

Map-based cloning and expression analysis of *BMR-6* in sorghum

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Abstract

Brown midrib mutants in sorghum are associated with reduced lignin content and increased cell wall digestibility. In this study, we characterized a *bmr-6* sorghum mutant, which shows reddish pigment in the midrib and stem after the fifth-leaf stage. Compared to wild type, Kalson lignin content of *bmr-6* is decreased significantly. We used histological analysis to determine that the mutant exhibited a modified pattern of lignin staining and found an increased polysaccharide content. We cloned *BMR-6* gene, a gene encoded a cinnamyl alcohol dehydrogenase (CAD), using a map-based cloning approach. Genetic complementation confirmed that CAD is responsible for the *BMR-6* phenotype. *BMR-6* gene was expressed in all tested sorghum tissues, with the highest being in midrib and stem. Transient expression assays in *Nicotiana benthamiana* leaves demonstrated cytomplasmic localization of BMR-6. We found that the expression level of *bmr-6* was significantly decreased in the mutant but expression of *SbCAD3* and *SbCAD5* were significantly increased. Our results indicate that *BMR-6* not only affects the distribution of lignin but also the biosynthesis of lignin in sorghum.

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Introduction

Sorghum (*Sorghum bicolor* L. Moench) is a C4 crop that displays improved photosynthetic activity at high temperatures and under drought conditions. Additionally, its small genome (~730 Mb) makes it an attractive model for functional genomics studies of Saccharinae and other C4 grasses (Paterson *et al.* 2009).

Lignin is an aromatic heteropolymer that is mainly present in the walls of secondarily thickened cells, making them rigid (Dauwe *et al.* 2007). Lignin is a complex polymer of aromatic alcohols that is formed through oxidative coupling. The main building blocks of lignin are hydroxycinnamyl alcohols (or monolignols) p-coumaryl, coniferyl and sinapyl alcohol, which give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin, respectively (Grabber *et al.* 1998; Dixon *et al.* 2001). The biosynthesis of lignin has become a key focus of research attention since lignin is an important limiting factor in the conversion of plant biomass required for a number of agro-industrial processes such as chemical pulping and forage digestibility (Baucher *et al.* 2003). Since the removal of lignin from plant biomass is costly, hence the development of cultivars with less lignin accumulation is an important strategy for improving the digestibility of forage. Such breeding efforts require understanding the lignin biosynthetic pathway and the gene products involved in this pathway. Developing mutations and analysing them is a powerful method to dissect the function of these genes. Mutations in the lignin biosynthetic pathway have been identified, in both maize and sorghum, as brown midrib mutations, which result in the formation of brown vascular tissue in the leaves and stems (Bout and Vermerris 2003). Compared with wild type (WT), brown midrib mutants have increased digestibility and are a focus of breeding efforts (Oliver *et al.* 2005). Six brown midrib mutants (*bm1*, *bm2*, *bm3*, *bm4*, *bm5* and *bm6*) were identified in maize with morphologic and genetic differences (Haney *et al.* 2008). Maize *bm1* and *bm3* were found to encode a cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT), respectively (Vignols *et al.* 1995; Halpin *et al.* 1998). Porter *et al.* (1978) used chemical treatment of seeds to produce 19 *bmr* sorghum mutants. After the field studies, three lines, *bmr-6*, *bmr-12* and *bmr-18*, were determined to be the highest performing strains and worthy of further study, but it was later that we found that two of these lines, *bmr-12* and *bmr-18*, were allelic and encoded COMT (Bout and Vermerris 2003).

In the present study, we characterize a *bmr-6* sorghum brown midrib mutant phenotypically and histologically. We mapped *bmr6* region and determine its molecular function in sorghum and found that *BMR-6* plays an important role in sorghum lignin biosynthesis.

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Keywords. *bmr-6*; cinnamyl alcohol dehydrogenase; lignin biosynthesis; sorghum.

Materials and methods

Plant materials and samples detection

The sorghum brown midrib (*bmr-6*) mutant and the isogenic WT were studied in the genetic background of the cultivar Early Hegari-Sart. When the plants began to flower, the underground parts were cut into small pieces, weighed and oven dried at 70°C for 48 h. The samples were then ground in a mill and passed through a 1 mm screen. Each sample was analysed for Kalson lignin content using the method described by Hatfield *et al.* (1994) with three experimental replicates. Data analysis was performed using Excel software (Microsoft, USA).

Histological sections of bmr-6

The midribs of mutant and WT were cut into 100 μm sections by an ultramicrotome EMUC7 (Leica, Germany). Fresh sections were subjected to histochemical analysis and observed under a stereo microscope MVX10 (Olympus, Japan). Wiesner staining was performed by incubating sections in 1% phloroglucinol in ethanol : water (7 : 3) with 30% HCl. Sections were incubated for 15 min in a Safranin O/Alcian Blue 1 : 1 (V : V) solution. Then they were washed and observed under UV light for detection of carbohydrates and phenolics.

Mapping of bmr-6

Hundred and twenty-two individuals with brown midrib phenotype were screened from the *bmr-6*/Sa F_2 population for linkage analysis. A total of 1321 F_2 individuals with brown midrib phenotype were used for fine mapping using SSR markers, which were designed based on sorghum genome (Ramu *et al.* 2010). To construct a high-density genetic map for fine mapping, new insertion/deletion (InDel) markers were developed according to the sequencing differences between parents, Sa and *bmr-6*. All primers were designed using the program Primer Premier 5.0 (table 1).

Transformation vector construction and genetic complementation of BMR-6

The cDNA fragment of entire *BMR-6* coding region was amplified (primer pair bmr-6cDNA, table 1) with KOD FX polymerase (Toyobo, Japan) from WT sorghum and cloned into the binary vector pCUbi1390 under the control of the cauliflower mosaic virus 35S promoter. The recombination plasmid was introduced into *Agrobacterium tumefaciens* EHA105 by heat shock and then used to infect seeds of the *bmr-6* mutant (Supartana *et al.* 2005). The transgenic plants were identified by hygromycin resistance assay and polymerase chain reaction (PCR). The PCR primers are listed in table 1 (primer pair bmr-6T).

Subcelluar localization

The primer pair bmr-6GFP was used to amplify the cDNA fragment encoding the full-length BMR-6 protein. The PCR fragment was inserted into the vector 1305GFP at the N-terminus of the green fluorescence protein (GFP) under the control of cauliflower mosaic virus 35S promoter. An *A. tumefaciens* strain EHA105 carrying the *35S::bmr-6-GFP* or *35S::GFP* plasmid was individually infiltrated into *N. benthaminana* leaves and analysed by confocal microscopy 48 h after agroinfiltration, as described previously (Goodin *et al.* 2002). Fluorescence of GFP was observed by Leica TCS-SP2 confocal laser scanning microscope.

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA of WT was extracted from leaves, spikelets, midribs, stems and roots after plant tillering stage using an RNA Prep Pure Plant kit (Tiangen, Beijing, China), and was reverse transcribed using a SuperScript II kit (Takara, Japan). Real-time PCR was performed using a SYBR Green supermix (Biorad, Hercules, USA) on an ABI prism 7900 real-time PCR system. The primers for genes related to lignin biosynthesis are listed in table 1. The sorghum *eIF4a1* gene (Sb04g003390) was used as the endogenous control in the experiment. All reactions were run in three replicates. The

Table 1. Primers used in the research.

Molecular marker	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
GS-198	CTTCCCTTCCTTCCATCTCCATCT	GCCTAGTATACGCTTGGCAGGAGA
GS-256	ATCCATCGAGATCAAGTGAAAGGC	GTCAATGGAGGTCCTTTTGCTGAG
GS-203	AACGCTTTTTCTATGCCGTGTGTC	TTACAACACCTTGCCACTACCGTG
GS-207	CACTCGATATTTCGATTCTTCGGG	AGCTGTGGGTGGGGGTTAAGAAAC
S-23	CCGTTTGCCAGGTGTTAC	ATGGGTGATTGGTTATGAAA
S-4	CGTTGCGGGTGAACAAAT	TATGGCTGGGCAGTCAGG
bmr-6cDNA	CGGGGTACCATGGGGAGCCTGGCG	ATTACTAGTGTTGCTCGGCGCATC
bmr-6T	GGCGGTCGTTCATTCGT	TGGCAGATCCCACAGTAGAG
bmr-6GFP	TGCTCTAGAATGGGGAGCCTGGCG	CGCGGATCCGTTGCTCGGCGCATC
bmr-6 (RT-PCR)	GGCTTCGCCTCCACCATG	GCCTTCGCCACCTTCACG

$2^{-\Delta\Delta CT}$ method was used to analyse relative changes in gene expression (Livak and Schmittgen 2001).

Results

The phenotype characterization of sorghum bmr-6

Consistent with the initial characterization (Porter *et al.* 1978), the sorghum *bmr-6* mutant displayed increased brown pigmentation in the midrib and stem as compared with WT after the fifth leaf stage (figure 1, a&b). Kalson lignin content of *bmr-6* mutant was significantly decreased relative to WT (figure 1c). The plant height and fresh weight per plant was significantly decreased in the mutant, but the stem diameter was significantly increased. However, no significant differences were observed in the tiller number and blade number (table 2). Therefore, the mutant of *bmr-6* not only showed brown pigment in stem and midrib but also affected the concentration of Kalson lignin and other agronomic traits.

The histological analysis of sorghum bmr-6

To further correlate these phenotypes with lignin formation, we performed a series of histological analysis using cross sections of midrib. Midrib sections of *bmr-6* and WT were stained with phloroglucinol-HCL, a reagent used to detect

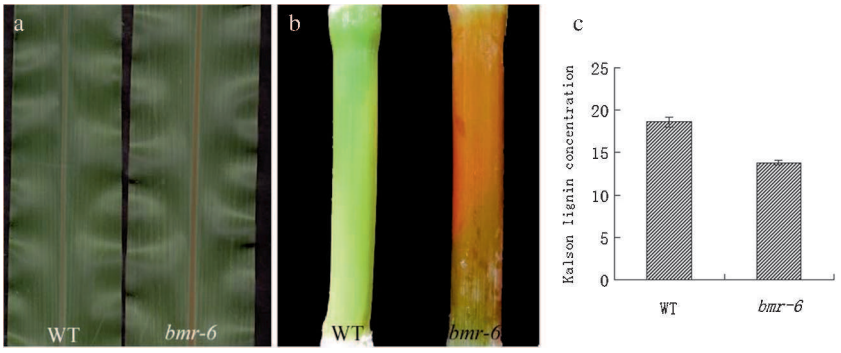

Figure 1. Phenotype of WT and *bmr-6* mutant. (a) Midrib, (b) stem, (c) Kalson lignin concentration.

Table 2. Comparison of agronomic traits between *bmr-6* and WT.

	Plant height (cm)	Stem diameter (cm)	Fresh weight per plant (kg)	Tiller number	Blade number
WT	222.67±10.50	1.87±0.06	1.19±0.17	2.67±0.57	13.67±0.57
bmr6	157.67±8.14	2.15±0.07	0.87±0.07	2.17±0.37	13.33±1.53
t value	8.47*	−6.07*	2.92*	2.1	0.4

*Significant difference at 0.05 probability level.

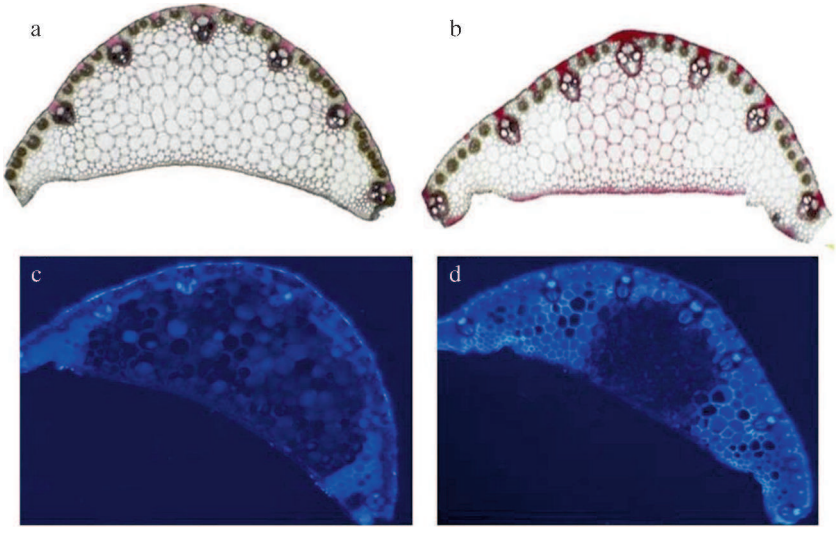

Figure 2. The histological sections of midrib between WT (a and c) and *bmr-6* (b and d).

Table 3. Segregation for green and brown midrib in F_2 populations from two crosses.

Cross	No. of green midrib	No. of brown midrib	$\chi^2_{3:1}$
bmr-6/Sa	398	122	0.58
S722/*bmr-6*	132	31	2.79
bmr-6/S722	143	62	2.73

Value for significant at 0.05 and $df = 1$ is 3.84.

cinnamyl aldehydes and lignin. In WT midrib, weak phloroglucinol staining was evident, whereas in *bmr-6* midrib, a strong staining occurred in the subepidermal sclerified parenchyma, in bundle sheath sclerenchyma, and in some parenchyma cells in the central pith region (figure 2, a&b). These results suggest that aldehyde content was increased in *bmr-6* mutant.

Safranin/Alcinan Blue staining allows polysaccharide elements to be distinguished from phenolic compounds. Compared with WT, the midrib section of *bmr-6* stained more intensely in bundle sheath sclerenchyma and in some parenchyma cells in the central pith region (figure 2, c&d). This result suggested that the lower lignin levels caused the increase of polysaccharide in *bmr-6* mutant.

Map-based cloning of bmr-6 gene

For genetic analysis of the *bmr-6* locus, three F_2 populations were generated from *bmr-6*/Sa, S722/*bmr-6* and *bmr-6*/S722. All F_1 plants displayed normal green midrib. The F_2 generation pattern fit to 3 : 1 (table 3), indicating that the mutant phenotype of *bmr-6* was controlled by a single recessive nuclear gene.

For linkage analysis of the *bmr-6* locus, 122 F_2 individuals showing brown midrib phenotype were selected. Using SSR markers covering all 10 chromosomes, we found that GS-198 and GS-256 from chromosome four linked with *bmr-6* phenotype (figure 3a). F_2 homozygous plants, 1321 with *bmr-6* phenotype were used for fine mapping of *bmr-6* locus. New InDel markers were developed and synthesized based on sequencing results of *bmr-6* and Sa in the targeted region, allowing us to localize the *bmr-6* gene between markers S-23 and S-4, 56.4 kb (figure 3b).

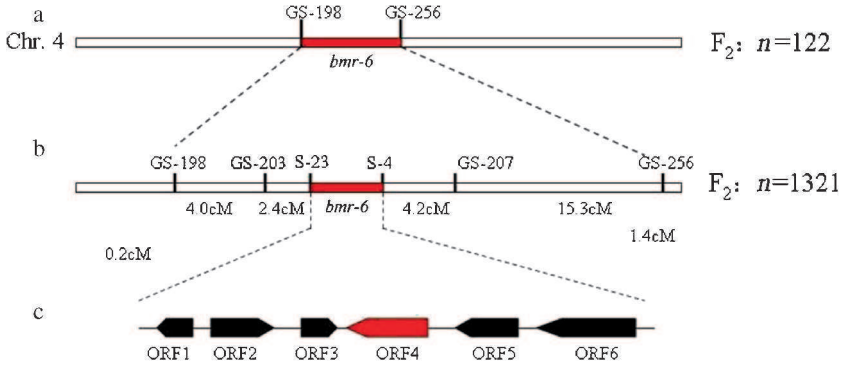

Figure 3. Map-based cloning of *bmr-6* gene. (a) The *bmr-6* locus was located in chromosome 4 between SSR markers GS-198 and GS-256. (b) The *bmr-6* locus was located in the region of newly developed InDel markers S-23 and S-4. (c) Six ORFs were predicted in the mapped region.

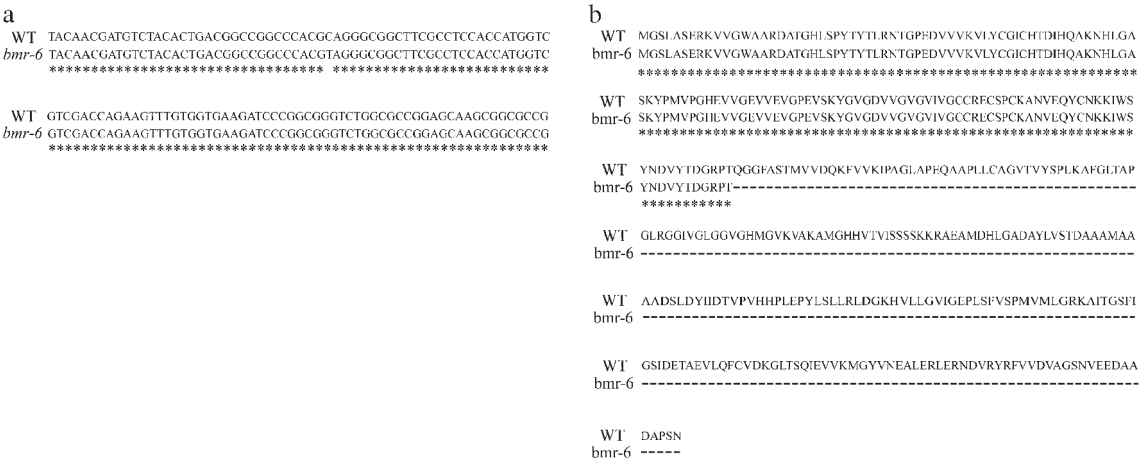

Figure 4. Comparison of DNA and protein sequence for WT and *bmr-6.* (a) DNA sequence; (b) protein sequence.

In this target region, we found six predicted open reading frames (ORFs) in the reference genome annotation database (figure 3c). Only one of these, ORF4 (*Sb04g005950*), a putative CAD, was associated with lignin biosynthesis. Therefore, we sequenced cDNA of this gene. A C–T transition mutation was found in the third exon of *Sb04g005950* in the mutant (figure 4a). This mutation caused premature translation in the mutant protein without changing other amino acids composition in the mutant (figure 4b). These results indicated that *Sb04g005950* could be the target gene.

Confirmation of BMR-6 function

To confirm whether the single base mutation in *BMR-6* was responsible for the brown midrib phenotype, we performed a complementation test. We confirmed 10 positive transgenic plants by the hygromycin resistance assay and PCR (figure 5, a&b). The average transformation efficiency was 1.875%. All these positive transgenic plants were completely reverted to green midrib (only one is seen in figure 5c). Therefore, *Sb04g005950* corresponds to the *BMR-6* gene. Additionally, the plant height, fresh weight per plant, stem diameter, tiller number, blade number and Kalson lignin content in *bmr-6*/*BMR-6* transgenic plants were no significant with WT (table 4).

The subcelluar localization of BMR-6

To determine the subcelluar localization of BMR-6 protein, a transient expression assay was performed in *N. benthamiana* leaves. GFP alone was expressed as a control compared with the full-length BMR-6 protein fused to the N-terminus of GFP. Free GFP was dispersed through the cytoplasm in the *N. benthamiana* epidermal cell and the green fluorescent signal of GFP was not merged with the autofluorescence of chlorophylls in chloroplasts. The localization pattern of BMR-6-GFP was similar to that of free GFP, indicating that the fusion protein was targeted to the cytoplasm (figure 6).

Expression analysis of BMR-6 and its paralogous genes

To characterize the expression pattern of *BMR-6*, we quantified *BMR-6* transcript levels in various plant organs, including leaves, spikelets, midribs, stems and roots. We found that the transcript of *BMR-6* was detected in all test tissues (figure 7). The expression level of *BMR-6* in root, stem and midrib was obviously higher than spikelet and leaves. These results are consistent with the patterns of lignin deposition in these organs. The same expression pattern was also observed in *BdCAD1* gene of tobacco (Bouvier *et al.* 2013).

Arabidposis contains nine CAD-like genes in its genome. Among them, *AtCAD-C* and *AtCAD-D* are classified as bona fide CAD genes (Sibout *et al.* 2005). BLASTP found nine

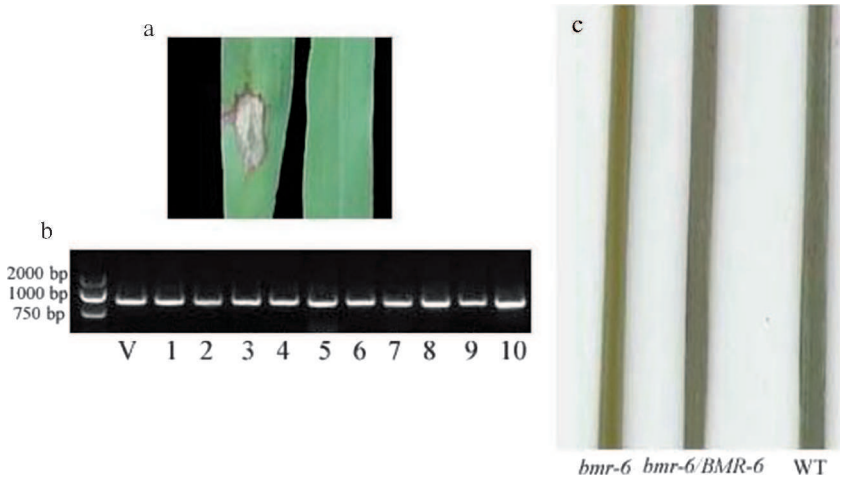

Figure 5. Functional complementation at *bmr-6* locus. (a) Hygromycin resistance assay identified positive transgenic plant at the third-leaf stage. (b) PCR identified positive transgenic plant. V, vector; 1–10, positive transgenic plants. (c) the midrib phenotype of *bmr-6*, complemented plant (*bmr-6*/*BMR*-6) and WT at heading stage.

Table 4. Comparison of agronomic traits between *bmr-6*/*BMR-6* transgenic plants and WT.

	Plant height (cm)	Stem diameter (cm)	Fresh weight per plant (kg)	Tiller number	Blade number	Kalson lignin content
WT	216.67±9.32	1.72±0.06	1.13±0.15	2.67±0.47	13.67±0.57	16.34±0.86
bmr6/BMR-6	210.35±6.12	1.80±0.07	1.01±0.18	3.0±0.30	14.00±1.55	17.41±1.12
t value	1.41	−1.77	1.04	−1.48	−0.35	−1.51

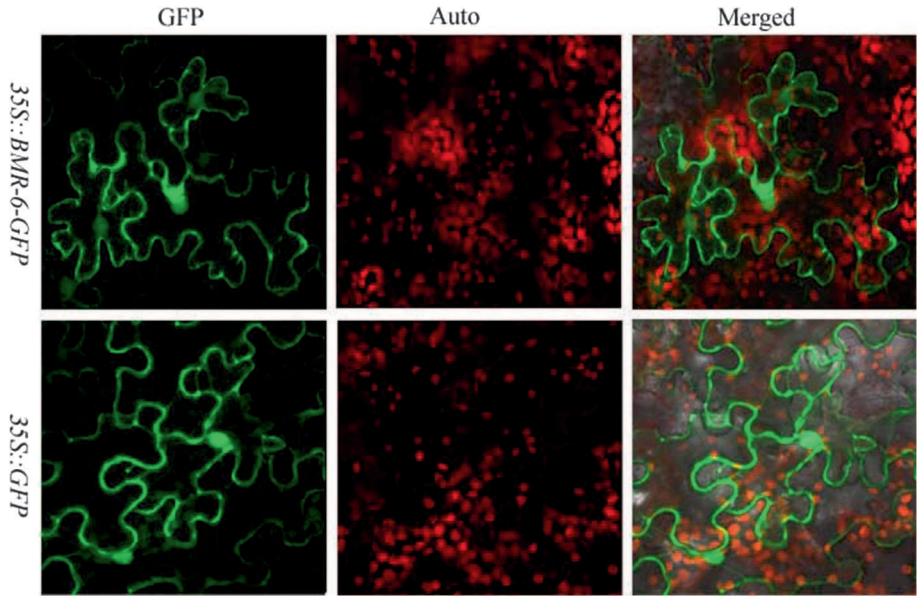

Figure 6. The subcellular localization of the BMR-6 protein. Transient expression of BMR-6-GFP fusion protein and GFP in *N. benthaminana* leaves; GFP fluorescence of BMR-6-GFP and GFP; auto, Chl autofluorescence; Merged image of GFP and auto in bright.

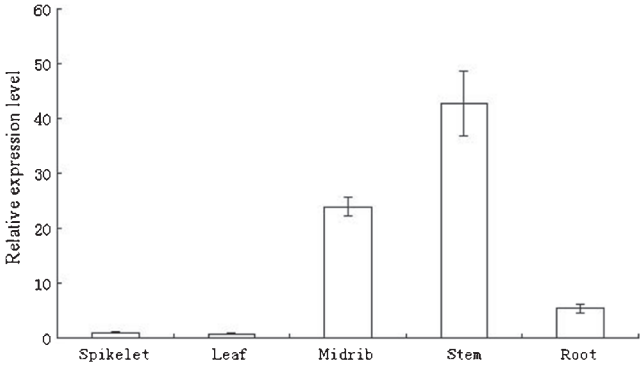

Figure 7. Expression level of *BMR-6* in different organs.

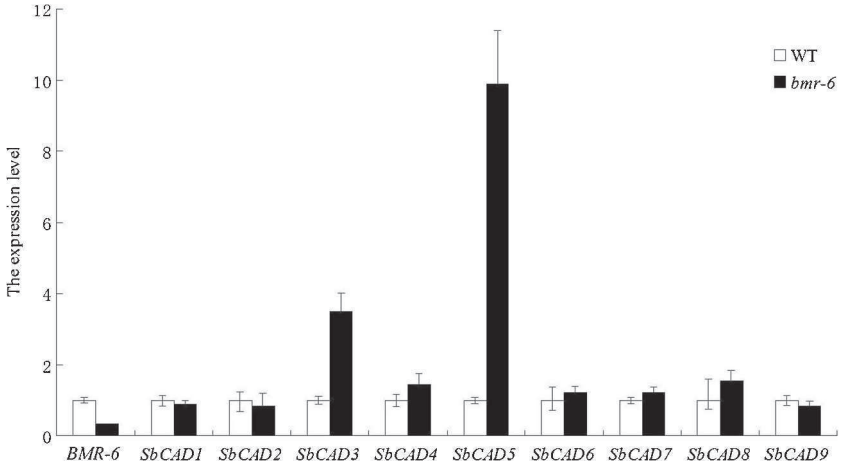

Figure 8. Expression level of *BMR-6* and its paralogous genes for WT and *bmr-6*. GenBank accession numbers are as follows: *BMR-6*, *Sb04g005950*; *SbCAD1*, *Sb02g 024220*; *SbCAD2*, *Sb02g024210*; *SbCAD3*, *Sb02g024190*; *SbCAD4*, *Sb10g006290*; *SbCAD5*, *Sb10g006280*; *SbCAD6*, *Sb10g006270*; *SbCAD7*, *Sb07g006090*; *SbCAD8*, *Sb06g001430*; *SbCAD9*, *Sb06g028240*.

orthlogous genes to *BMR-6* in sorghum genome. To identify whether other CAD-like genes are involved in the lignin biosynthesis, we performed expression analysis of *BMR-6* and its orthlogous genes between mutant and WT. Real-time PCR analysis showed that the expression level of *BMR-6* was significantly decreased in mutant compared to WT (figure 8). It is likely that the mutation of *BMR-6* caused the lower mRNA level. Interestingly, the levels of *SbCAD3* and *SbCAD5* were significantly increased in the mutant, with the expression level of *SbCAD5* almost 10 times higher than the level in WT. Other paralogous genes had no obvious changes in expression level between mutant and WT (figure 8). Therefore, we speculated that *SbCAD3* and *SbCAD5* may also be involved in the biosynthesis of lignin.

Discussion

Brown midrib mutants are characterized by brown-reddish colouration of their leaf midrib and stalk pith, and have reduced lignin content, making them desirable for forage breeding (Marita *et al.* 2003; Lorenz *et al.* 2009; Chen *et al.* 2012; Yan *et al.* 2012). Here, we showed that a sorghum brown midrib mutant *bmr-6* showed brown midrib phenotype and has decreased Kalson lignin concentration. Additionally, we found that *bmr-6* gene has a negative agronomic impact on biomass, as observed for other brown midrib mutants (Porter *et al.* 1978; Oliver *et al.* 2004).

CAD catalyzes the biosynthesis of three lignin monolignols, *ρ*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin. The Kalson content was significantly decreased in *bmr-6* mutant and the levels of aldehyde and polysaccharide increased in the cell wall of the *bmr-6* midrib, suggesting that aldehyde and polysaccharide replaced lignin monolignols in *bmr-6* cell wall. Recent work found that MYB (v-myb avian myeloblastosis viral oncogene homolog) and bHLH transcription factors were involved in feedback v-myb avian myeloblastosis viral oncogene homolog regulation in the biosynthesis of lignin (Yan *et al.* 2013; Zhu *et al.* 2013). Zhu *et al.* (2013) identified that a *SbbHLH1* downregulated the lignin synthesis genes *4CL1*, *HCT*, *COMT*, *PAL1* and *CCR1*, and upregulated the transcription factor *MYB83*, *MYB46* and *MYB63*. These transcription factors involved in the regulation of secondary metabolism pathways, such as alkaloid synthesis, flavonoid synthesis and anthocyanin accumulation. MYB and bHLH transcription factors may also be involved in the feedback regulation of lignin biosynthesis in the *bmr-6* mutant. A more detailed study of the feedback regulation pathway is required.

Lignin monolignols are synthesized in the cytoplasm and transported to the cell wall where they are oxidized prior to their incorporation into the polymer (Vanholme *et al.* 2008). Therefore, CAD protein should localize to the cytoplasm. Bukh *et al.* (2012) analysed seven putative *BdCAD* genes and found that *BdAD4* was predicted by TargetP and ChloroP to have a chloroplast signal in *Brachypodium distachyon*. We also used TargetP and ChloroP and found that *SbCAD1* are also predicted to have a chloroplast signal (data not shown). This suggests that CAD paralogous genes play different roles in plant cells. We found that BMR-6 was targeted to cytoplasm, consistent with the *BMR-6* gene being involved in the biosynthesis of lignin.

CAD-like genes may exist in multiple isoforms, as revealed by genomewide analyses from *Arabidposis* and rice, but the numbers of bona fide CAD enzymes known to be involved in lignin biosynthesis are rather limited (Kim *et al.* 2004; Eudes *et al.* 2006; Hirano *et al.* 2012). There is one CAD enzyme in gymnosperms, and two or three CAD isoforms in dicot species, such as *Arabidposis*. AtCAD5, AtCAD4 and AtCAD1 are the three main CADs that are responsible for lignin biosynthesis (Sibout *et al.* 2005; Eudes *et al.* 2006). In this study, we determined that the *BMR-6* gene is involved in the biosynthesis of lignin and also possibly one or two CAD-like genes. Two reasons support our hypothesis. First, CAD catalyzes the last step of three main monolignols (G, S and H), but the Kalson lignin content of *bmr-6* decreased 23.4%. Compared with WT, the content of lignin decreased 94% in the double mutant of AtCAD-4 and AtCAD-5 in *Arabdiposis*. Therefore, one or two CAD paralogous genes maybe partly complement the loss of *BMR-6* gene function in the *bmr-6* mutant. Secondly, RT-PCR showed that *SbCAD3* and *SbCAD5* were significantly increased in *bmr-6* mutant. Although, *SbCAD3* and *SbCAD5* may be involved in the lignin biosythesis, *SbCAD3* and *SbCAD5* cannot substitute the function of *BMR-6*. The Kalson lignin content of *bmr-6* decreased 23.4% even when the expression level of *SbCAD3* and *SbCAD5* increased about four-fold and ten-fold in *bmr-6* mutant. Our results suggest that *BMR-6* should be the predominant CAD in lignin biosynthesis, at least in the culm. We speculated that *SbCAD3*, *SbCAD5* and *BMR-6* may play the same function in different tissues. The hypothesis was supported by the researches of rice *gh2* (*golden hull* and *internode 2*) and *fc1* (*flexible culm 1*) mutants (Zhang *et al.* 2006; Li *et al.* 2009). Rice *gh2* and *fc1* were a mutation of *OsCAD2* and *OsCAD7* gene, respectively (Zhang *et al.* 2006; Li *et al.* 2009). Both mutants showed a reduction in Kalson lignin content. Expression of *OsCAD7* in the culm was more than three orders of magnitude, lower than that of *OsCAD2* (Hirano *et al.* 2012). Another possibility is that different CADs have different affinity ability to substrates. Kim *et al.* (2004) found that *Arabidposis* AtCAD-C protein showed higher affinity to coniferaldehyde than AtCAD-D.

Acknowledgements

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