

# Identification and Characterization of Four Missense Mutations in *Brown midrib 12* (*Bmr12*), the Caffeic *O*-Methyltransferase (COMT) of Sorghum

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Published online: 22 April 2012

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**Abstract** Modifying lignin content and composition are targets to improve bioenergy crops for cellulosic conversion to biofuels. In sorghum and other C4 grasses, the *brown midrib* mutants have been shown to reduce lignin content and alter its

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**Electronic supplementary material** The online version of this article (doi:10.1007/s12155-012-9197-z) contains supplementary material, which is available to authorized users.

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composition. *Bmr12* encodes the sorghum caffeic *O*-methyltransferase, which catalyzes the penultimate step in monolignol biosynthesis. From an EMS-mutagenized TILLING population, four *bmr12* mutants were isolated. DNA sequencing identified the four missense mutations in the *Bmr12* coding region, which changed evolutionarily conserved amino acids Ala71Val, Pro150Leu, Gly225Asp, and Gly325Ser. The previously characterized *bmr12* mutants all contain premature stop codons. These newly identified mutants, along with the previously characterized *bmr12-ref*, represent the first allelic series of *bmr12* mutants available in the same genetic background. The impacts of these newly identified mutations on protein accumulation, enzyme activity, Klason lignin content, lignin subunit composition, and saccharification yield were determined. Gly225Asp mutant greatly reduced protein accumulation, and Pro150Leu and Gly325Ser greatly impaired enzyme activity compared to wild type (WT). All four mutants significantly reduced Klason lignin content and altered lignin composition resulting in a significantly reduced S/G ratio relative to WT, but the overall impact of these mutations was less severe than *bmr12-ref*. Except for Gly325Ser, which is a hypomorphic mutant, all mutants increased the saccharification yield relative to WT. These mutants represent new tools to decrease lignin content and S/G ratio, possibly leading toward the ability to tailor lignin content and composition in the bioenergy grass sorghum.

**Keywords** *Brown midrib* · *bmr12* · Caffeic *O*-methyltransferase (COMT) · Lignin · Sorghum

## Introduction

Photosynthetic carbon fixation is a key global consumer of atmospheric carbon dioxide, and the world community has

started to look toward plant-based sustainable fuels and chemicals, which would mitigate carbon dioxide emission and reduce petroleum dependence [1, 2]. Lignocellulosic bioenergy conversion requires decomposition of the cell wall polysaccharides cellulose and hemicellulose into monomeric sugars. Lignin, the third cell wall component, is an ubiquitous polymer whose abundance in the world is exceeded only by cellulose [3]. It accounts for approximately 30 % of organic carbon in the biosphere [4]. Lignin subunits are derived from the amino acid phenylalanine, hence both phenylalanine metabolism and lignin biosynthesis occupy central roles in plant metabolism. Lignin is polymerized from monolignols (*p*-coumaryl, coniferyl, sinapyl alcohols) which form *p*-hydroxyphenol (H), guaiacyl (G), and syringyl (S) subunits in the lignin, respectively [4]. The lignin polymer cross-links these polysaccharides, rigidifying and reinforcing the secondary cell wall structure [4], resulting in a barrier that is chemically and microbially resistant. Reducing lignin has also become an important target for bioenergy feedstock improvement [5–7]. Although lignin makes liberating sugars from biomass more difficult, lignin also serves critical functions for vascular plants. Lignin is required for vascular elements to transport water under negative pressure, and in severely lignin deficient plants, vascular collapse has been observed [8–10]. Therefore, there is a limit to the extent to which lignin may be manipulated without significantly impacting plant fitness.

In the C4 grasses maize [*Zea mays* L.], sorghum, and pearl millet [*Pennisetum glaucum* (L.) Leek], the visible *brown midrib* phenotype has been useful for identifying mutants impaired in lignin synthesis (reviewed in [11]). In maize, there are at least six *brown midrib* (*bm1-6*) loci [12], and in sorghum, there are at least four *brown midrib* (*bmr2*, *6*, *12*, *19*) loci [13]. Both the maize *Bm3* and the sorghum *Bmr12* loci encode orthologous caffeic *O*-methyltransferases (COMT) [14, 15], which catalyze the penultimate step in monolignol biosynthesis, the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to the 5-hydroxyl group of 5-hydroxyconiferyl aldehyde to form sinapyl aldehyde. *bmr12-ref* contains a premature stop prior to the sequence encoding the SAM domain [15], and the Bmr12 protein was undetectable in *bmr12-ref* plant extracts [16]. Together these results indicate that *bmr12-ref* is likely a null allele. *bmr12-ref* plants had significantly altered lignin composition and had a significantly reduced amount of lignin compared to their wild type (WT) near isogenic parents in multiple grain and forage sorghum varieties [6, 13, 15–18]. The reduction in lignin deposition was also observed at the cellular level through histochemically stained internode sections and light microscopy. *bmr12-ref* showed reduced lignin staining and altered cell morphology relative to WT, but vascular collapse was not observed in

*bmr12-ref* [16], indicating that the structural role of lignin was still maintained. In addition, sorghum lines carrying *bmr12-ref* mutation had significantly increased ethanol conversion efficiency of lignocellulosic tissues relative to WT tissues using standard dilute acid pretreatment and hydrolytic enzymes [6, 13], which supports targeting this locus to improve sorghum biomass for conversion.

The previously characterized *bmr12* mutants: *bmr12-ref*, *bmr12-18*, *bmr12-25*, and *bmr12-26* all contain premature stop codons [13, 15, 19]. Hypomorphic alleles of *bmr12* would potentially retain residual function, which may be useful to modulate enzymatic activity and fine tune biomass composition. Here we characterize the effects of four EMS-derived *bmr12* alleles have on gene expression, COMT enzymatic activity, lignin content and composition, and stover saccharification in the same genetic background B Tx623. These findings make some of the *bmr12* alleles characterized in this article promising tools to tailor lignin content and composition in sorghum for cellulosic bioenergy uses.

## Materials and Methods

### Preparation of Plant Materials

The near isogenic *bmr12-ref* genetic stock was developed by crossing the *brown midrib* sources F220 or F324 (*bmr12-ref*, a gift from Robert Kalton) to the recurrent parent B Tx623 and followed by three to four backcross cycles as described previously [20]. The other *bmr12* alleles were isolated from an EMS-mutagenized TILLING population (B Tx623) based on visible *brown midrib* phenotype [21, 22]. These lines were self-pollinated for at least two generations, but were not backcrossed to B Tx623. Allelism to *bmr12* was determined by complementation testing; *bmr12* missense alleles herein described failed to complement *bmr12-ref*. Seeds were sown in a soil mix consisting of soil to peat moss to vermiculite to perlite to sand (4:1:1:1), grown in 28-cm pots under a 16-h daylight regimen supplemented with high-pressure sodium lights and fertilized as needed (approximately every 7 days, 5 mL per pot) with a fertilizer containing 11:15:11 (N to P to K; Ferti-lome Gardener's Special). Plants were grown in the greenhouse during November 2009 and January 2012; temperatures were maintained at 29 to 30 °C during the day and 26 to 27 °C during the night. Upon seedling emergence, the plants were thinned to two plants per pot. The stalks were harvested with the leaf tissue removed approximately 5 to 6 weeks after planting for immunodetection and RT-qPCR.

Alternatively, plants were grown to full maturity in 2009 and 2010 at the University of Nebraska Field Laboratory,

Ithaca, NE (Sharpsburg silty clay loam; fine, smectitic, mesic Typic Argiudoll). Nitrogen fertilizer was applied prior to planting at 157 kg ha<sup>-1</sup> both years. Individual plots consisted of two 7.6-m rows spaced 76 cm apart. Plots were seeded with a precision vacuum planter calibrated to deliver 240 seeds per plot. Material was planted on 21 May 2009 and 25 May 2010. In 2009, 3.8 cm of supplemental irrigation was applied via overhead sprinklers on 6 August and on 29 August. In 2010, 3.8 cm of supplemental irrigation was applied on 9 August. Days to flowering were recorded at 50 % anthesis. Height was measured to the top of the mature panicle before harvest. The lignocellulosic tissues were harvested 7 October 2009 and 4 October 2010. Three individual plants were harvested at random from the center row of each block. Panicles were removed at maturity, and lignocellulosic tissue was harvested using a commercial silage cutter modified for small plot use and dried at 50 °C for further analysis. These materials were used for thioacidolysis, Klason lignin, and saccharification analyses.

#### RNA Expression Analysis

RNA was extracted from 5-week-old greenhouse-grown plant stalks as previously described [23]. One microgram of DNase-treated total RNA from each sample was reverse transcribed using a Transcriptor First Strand cDNA kit (Roche Diagnostics, Indianapolis, IN) for real-time PCR. Three biological replicates were analyzed for each mutant and WT. Real-time PCR was performed using Quantitect® SYBR Green (Qiagen, Valencia, CA) and an Eppendorf Mastercycler EP Realplex PCR module (Hamburg, Germany). Primers for *Bmr12* (Sb07g003860.1), and  $\alpha$ -tubulin (Sb01g009570.1) were designed using Invitrogen Vector NTI software (Life Technologies Corporation, Grand Island, NY), and were 5'-GCTCACCCCTAACGAGGACGG-3' (forward), 5'-GCACCGCGTCTTCAGGTAGTA-3' (reverse), and 5'-TCGGAAACGCGTGCTGGAG-3' (forward), 5'-AGCATCGTCACCTCCCCAA-3' (reverse), respectively. Thermal cycling parameters consisted of a 15-min hold at 95 °C to activate the enzyme, followed by 40 cycles of denaturing at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Relative expression of mRNA was calculated using the  $\Delta\Delta C_t$  method. The values for *Bmr12* were determined by normalizing their  $C_t$  values to  $\alpha$ -tubulin, a housekeeping gene. Controls containing RNA without reverse transcription and no template were performed to validate the detection and ensure the absence of DNA contamination within the RNA samples. Sample efficiencies and thresholds were determined by LinRegPCR software (<http://www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR>).

#### Western Blotting and Immunodetection

Immunodetection of Bmr12 followed our previous report [16] with some minor modifications. Proteins were extracted in protein extraction buffer containing 1 % protease inhibitor (Sigma; P9599) from tissue ground in liquid nitrogen. Protein extracts were separated on a 12 % SDS Bio-Rad criterion XT precast gel. Proteins were transferred to nitrocellulose membrane in a wet blot system for 45 min at 65 V in 10 mM Tris, 100 mM glycine, and 8 % (v/v) methanol. Following transfer, the membrane was stained with Ponceau S stain to verify the quality of transfer and then blocked with 3 % nonfat dry milk in TBST (TBS+0.5 % Tween 20) for 1 h. The membrane was probed with the primary antibody (polyclonal rabbit antisera against Bmr12 and polyclonal rabbit antisera against ascorbate peroxidase, a loading control) diluted 1:5,000 for 1 h followed by three washes with TBST for 5 min each. The secondary antibody (goat antirabbit IgG + horseradish peroxidase; Sigma-Aldrich A-0545) was diluted 1:10,000 and incubated for 1 h. The membrane was washed in TBST for 5 min twice and then washed in TBS+0.5 M NaCl for 5 min. Secondary antibody was detected through chemiluminescence using Amersham ECL western blotting reagent (GE Healthcare). Each lane of the gel represents one individual plant. The immunoblotting was performed on at least three different plants. We displayed representative blots from all analyzed blots.

#### Bmr12 Recombinant Proteins

The coding region of Bmr12 was PCR amplified and cloned into the pET30a vector as previously described [24]. The corresponding missense mutations described within this article were created in the expressed Bmr12 coding region through site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit and following the manufacturer's protocol (Stratagene, now Agilent). The presence of these point mutations was confirmed by automated DNA sequencing. Vectors were introduced into Rossetta R2 *Escherichia coli* cells for protein expression. Cultures were grown to log phase from a single colony at 37 °C, and then protein expression was induced using 0.1 mM isopropylthio  $\beta$ -galactoside at 20 °C for approximately 18 h. Soluble protein was extracted using sonication at 20 W with a 10-s pulse following a 30-s rest interval for 3 min. The expressed protein, which contained N-terminal 6 His tag, was isolated by affinity purification on a nickel column and eluted with imidazole. Induction of the expressed protein and protein purification was monitored by SDS-PAGE.

## Enzyme Assays

COMT enzymatic activity was quantified using a continuous coupled fluorescent assay previously developed and validated [24]. *S*-Adenosyl homocysteine hydrolase (SAHH) was used as a coupling enzyme and the thiol-specific fluorophore, Thioglo1 (Covalent Associates Inc., Corvallis, OR), as detection reagent to monitor the conversion of the methyl donor SAM to a fluorescent product [24]. The reaction conditions were 100 mM Tris buffer pH 7.5, 1  $\mu$ M SAHH, 85  $\mu$ M SAM, 15  $\mu$ M Thioglo1, and 40  $\mu$ M 3,4-dihydroxy-5-methoxybenzaldehyde at 37 °C. The concentrations of the recombinant Bmr12 proteins were determined using a colorimetric assay (Pierce 660 nm Protein Assay; Pierce Biotechnology, Rockford, IL), and bovine serum albumin was used as a protein standard. Neither the maleimido form nor the hydrolyzed unconjugated Thioglo1 exhibits intrinsic fluorescence (<http://www.covalentassociates.com/thioglo.htm>). Assays were performed in a black 96-well microtiter plate (Micro Fluor 2 black, flat well; ThermoFisher Scientific, Waltham, MA) using a Bio Tek Synergy HT fluorescence plate reader (Bio Tek Instruments, Winooski, VT). Fluorescence was monitored using an excitation filter 360 $\pm$ 40 nm and an emission filter 528 $\pm$ 20 nm. Fluorescence signal was measured every minute for 45 min with an instrument pre-designated “moderate shake” for 5 s between each read. Raw fluorescence data were imported into Microsoft Excel (MS Office 2007) and analyzed. Fluorescence data were converted into picomoles of –SH groups released based on a standard curve constructed using glutathione.

## Relative Lignin Composition

Thioacidolysis followed by GC-MS was used to determine the relative lignin composition, and the H, G, and S subunits were identified with other minor lignin components based on mass spectra, and quantified using major ions (*m/z*) as previously published [16]. The ground lignocellulosic material (Wiley mill, 1-mm screen) was washed, extracted, derivatized, and analyzed as previously described [16]. Two hundred microliters of 1.25 mg/mL docosane in dichloromethane was added as an internal standard at the time of tissue extraction as previously described [16]. Lignin components were measured based on the peak area of major ions (*m/z*) as follows: H lignin 239, G lignin 269, S lignin 299, and hG lignin 357 as previously described [16]. The peak area of the internal standard docosane (*m/z* 57) was used for the normalization of peak areas between samples as previously described [16]. Analysis was performed on four biological individuals representing one individual from each block.

## DNA Sequencing and Sequence Analysis

Eurofins MWG Operon (Huntsville, AL) performed automated DNA sequencing of amplification products. DNA sequence assembly and bioinformatic analysis were performed using the MacVector version 12.0 software package (Cary, NC).

## Klason Lignin Analysis

Stover was dried and ground in a Wiley mill to pass a 1-mm screen, then extracted in warm (60 °C) 50 % (*v/v*) ethanol for 45 min to remove soluble sugars, minerals, and soluble phenolics and dried at 50 °C. One hundred-milligram extractive-free sorghum stover was used to determine the Klason lignin concentration. Klason lignin is defined as the ash-corrected residue remaining after the cell wall polysaccharides have been removed via a two-stage hydrolysis in concentrated (12 M) and dilute (0.4 M) sulfuric acid. The procedure was based on the method described by Theander and Westerlund [25], with the modifications of Hatfield et al. [26]. Means were calculated based on a minimum of nine plants, sampled from a randomized complete block design with four replicates with a least one plant from each replicate.

## Pretreatment and Enzymatic Saccharification

Dilute sulfuric acid pretreatment was performed by autoclaving 300 mg extractive-free stover at a 10 % solids loading in 1.3 % (*w/v*) sulfuric acid for 40 min at 121 °C, as described in [13]. The residue was collected on a Whatman GF/A filter, washed in 50 mL deionized water (65 °C), and transferred to a 15-mL tube. Enzymatic saccharification was carried out by adding a volume of 9.7 mL enzyme cocktail containing a 1:1 mix of *Trichoderma reesei* cellulase and Novozyme 188 (both from Sigma-Aldrich, St. Louis, MO) in 50 mM sodium citrate buffer pH 4.8 to 300 mg of extractive-free unpretreated stover or to the filtrate remaining after pretreatment. Novozyme 188 is a  $\beta$ -glucosidase (cellobiase) enzyme preparation from *Aspergillus niger* that results in the hydrolysis of cellobiose to glucose. The enzyme cocktail had a cellulase activity of 60 FPU/g cellulose based on NREL standard activity measurement LAP006 [27], and the cellobiase activity in the cocktail was 2.0 CBU/mL (based on information from Sigma-Aldrich). The cocktail also contained tetracycline at a final concentration of 20  $\mu$ g/mL to prevent microbial growth. The tubes containing the saccharification reactions were incubated at 50 °C in a shaker incubator shaking at 100 rpm. Hydrolysate samples (100  $\mu$ L) were collected at 4, 20, and 96 h (unpretreated stover) or 24 h (pretreated stover), placed in boiling water to inactivate the enzymes, cooled,

centrifuged, and used to determine the glucose concentration using a calibrated OneTouch UltraSmart blood glucose meter (Roche Diagnostics, Indianapolis, IN) according to the procedure described in [7]. Means were calculated based on a minimum of nine plants, sampled from a randomized complete block design with four replicates with at least one plant from each block.

### Statistical Analysis

Fisher's protected least significant difference (LSD) test was used to determine if there were statistically significant differences in Klason lignin content and glucose yields between genotypes. Pairwise comparisons among genotypes were made using Tukey's honest significant difference (HSD) with an experiment-wise error rate of  $\alpha=0.05$  using the statistical software package SAS (Cary, NC). For the other datasets, the data were analyzed using SAS (Cary, NC) PROC MIXED v. 9.2 (SAS, 2002–2008). Heterogeneous variances were detected using Levene's test for homogeneity of variance and accounted for using PROC MIXED. *F*-protected least significant differences were used to determine differences among genotypes, which were considered significant at *P* 0.05 (SAS, 2002–2008).

## Results

### Mutation Identification

From an ethyl methane sulfonate (EMS)-mutagenized sorghum population [22], four *brown midrib* mutants (*bmr12-30*, *bmr12-34*, *bmr12-35*, and *bmr12-820*) were isolated for their characteristic tan to brown-colored midribs in contrast to green midrib found in WT B Tx623 sorghum lines. Their allelism to *brown midrib 12* (*bmr12*) was confirmed through complement testing to plants carrying the *bmr12-ref* reference allele [15]. In the field, *bmr12-30*, *bmr12-34*, and *bmr12-820* flowered significantly later than WT, *bmr12-ref*, or *bmr12-35* (Supplementary Table 1). At maturity, the plant heights of all the *bmr12* mutants were significantly shorter than WT, and *bmr12-30* was shorter than other *bmr12* mutants (Supplementary Table 1). The delayed flowering relative to WT or *bmr12-ref* for some *bmr12* mutants suggests that these mutants may harbor effects of additional mutations from the generation of the TILLING population.

The DNA lesions responsible for the *bmr12* phenotype were identified through PCR amplification and DNA sequencing the *Bmr12*, the sorghum COMT (Sb07g003860.1) gene from these mutants and compared to WT B Tx623 [28]. Four transition (G to A or C to T) mutations were identified within the coding region of the gene, which was consistent

**Table 1** Mutations identified in the *Bmr12* gene and their corresponding amino acid changes

<i>bmr12</i> mutant	Mutation (position)	Amino acid change
<i>bmr12-30</i>	G2364A	Gly225Asp
<i>bmr12-34</i> <sup>a</sup>	C518T	Ala71Val
	C2139T	Pro150Leu
<i>bmr12-820</i> <sup>a</sup>	C518T	Ala71Val
	C2139T	Pro150Leu
<i>bmr12-35</i>	G2663A	Gly325Ser

Positions are based on sorghum COMT gene (*Bmr12*; Sb07g003860.1), Genbank accession number AY217766 [15]

<sup>a</sup> *bmr12-34* and *bmr12-820* contain two mutations in *Bmr12*, which are identical

with the mode of action of the mutagen EMS (Table 1). Interestingly, the same two missense mutations within the *Bmr12* coding region were identified in *bmr12-34* and *bmr12-820*, which most likely resulted from the same mutation events and shared a pollen parent. These four base pair transitions caused missense mutations resulting in changes in conserved amino acids throughout the protein (Table 1 and Fig. 1). In all four cases, the amino acid residue was nearly invariant between similar *O*-methyltransferase proteins across vascular plant including monocots, dicots, gymnosperms, and the primitive vascular plant *Selaginella moellendorffii* (Fig. 1). The one exception is Ala71, which is a Ser in *Selaginella*. Based on previously published crystal structures of COMT proteins from other plants, these mutations did not affect amino acids involved in substrate binding, SAM binding, catalysis, or that are present in the active site. However, Gly225Asp was proximal to Asp229 and Leu230, which were shown to be involved in SAM binding (Fig. 1) [29, 30]. Gly325Ser was proximal to Asn323 and Glu328, which were shown to be involved in binding and positioning of the phenolic substrate; Asn323 has been shown to H bond with the 4-hydroxyl group (Fig. 1) [29, 30]. Although Ala71 and Pro150 are not directly involved in dimerization, Ala71Val and Pro150Leu occurred within the dimerization domain of protein [29], which might be relevant to their effect on enzymatic activity.

### *Bmr12* Gene Expression

To examine the effect these mutations have on *Bmr12* gene expression, total RNA was extracted from 5-week-old sorghum stalks, and the gene expression of *Bmr12* was determined using RT-qPCR. The mutants examined did not have a significant impact on *Bmr12* gene expression compared to WT with the exception of *bmr12-ref* (Fig. 2). In *bmr12-ref* stalks, *Bmr12* expression was reduced approximately 77-

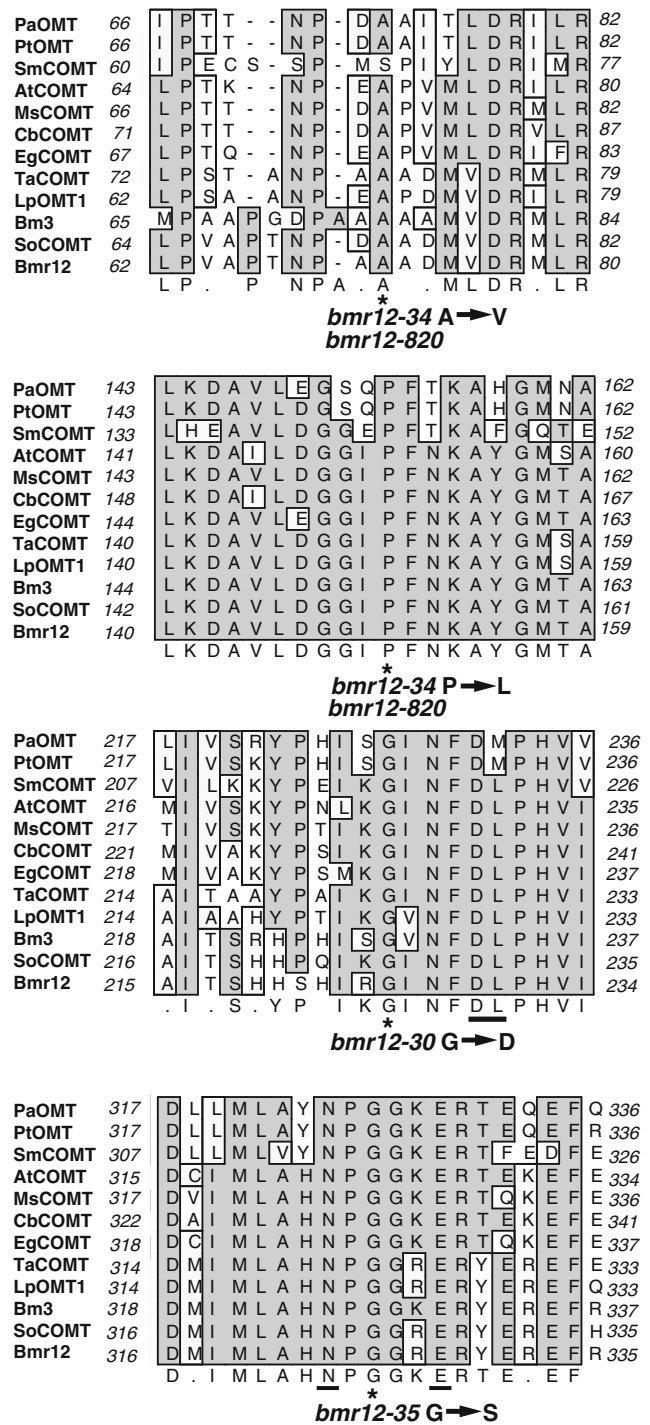
**Fig. 1** Amino acid sequence alignment of caffeic *O*-methyltransferase genes. Predicted amino acid sequences for caffeic *O*-methyltransferase genes from a range of plant species (Genbank accession no. or alternative gene identifier): *Picea abies* PaOMT (CAI30878), *Pinus taeda* PtOMT (TC98491; DFCI Gene Indices), *Selaginella moellendorffii* SmCOMT (438615; Phytozome), *Arabidopsis thaliana* AtCOMT (At5g54160), *Medicago sativa* MsCOMT (AAB46623), *Clarkia breweri* CbCOMT (O23760), *Eucalyptus gunnii* EgCOMT (CAA52814), *Triticum aestivum* TaCOMT (AAP23942), *Lolium perenne* LpOMT1 (AAD10253), *Z. mays* Bm3 (AAQ24340.1), *Saccharum officinarum* SoCOMT (O82054), and *Sorghum bicolor* Bmr12 (Sb07g003860.1; AA043609) were aligned using ClustalW (see the “Materials and Methods” section). The aligned amino acid sequences include the 20 amino acids surrounding the sorghum *bmr12* missense mutations described in this article, which are denoted by an asterisk. Dark gray shading denotes conserved amino acids and light gray shading denotes similar amino acids. Underlined segments denote conserved amino acid residues involved in substrate binding or catalysis

fold relative WT, which is consistent with the previously published Northern blot results [15]. Nonsense mediated decay likely explained the major reduction of these transcripts in *bmr12-ref*, which encodes a nonsense mutation within the coding region [15]. Overall, absence of effects on *Bmr12* gene expression is consistent with missense mutants, which rarely alter gene expression of their own gene.

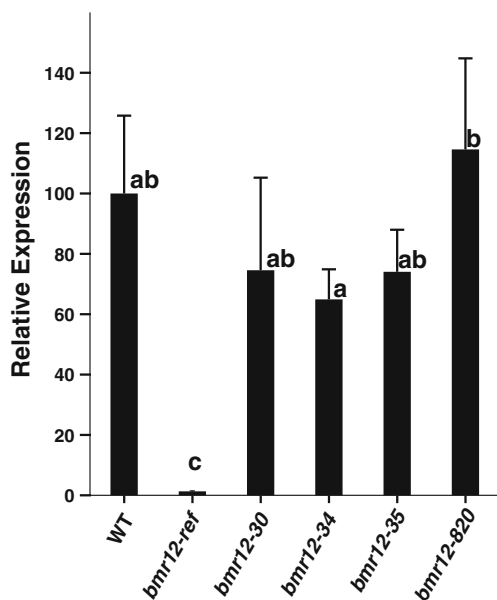
### Bmr12 Protein and Enzymatic Activity

To examine the effect these mutations have on the Bmr12 protein, total proteins were extracted from 6-week-old sorghum stalks, separated by SDS-PAGE, and Bmr12 was detected on an immunoblot using a polyclonal antibody against Bmr12. Bmr12 protein was not detected in *bmr12-ref* protein extracts as previously shown using this antibody [16] (Fig. 3). The Bmr12 protein was nearly undetectable in *bmr12-30* extracts (Fig. 3), which suggests the Gly225Asp change caused the protein to be misfolded and proteolyzed. This conclusion is consistent with the *Bmr12* gene expression in *bmr12-30*, which was comparable to WT (Fig. 2). The Bmr12 protein was also reduced in *bmr12-820* extracts (Fig. 3). Bmr12 was detected at levels comparable to WT in *bmr12-34* and *bmr12-35* based on detection of the loading control with a polyclonal antibody against ascorbate peroxidase, which recognizes both cytosolic and plastidic protein isoforms (Fig. 3).

To determine how these mutations affect enzymatic activity, the WT Bmr12 protein and the mutated forms of Bmr12 corresponding to the four missense mutations were expressed in *E. coli* and purified, because previously sorghum extracts were shown to be incompatible to the COMT enzyme assay [24]. Even when an excess of purified recombinant Bmr12 protein was added to sorghum extracts, the assay failed to detect enzyme activity [24]. However, in switchgrass crude extracts, COMT enzyme activity was



fully detectable [24]. As such, we chose to perform the assay using *E. coli*-expressed proteins. All the mutated forms of the protein were expressed and purified except for Gly225Asp (*bmr12-30*), which resulted in a truncated protein, which was insoluble (Supplementary Fig. 1). This result suggests that Gly225Asp (*bmr12-30*) caused the protein to be misfolded and cleaved, which is consistent with the immunoblot results (Fig. 3). Enzymatic activity was quantified using a previously



**Fig. 2** Relative RNA expression of *Bmr12* in 5-week-old sorghum stalks. Total RNA was extracted from wild-type (*WT*) and *bmr12* stalks. RT-qPCR was used to quantify RNA expression of *Bmr12* and relative expression was determined using  $\Delta\Delta C_t$  method (see the “Materials and Methods” section for further details). *Bmr12* gene expression was expressed relative to WT levels. Means with differing letters differ at  $P=0.05$  using an *F*-protected LSD ( $n=3$ )

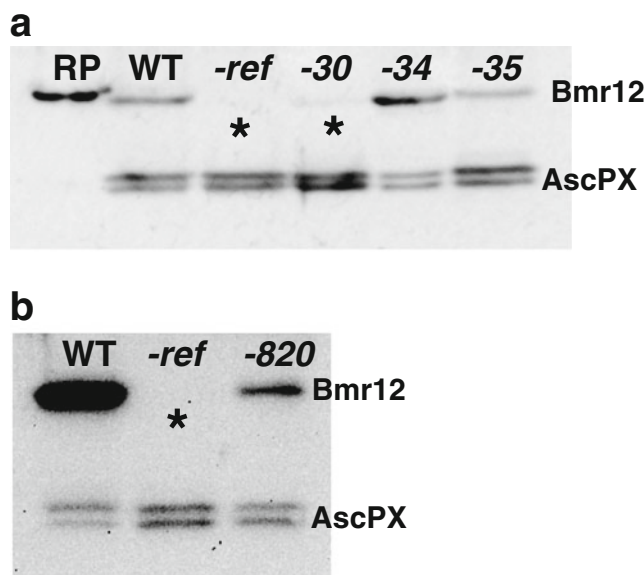
described continuous assay [24]. Enzymatic activity was reduced slightly to 82 % of WT activity for the Ala71Val (first mutation in *bmr12-34/820*, Fig. 4). Pro150Leu (second mutation in *bmr12-34/820*) and Gly325Ser (*bmr12-35*) severely affected enzyme activity relative to the WT activity (Fig. 4). Gly325Ser (*bmr12-35*) recombinant protein had approximately 1.2 % of WT activity. Pro150Leu recombinant protein had 800-fold less activity than WT, and combining Ala71Val and Pro150Leu together, which occurred together in *bmr12-34* and *bmr12-820*, further reduced enzymatic activity to approximately 4,000-fold less than WT activity (Fig. 4). Overall, the missense mutants significantly impaired assayed enzymatic activity, with the exception of Ala71Val (first mutation in *bmr12-34/820*).

**Lignin Content and Enzymatic Saccharification**

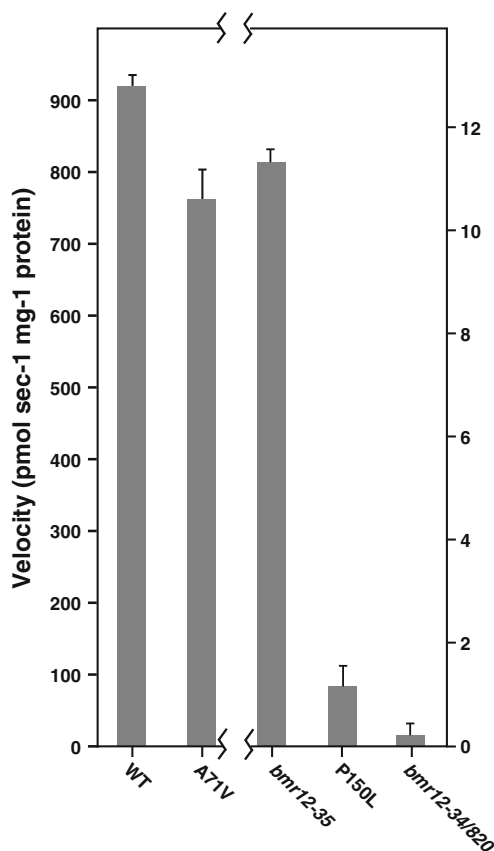
To determine the effect these mutations had on lignin content, Klason lignin content in lignocellulosic biomass from field-grown plants was measured (Table 2). All the *bmr12* mutants examined had statistically significantly reduced Klason lignin content in their biomass relative to WT BTx623. The greatest reduction in lignin content relative to WT (–21.5 %) was observed in *bmr12-820* biomass. The lignin content in *bmr12-30* and *-34* biomass was intermediate; Klason lignin was reduced by 13.2 and 9.0 %, respectively. The biomass from *bmr12-35* had the smallest reduction in

lignin content relative to WT (–7.5 %) for this allelic series. Although *bmr12-820* and *bmr12-34* contain the same two mutations within the *Bmr12* gene, their lignin contents were significantly different, which suggests that a mutation at another locus in either *bmr12-34* or *bmr12-820* has influenced lignin content. The observed reduction in Klason lignin content in these *bmr12* mutants is consistent with the previously described effects of *bmr12* on cell wall lignification.

To assess the effect these mutants had on lignin subunit composition, washed cell wall material from mature stalks was analyzed by thioacidolysis. Thioacidolysis cleaves  $\beta$ -O-4 bonds within the lignin polymer. The resulting monomer constituents were derivatized for analysis by GC-MS. Relative to WT stalk tissue, monomer levels and monomer composition were altered in all of the *bmr12* alleles (Fig. 5a). The content of S subunits was severely reduced in all *bmr12* mutants; the lowest level was observed in *bmr12-ref*, followed by *bmr12-30*, *bmr12-820*, and *bmr12-34*. The *bmr12-35* mutant retained a modest level of S subunits, roughly half the level observed in WT tissue. The content of G subunits was also reduced in *bmr12* alleles relative to WT, albeit modestly. These changes in subunit composition translated into considerably reduced S/G ratios in the *bmr12* mutants. The smallest ratio was observed in *bmr12-ref* (0.0304); *bmr12-820*



**Fig. 3** Immunoblot detection of Bmr12 from 6-week-old sorghum stalks. Protein extracts from wild-type (*WT*) and *bmr12* stalks were separated by SDS-PAGE, transferred to membrane, and probed with polyclonal antibodies raised against the N-terminus of recombinant Bmr12 protein in rabbits [16]. AscPx antibody was raised against purified soybean root nodule ascorbate peroxidase in rabbits. This antibody was used as a loading and transfer control. **a** Recombinant Bmr12 (*RP*), *WT* extracts (*WT*), *bmr12-ref* (*-ref*), *bmr12-30* extracts (*-30*), *bmr12-34* extracts (*-34*), and *bmr12-35* extracts (*-35*). **b** *WT* extracts (*WT*), *bmr12-ref* (*-ref*), *bmr12-820* extracts (*-820*). Asterisks denote the absence of the Bmr12 protein in *bmr12-ref* and *bmr12-30* extracts. See the “Materials and Methods” section for further details



**Fig. 4** Enzyme activity of Bmr12 and mutated versions of protein. COMT enzymatic activity from recombinant proteins was determined using a continuous fluorescent assay previously developed and described [24]. 3,4-Dihydroxy-5-methoxybenzaldehyde at 40  $\mu$ M was used as the substrate. The enzyme velocity was normalized to the amount of protein. The scale on the left corresponds to the first two columns and the scale on the right corresponds to the last three columns. The recombinant protein containing both Ala71Val and Pro150Leu amino acid changes corresponds to *bmr12-34/820*. The recombinant protein containing Gly325Ser corresponds to *bmr12-35*. Error bars denote SE ( $n=4$ ). See the “Materials and Methods” section for further details

(0.0750), *bmr12-30* (0.0752), and *bmr12-34* (0.0806) were intermediate; and *bmr12-35* (0.424) had the highest ratio among the mutants, closest to WT (0.625, Fig. 5a). The content of H subunits was also modestly reduced relative to WT in all of the *bmr12* mutants examined (Fig. 5b). The lignin polymers of the *bmr12* alleles contained elevated 5-hydroxy guaiacyl lignin levels relative to WT, because the COMT substrate 5-hydroxyconiferylalcohol was incorporated in the lignin polymer. The highest levels were observed in *bmr12-ref* and *bmr12-34*, and intermediate levels were observed in *bmr12-820*, *bmr12-30*, and *bmr12-35* (Fig. 5b). 5-Hydroxy guaiacyl residues were detected in WT tissue, but at levels that were over fourfold lower relative to the intermediate levels in *bmr12-30* and *bmr12-35*, and over eightfold lower compared to the high levels observed in *bmr12-ref*, *bmr12-35*, and *bmr12-820*. Overall, these data indicated that the *bmr12*

missense alleles altered lignin composition in a manner consistent with reduced enzymatic activity.

To assess the potential these alleles have to improve cellulosic conversion, 300 mg of ground lignocellulosic material from mature stalk tissue was washed and enzymatically saccharified using cellulases at a loading of 60 FPU/g. The yield of glucose released was measured after 4, 20, and 96 h of saccharification (Table 2). All the *bmr12* alleles examined had statistically greater glucose release than WT at 20 and 96 h, except for *bmr12-35*. The highest release of glucose was observed from *bmr12-820* tissue followed by *bmr12-34*, *bmr12-30*, *bmr12-ref*, and WT at 20 and 96 h. The improved glucose yields were maintained after pretreatment in dilute sulfuric acid (Table 2). Based on these assays, all *bmr12* alleles except *bmr12-35* appear to improve cellulosic conversion rates.

## Discussion

We have identified and characterized four novel missense mutations in *Bmr12*, the sorghum COMT gene. These mutations resulted in changes to amino acid residues that are nearly invariant across vascular plants. The previously characterized *bmr12* mutants: *bmr12-ref*, *bmr12-18*, *bmr12-25*, and *bmr12-26* all contain premature stop codons [13, 15, 19], and are likely null alleles encoding nonfunctional forms of the enzyme. These newly discovered alleles represent the first allelic series of *bmr12* mutants derived from the same varietal background to investigate the impact of these mutants on *Bmr12* gene expression, enzymatic activity, lignin content, and composition and biomass conversion. These missense mutations in *Bmr12* (Ala71Val, Pro150Leu, and Gly325Ser) were also identified in recent studies [22].

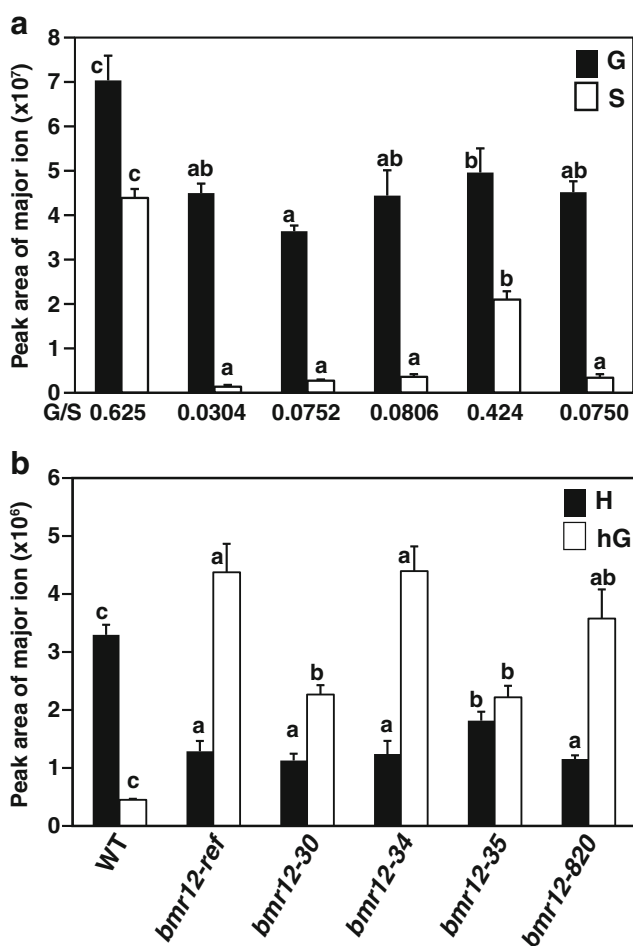
The effects of these missense mutations were examined in the context of protein function. Changing an evolutionarily conserved amino acid residue to a dissimilar amino acid (an Asp replaced Gly225) substantially decreased the steady-state level of the Bmr12 protein in *bmr12-30* (Fig. 3). The premature stop codon in the *bmr12-ref* allele resulted in undetectable levels of the Bmr12 protein in plant extracts (Fig. 3) [16], and therefore, this allele likely represents a null allele of *Bmr12*. The gene expression results (Fig. 2) indicated that neither a transcriptional nor a posttranscriptional reduction of *Bmr12* transcripts was responsible for the near absence of the protein in *bmr12-30* (Fig. 3). Hence, the Gly225Asp change in *bmr12-30* likely destabilized the protein causing misfolding and subsequent protein degradation. Interestingly, we were unable to express Gly225Asp altered COMT protein in *E. coli* cells; this amino acid change resulted in a truncated protein product (Supplementary Figure 1). This result suggested that the mutated form of the protein was also misfolded in *E. coli*, and an endogenous



**Table 2** Variation in Klason lignin content and glucose yields obtained after enzymatic saccharification at 50 °C at 60 FPU/ g cellulase of native (unpretreated) stover after 4, 20, and 96 h, and of pretreated stover after 24 h

	Klason lignin (mg/g)	Glucose yield (mg/g stover)			
		Unpretreated		Pretreated	
		4 h	20 h	96 h	24 h
Mean (SD)	Mean (SD) <sup>a</sup>	Mean (SD) <sup>a</sup>	Mean (SD)	Mean (SD) <sup>a</sup>	
WT BTx623	21.2 (1.9)a	38 (6)bc	54 (5)d	55 (4)d	174 (25)c
<i>bmr12-ref</i>	17.1 (0.8)cd	42 (6)abc	62 (5)cd	64 (8)bcd	212 (19)b
<i>bmr12-30</i>	18.4 (0.9)bc	43 (13)abc	64 (7)bc	70 (8)abc	230 (27)ab
<i>bmr12-34</i>	19.3 (1.5)b	51 (10)ab	74 (9)ab	74 (9)ab	213 (22)b
<i>bmr12-820</i>	16.7 (1.7)d	54 (14)a	79 (12)a	82 (13)a	238 (35)a
<i>bmr12-35</i>	19.6 (0.5)ab	32 (7)c	54 (6)d	59 (12)cd	178 (12)c
HSD (0.05)	2.2	10	8.3	9.9	22

<sup>a</sup>Means in rows with different letters are statistically significant based on Tukey’s HSD with an experiment-wise error rate of 0.05 (*n*=9)



**Fig. 5** Lignin composition in wild-type (*WT*) and the *bmr12* mutants. **a** Distribution of major lignin subunits G lignin (*G*; black bars) and S lignin (*S*; white bars) in stalks from *WT* and *bmr12* alleles were quantified by GC-MS following thioacidolysis. **b** Minor lignin subunits were determined by abundance of ions diagnostic of H lignin (*H*; black bars) and 5-hydroxy G lignin (*hG*; white bars) in stalks. Error bars denote SE (*n*=4). Means with differing letters differ at *P*=0.05 using an *F*-protected LSD. See the “Materials and Methods” section for further details

protease subsequently cleaved this protein. The size of the protein fragment observed on SDS-PAGE differed from the expected peptide fragments if the cleavage occurred at Gly225Asp, which suggested the cleavage occurred at another site. Based on protein accumulation, *bmr12-30* is likely another strong mutant allele similar to *bmr12-ref* as opposed to *bmr12-34* and *bmr12-35*, which did not affect steady-state Bmr12 level. Examining COMT activity of the recombinant proteins containing the amino acid changes identified in *bmr12-34/820* and *bmr12-35* showed that these three mutations (Ala71Val, Pro150Leu, Gly325Ser) clearly reduced enzyme activity compared to WT. Ala71Val reduced COMT activity by 18 %, but in *bmr12-34/820*, the protein contained both Ala71Val and Pro150Leu changes. Combined together Ala71Val with Pro150Leu reduced enzyme activity to approximately 4,000-fold less than WT activity, but the residual activity of this recombinant protein was still detectable relative to the negative control. In contrast, Gly325Ser (*bmr12-35*) retained approximately 1.2 % of WT activity, 48-fold more activity than Ala71Val/Pro150-Leu protein (*bmr12-34/820*). Based on protein and enzyme analysis, *bmr12-35* should retain significantly more enzyme activity than *bmr12-30*, *bmr12-34*, and *bmr12-820*.

The impact these missense mutations had on lignin content and composition is consistent with either absence of Bmr12 protein or reduction of enzymatic activity. All the mutants examined significantly reduced Klason lignin content relative to WT. All their S/G ratios are drastically decreased compared to WT due to the reduced synthesis of sinapyl alcohol. The *bmr12-30*, *bmr12-34*, and *bmr12-820* mutants had S/G ratios that were very small, but still more than twofold higher than *bmr12-ref*. *bmr12-30* and *bmr12-34* biomass also had significantly higher Klason lignin relative to *bmr12-ref*. Together, these results suggest that *bmr12-30*, *bmr12-34*, and *bmr12-820* may retain very minute amounts of enzyme activity, which is completely absent from *bmr12-ref*. The enzyme assays also support this

hypothesis for *bmr12-34/bmr12-820*. The one clear discrepancy is the lignin content of *bmr12-820*, which was slightly lower than *bmr12-ref* but significantly lower than *bmr12-34*, even though both *bmr12-34* and *bmr12-820* contained the same two *bmr12* mutations. A plausible explanation is that *bmr12-34* and *bmr12-820* differ at another locus, resulting in even lower lignin content in *bmr12-820*. Clearly, *bmr12-35* is a hypomorphic (weak) allele, because it has an intermediate lignin content relative to WT and *bmr12-ref*, an intermediate S/G ratio compared to other *bmr12* mutants and WT and retained some enzyme activity. The 83-fold reduction in enzyme activity of the Gly325Ser recombinant protein translated into a modest reduction in Klason lignin and intermediate S/G in *bmr12-35*, which suggests this enzyme assay may magnify the effects of the missense amino acid change on enzyme activity. Alternatively, the Bmr12 (COMT) capacity of the plant could greatly exceed its requirement for sinapyl alcohol synthesis. Even in the putative null allele *bmr12-ref*, which lacks any detectable Bmr12 protein, S lignin subunits were still detectable [16]. These results indicate that other *O*-methyltransferases within sorghum are capable of catalyzing this reaction in the absence of Bmr12, and one potential candidate is the enzyme caffeoyl-CoA *O*-methyltransferase, which has been shown to perform a similar role in Arabidopsis COMT-deficient plants [31].

Except for *bmr12-35*, all *bmr12* mutants examined significantly increased glucose yield from biomass following enzymatic saccharification relative to WT, even after dilute sulfuric acid pretreatment. Even though the lignin content and S/G ratio are significantly reduced in *bmr12-35*, its glucose yield was not significantly different from WT. Although the *bmr12-34* mutant has a similar reduction in Klason lignin content compared to *bmr12-35*, it has a significantly lower S/G ratio as well as lower enzyme activity. These observations suggest that in order to achieve higher saccharification yields, lignin content and/or S/G ratio must be reduced below certain thresholds. The increased glucose yield appears to primarily result from the greater reduction of S/G ratio due to reduced sinapyl alcohol content. This observation is consistent with other *bmr12* mutants with low S/G ratios. Based on these data, the S/G ratio necessary to improve glucose yields is between 0.081 and 0.423, and additional research is needed to narrow the range down further. The reductions in Klason lignin content observed were much smaller compared to changes in lignin composition, so S/G ratio is a likely more sensitive metric for evaluating these mutants. In *bmr12*, the reduced S/G and reduced lignin content are linked, because *bmr12* mutants are primarily reduced in lignin content through the reduction of S lignin. More importantly, these results suggest that improvement of conversion of grass cell walls may require a fundamentally different approach than in angiosperm dicots, where reduced lignin content

[5] or a high S/G ratio, the opposite of *bmr12* mutants, was shown to be favorable [32].

In summary, these missense alleles of *bmr12* represent new tools to modify lignin content and composition to increase biomass conversion efficiency. Multiple rounds of backcrossing will be required to remove other mutations generated by the mutagen and understand the impact that these *bmr12* missense mutations have agronomic traits such as yield. Going beyond this study, these alleles could be combined with each other or with the previously characterized *bmr12* alleles to form interallelic hybrids in the same or in a hybrid background. This approach has the potential to modulate both lignin content and composition (S/G ratio), similar to transgenic approaches to downregulate COMT in plant species [9, 33, 34], but with relatively greater stability. The thresholds for altering lignin composition and content to increase bioenergy conversion for sorghum remain to be determined.

**Acknowledgments** We thank Tammy Gries, Nathan Peroutka-Bigus and John Toy for their technical assistance with experimental data presented in this manuscript, Dr. Lisa Durso for the use of her real-time PCR instrument, and Dr. Heather Van Buskirk for critically reviewing the manuscript. This research was supported by National Institute of Food & Agriculture, Grant 2011-67009-30026 (S.E.S, J.F.P), the Office of Science (BER), U.S. Department of Energy, grant DE-FG02-07ER64458 (W.V., S.E.S, J.F.P), and additional funding from USDA-ARS, CRIS project 5440-21220-024-00D (S.E.S, J.F.P).

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