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Efficacy of Singular and Stacked *brown midrib 6* and *12* in the Modification of Lignocellulose and Grain Chemistry

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In sorghum, *brown midrib* (*bmr*) *6* and *12* impair the last two steps of monolignol synthesis. *bmr* genes were introduced into grain sorghum to improve the digestibility of lignocellulosic tissues for grazing or bioenergy uses following grain harvest. Near-isogenic grain sorghum hybrids (AWheatland × RTx430) were developed containing *bmr6*, *bmr12*, and the *bmr6 bmr12* double mutant (stacked), and their impacts were assessed in a two-year field study. The *bmr* genes did not significantly impact grain or lignocellulosic tissue yield. Lignocellulosic tissue from *bmr6*, *bmr12*, and stacked hybrids had reduced lignin content and increased in vitro dry matter digestibility (IVDMD) compared to those of the wild type (WT). The lignin content of the stacked lignocellulosic tissue was further reduced compared to that of *bmr6* or *bmr12*. Surprisingly, *bmr12* modestly increased carbohydrates in lignocellulosic tissue, and *bmr6* increased fiber and lignin content in grain. These data indicate that *bmr6* and *bmr12* have broader effects on plant composition than merely lignin content, which has promising implications for both livestock utilization and bioenergy conversion.

KEYWORDS: Sorghum; *brown midrib*; digestibility; lignocellulosic tissues

INTRODUCTION

Photosynthetic carbon fixation constitutes a key global consumer of atmospheric carbon dioxide. The world community has started to look toward plant-based sustainable fuels, which would mitigate carbon dioxide emission and reduce petroleum dependence (*1*, *2*). Sorghum [*Sorghum bicolor* (L). Moench] because of its wide varietal diversity (grain, forage, sweet, and sudangrass) and its diverse end-uses has the potential to play several roles in the developing bioenergy sector. In the current generation of biofuels, ethanol is being synthesized via the fermentation of grain starch. Twenty-nine percent of the U.S. grain sorghum harvested in 2008 was fermented into ethanol (*3*). In addition, sweet sorghum varieties have the potential to be utilized in place of sugar cane in ethanol fermentation (*1*). For the next generation of biofuels, research is being directed toward the conversion of lignocellulosic biomass into biofuels (*4*), and several types of sorghum have potential as feedstocks. As bioenergy technologies progress, the conversion of biomass to biofuels could involve a range of chemical, biochemical, and fermentation processes to produce biofuels; alternate biofuels, such as butanol or dimethylfuran, are also on the horizon (*5*, *6*). Traditionally, sorghum grain is used in foods and beverages for humans (*7*, *8*) and in feed for livestock, companion animals, and poultry (*3*). Sorghum lignocellulose in various forms (grain stover, forage, silage, and sudangrass) is used as feed for cattle (*Bos tarus*) and other ruminant animals (*9*, *10*).

Both lignocellulosic bioenergy conversion and forage utilization by livestock require decomposition of the cell wall polysaccharides cellulose and hemicellulose into monomeric sugars. Plant cell walls consist of a complex polysaccharide moiety containing cellulose microfibrils, composed of β-1,4-linked glucose polymers (*11*). Connecting the cellulose microfibrils to each other is a hemicellulose network, which in sorghum is mainly composed of glucuronoarabinoxylans. Lignin, a heterogeneous polymer derived from aromatic amino acid precursors, cross-links these polysaccharides, rigidifying and reinforcing the cell wall structure (*11*). The addition of lignin polymers to the polysaccharide matrix creates a barrier that is chemically and microbially resistant, and can block the liberation of sugars from cell wall polysaccharide moieties. Hence, lignin is a major factor, reducing the digestibility of sorghum and other forages (*12*). Reducing lignin has also become an important target for bioenergy feedstock improvement (*13*, *14*).

In C4 grasses, the visible brown midrib phenotype has been useful for identifying mutants impaired in lignin synthesis. Spontaneous brown midrib mutants were first discovered in maize [*Zea mays* L.; (*15*)] and were subsequently generated in sorghum using diethyl sulfate mutagenesis (*16*). Brown midrib forage from maize, sorghum, and pearl millet [*Pennisetum glaucum* (L.) Leek] has a decreased amount of lignin and increased digestibility (*9*, *12*, *17*, *18*). In maize, there are at least five brown midrib (*bm1*−*5*) loci (*19*), and in sorghum, there are at least four brown midrib (*bmr2*, *6*, *12*, and *19*) loci (*20*). *Bmr6* has been shown to encode a cinnamyl alcohol dehydrogenase (CAD) (*21*, *22*), which catalyzes the last step in the synthesis of lignin subunits (monolignols) prior to the polymerization of lignin. *Bmr6* reduces cinnamyl aldehydes (coniferyl, coumaryl, and sinapyl aldehyde) to their corresponding cinnamyl alcohols

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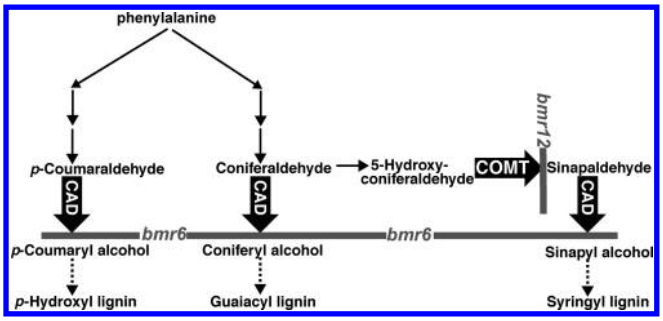

Figure 1. COMT and CAD enzymes and their roles in the monolignol biosynthetic pathway. A simplified model of the lignin biosynthetic pathway where COMT catalyzes the addition of a methyl group to 5-OH-conferyl alcohol and CAD catalyzes the reduction of cinnamyl (coumaryl, coniferyl and sinapyl) aldehydes to alcohols in the final step of monolignol biosynthesis. *bmr12* impairs COMT activity, and *bmr6* impairs CAD activity.

using NADPH as a cofactor prior to their incorporation into lignin polymers (*22*) (**Figure 1**). Evidence indicates that *bmr6* is most likely a null allele (*21*, *22*). In *bmr6* lignocellulosic tissues, lignin composition was altered (*20*, *22*−*25*), and the amount of lignin was significantly reduced relative to their WT isogenic parent as determined by either acid detergent lignin (ADL) or Klasson lignin (*9*, *10*, *26*, *27*) in multiple grain and forage sorghum varieties. *Bmr12* encodes a caffeic *O*-methyltransferase (COMT) (*28*), which catalyzes the penultimate step in monolignol biosynthesis, the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to the 5-hydroxyl group of 5-hydroxy-coniferyl substrates to form sinapyl products (**Figure 1**). Data indicate that *bmr12* is probably a null allele (*24*, *28*). The lignin composition was altered (*24*, *28*), and the amount of lignin was significantly reduced in multiple *bmr12* grain and forage sorghum varieties (*9*, *10*, *26*, *27*) compared to that of their WT isogenic parents.

In maize *bm3* lines, which are orthologous to *bmr12* (*29*), there have been significant reductions in both grain and forage yields (*30*, *31*). However, only one study utilized near-isogenic inbred lines (*30*); thus, the contributions of the genetic background in the other study cannot be estimated. *bmr6* and *bmr12* were isolated from a chemically mutagenized population (*16*); thus, their genomes contained multiple mutations in addition to the *bmr* locus. To determine the effects due to the *bmr* locus alone, near-isogenic lines were constructed by successively backcrossing to an inbred sorghum line and selecting for *bmr* progeny each subsequent generation for four or five generations in order to obtain a common genetic background for experimentation and evaluation. Previously, a 19−35% reduction in grain yield was observed in the near-isogenic *bmr6* and *bmr12* inbred lines of BWheatland and RTx430 backgrounds (*27*). In *bmr6* near-isogenic inbred lines, there were also significant reductions (12 and 18%) in lignocellulosic residue yields, while the lignocellulosic residue yields of *bmr12* were either similar to WT (Wheatland) or significantly increased relative to that of WT (*27*). However, analysis of the *bmr6* and *bmr12* near-isogenic grain hybrids (AWheatland × RTx430) indicated that heterosis could overcome yield loss associated with *bmr12* in sorghum (*27*). In addition, *bmr6 bmr12* double mutant (stacked) lines have been developed from the near-isogenic single mutation lines previously characterized as a strategy to further reduce lignin content in sorghum (*32*). The objective of the present study was to determine the impact of *bmr6* (impaired CAD activity), *bmr12* (impaired COMT activity), and the *bmr6 bmr12* double mutant on the modification of lignocellulose tissue and grain chemistry in a common commercial hybrid, and to determine the extent of potentially detrimental effects of the single and double mutations on biomass and grain yield in this heterotic hybrid background.

MATERIALS AND METHODS

Plant Materials. Near-isogenic *bmr6*, *bmr12*, *bmr6 bmr12*, and wild-type (WT) versions of the common grain sorghum hybrid AWheatland × RTx430 were created by crossing previously described near-isogenic versions of these two parental lines (*32*, *33*). Field trials using *bmr6*, *bmr12*, stacked, and wild-type (WT) near-isogenic hybrids were conducted in 2006 and 2007 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^{-1} 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 18 May, 2006 and 21 May, 2007. The experiment was replicated four times in each year. In 2006, 1.3 cm of supplemental irrigation was applied via overhead sprinklers on 26 May, and 3.8 cm was applied 26 June, 28 July, 4 August, and 11 August. In 2007, 3.8 cm of supplemental irrigation was applied on 6 June, 6 July, 16 July, and 26 July.

Days to flowering were recorded at 50% anthesis. Height was measured to the top of the mature panicle before harvest. Panicles were hand-harvested at maturity, then lignocellulosic tissue was harvested using a commercial silage cutter modified for small plot use (*34*). Grain and lignocellulosic tissue were harvested 3−4 October, 2006 and 20−21 September, 2007.

Sample Analysis. Grain samples were air-dried to uniform 14% moisture, threshed and weighed using a small plot combine as a stationary thresher. Acid detergent fiber (ADF) (*35*), acid detergent lignin (ADL) (*36*), fat (*37*), starch (*38*), and crude protein (CP) (*39*) concentrations were determined by Ward Laboratory, Kearney, NE. In vitro dry matter digestibility (IVDMD) was conducted for 12 h to estimate the rate of digestion (*40*).

Lignocellulosic tissue samples were collected and oven-dried (60 °C), dry matter (DM) content determined, and samples analyzed sequentially for ADF and ADL using an ANKOM 200 fiber analyzer (ANKOM Tech. Corp., Fairport, NY). IVDMD was performed using ANKOM rumen fermenters (Model No: Daisy II; ANKOM Tech. Corp., Fair-port, NY) (*35*, *36*). Crude protein concentration was calculated as %N × 6.25 (*37*). In vitro dry matter digestibility (IVDMD), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined using ANKOM Fiber Analyzer (ANKOM Technology Corp., Fairport, NY) using the procedures described by Vogel et al. (*35*) and the ANKOM ADL procedure (*36*).

Carbohydrate Composition. Approximately 20 mg of ground lignocellulosic tissue was dried overnight at 60 °C for compositional analysis. Soluble sugars were extracted from samples in 5 mL of 80% ethanol (v/v) for 60 min at room temperature. Samples were then filtered through polyethylene frits using a vacuum manifold and washed twice. Two hundred fifty milligrams of 2-deoxy-D-glucose (Sigma-Aldrich, St. Louis, MO) was added as an internal standard, and the final volume was adjusted to 10 mL for HPLC analysis. Then 800 μL of 6 M sulfuric acid was added to ground tissue samples and incubated at room temperature for 30 min to hydrolyze glucuronoarabinoxylans to their corresponding sugar monomers. Samples were diluted with 4.2 mL of water and autoclaved at 121 °C for 30 min. Internal standard was added, and the samples were filtered and washed twice. The filtrate was neutralized with 5 M sodium hydroxide, and the volume was adjusted to 10 mL for HPLC analysis. The plant material remaining on the frit was dried overnight at 60 °C. Then 300 μL of 18 M sulfuric acid was added to the remaining plant material and incubated at room temperature for 30 min to hydrolyze the cellulose into glucose monomers. Samples were diluted with water (3.6 mL), then autoclaved at 121 °C for 30 min. Internal standard was added, and the samples were filtered, washed, neutralized, and diluted as described above for the glucoarabinoxylan hydrolysis.

Each sample was filtered through a 0.45 μm nylon membrane (Pall #4426T; Port Washington, NY) and diluted 50-fold prior to HPLC analysis. Sugars were separated isocratically with 5 mM sodium hydroxide at a flow rate of 0.5 mL min^{-1} on a Dionex ICS3000 HPLC system with a Dionex CarboPac PA20 column (Dionex; Bannockburn, IL) at 30 °C. Sugars were detected using a pulsed amperometric detector, normalizing

Table 1. Effects of Brown Midrib (*bmr*) Genes on Grain and Lignocellulose Traits in the Grain Sorghum Hybrid AWheatland × RTx430[a]

	WT	*bmr6*	*bmr12*	*stacked*	SEM
days to 50% anthesis	67 a	68 a	71 b	71 b	1
height (cm)	133 a	126 b	148 c	136 d	2
grain yield (T ha^{-1})	8.1	7.7	8.0	7.7	0.2
lignocellulose yield (T ha^{-1})	5.3	5.0	5.5	5.2	0.3

[a] Means in rows with differing letters differ at $P = 0.05$ using an *F*-protected LSD.

Table 2. Effects of Brown Midrib (*bmr*) Genes on Grain Composition (Dry Basis) in the Grain Sorghum Hybrid AWheatland × RTx430[a]

	WT	*bmr6*	*bmr12*	*stacked*	SEM
ADF (g kg^{-1})	44 b	48 a	42 b	45 ab	3
ADL (g kg^{-1})	16 bc	18 a	15 c	17 ab	1
fat (g kg^{-1})	31	30	32	31	2
starch (g kg^{-1})	720	718	717	712	8
crude protein (g kg^{-1})	113	115	117	118	3
12-h IVDMD (g kg^{-1})	183	176	172	176	9

[a] Means in rows with differing letters differ at $P = 0.05$ using an *F*-protected LSD.

to the internal standard, and quantified by standard curves of known concentrations.

Statistical Analysis. The experimental design was a randomized complete block replicated four times in each of two years. Genotypes (wild-type, *bmr6*, *bmr12*, and stacked) were considered fixed, and year and replication within the year were considered random. The data were analyzed using PROC MIXED of SAS (2004). The REPEATED function of PROC MIXED was used to account for the potential lack of homogeneity of variance among the years. F-protected least significant differences were used to determine differences among genotypes, which were considered significant at $P \leq 0.05$ (SAS, 2000−2004).

RESULTS

Yield, Maturity, and Height. Maturity, measured as the number of days from planting to 50% anthesis, was delayed by 3 to 4 days in *bmr12* and stacked hybrids compared to that of WT or *bmr6* (**Table 1**). Plant heights were significantly different in all four hybrids (**Table 1**). The *bmr12* hybrid was the tallest followed by stacked, WT, and *bmr6*. In the stacked hybrid, the effects of *bmr6* and *bmr12* on plant height appeared to be additive. These differences in height were not large enough to override the effect of the major sorghum height genes, and all four hybrids still fit height expectations for 3-dwarf (combine-height) sorghum hybrids. Significant differences in grain yield were not detected among the near-isogenic hybrids (**Table 1**) and ranged from 8.1 T ha^{-1} for WT to 7.7 T ha^{-1} for stacked. Interestingly, the addition of *bmr12* to *bmr6* in the stacked hybrid did not reduce grain yield (**Table 1**) in this hybrid background. The three to four day delay in maturity observed in *bmr12* and stacked hybrids may partially ameliorate the expected reduction in grain yield. However, the combined effects of heterosis and a high performing genetic background likely contributed to the lack of observed statistically significant differences in grain yields among the near-isogenic hybrids. Differences in lignocellulosic tissue yields (residue left after grain harvest) were also not significantly different among the near-isogenic hybrids (**Table 1**), ranging from 5.0 T ha^{-1} for *bmr6* to 5.5 T ha^{-1} for *bmr12*. Together, these grain and lignocellulosic tissue yield data indicate that the agronomic characteristics of *bmr* hybrids are comparable to those of the WT.

Grain Composition. Fat, starch, and crude protein content, and 12-h IVDMD of grain were not appreciably modified by the individual or stacked *bmr* mutants (**Table 2**) with content averaging 31 g kg^{-1} for fat, 716 g kg^{-1} for starch, 116 g kg^{-1} for crude protein, and 177 g kg^{-1} for 12-h IVDMD. ADF and ADL were, however, modified by the *bmr* mutations. The *bmr12* hybrid had statistically equivalent ADF and ADL as compared to those of the near-isogenic WT hybrid. The *bmr6* mutant had 9% greater ADF and 13% greater ADL than the WT hybrid. The stacked hybrid was intermediate in ADF and was not significantly different from any other near-isogenic hybrids, and ADL content was equivalent to that of WT and *bmr6*, but higher than that of *bmr12*. This result is the first reported incidence of *bmr* genes modifying grain chemistry, and the *bmr* genes did so in near-isogenic hybrids with equivalent grain and lignocellulosic tissue yields as reported above.

Table 3. Effects of Brown Midrib (bmr) Genes on Lignocellulose Composition (Dry Basis) in the Grain Sorghum Hybrid AWheatland × RTx430[a]

	WT	*bmr6*	*bmr12*	*stacked*	SEM
ADF (g kg^{-1})	399 a	379 c	392 ab	386 bc	6
ADL (g kg^{-1})	41 a	31 b	27 b	21 c	3
crude protein (g kg^{-1})	81 a	79 a	73 b	79 a	5
IVDMD(g kg^{-1})	566 c	596 b	642 a	639 a	22

[a] Means in rows with differing letters differ at $P = 0.05$ using an *F*-protected LSD.

Lignocellulosic Tissue Composition. The *bmr6* and *bmr12* genes were introduced into the hybrid AWheatland × RTx430 to reduce lignin levels and improve the bioenergy conversion efficiency and digestibility of the lignocellulosic tissue by livestock. ADL levels of the lignocellulosic tissue were significantly reduced by roughly a third in *bmr6* and *bmr12* hybrids compared to that of the WT (**Table 3**). Stacking the mutant genes further reduced ADL to roughly half of that of the WT, which indicated that *bmr6* and *bmr12* acted additively to reduce lignin content in the lignocellulosic tissue. ADF was reduced in *bmr6* and stacked near-isogenic hybrid tissues relative to that of WT (**Table 3**). Because ADF estimates the amount of cellulose and lignin, the reduction associated with *bmr6* and stacked hybrids suggests that *bmr6* may reduce the amount of cellulose present within cell walls. Crude protein was significantly reduced in *bmr12* relative to that of other hybrids (**Table 3**). Interestingly, *bmr12* and stacked lignocellulosic tissues had the highest IVDMD (**Table 3**). *bmr6* had significantly higher IVDMD levels than the WT, but lower levels than the *bmr12* or stacked lignocellulosic tissues. These data indicated that *bmr6* and *bmr12* have similar effects on the amount of lignin in cell walls but different effects on IVDMD.

Carbohydrate Composition. To address whether *bmr6*, *bmr12*, or their stacked combination affected soluble sugar or polysaccharide composition of the lignocellulosic tissue, soluble sugars were directly extracted, and polysaccharides were acid hydrolyzed to their constituent sugar monomers, then the sugars were separated by HPLC and quantified using pulsed amperometric detection. The dilute acid treatment liberated glucose, arabinose, and xylose from the glucuronoarabinoxylans, and the subsequent concentrated acid treatment liberated glucose from cellulose. The soluble sugars were fairly similar among the hybrids except for *bmr12*, which had significantly higher levels of arabinose and soluble glucose relative to those of the WT or *bmr6* hybrids (**Table 4**). Soluble glucose levels were 28% higher in *bmr12* tissue than in the WT. Likewise, sugars liberated by dilute acid treatment from glucuronoarabinoxylan (hemicellulose) were fairly similar for all of the hybrids except for *bmr12*, which contained 21% higher levels of arabinose than the WT (**Table 4**). In the *bmr12* hybrid, the level of arabinose was not significantly elevated in comparison to that of the stacked hybrid. Similarly, the amount of glucose liberated by the concentrated acid treatment from cellulose was comparable for all of the hybrids except for *bmr12*, which contained higher levels relative to that of the WT, *bmr6*, or stacked hybrids (**Table 4**). This

Table 4. Effects of Brown Midrib (*bmr*) Genes on Lignocellulose Carbohydrate Composition (Dry Basis) in the Grain Sorghum Hybrid AWheatland × RTx430[a]

	WT	*bmr6*	*bmr12*	*stacked*	SEM
soluble glucose (g kg^{-1})	12.7 b	11.4 b	15.4 a	13.0 ab	4.0
soluble fructose (g kg^{-1})	26.4	25.3	28.2	27.3	5.6
soluble sucrose (g kg^{-1})	56.3	58.5	64.9	59.3	7.1
arabinose (g kg^{-1})	15.3 b	16.0 b	19.6 a	17.1 ab	2.4
xylose (g kg^{-1})	175.8	179.8	192.0	174.0	11.3
dilute acid glucose (g kg^{-1})	87.7	91.2	81.7	83.7	10.8
conc. acid glucose (g kg^{-1})	172.5 b	163.3 b	196.5 a	161.0 b	6.1

[a] Means in rows with differing letters differ at $P = 0.05$ using an *F*-protected LSD.

carbohydrate compositional analysis of hybrid lignocellulosic tissue suggested that *bmr12* had significantly higher levels of soluble sugars, hemicellulose, and cellulose compared to those of the WT (**Table 4**). In contrast, *bmr6* tissue had levels of soluble sugars, hemicellulose, and cellulose that were comparable to those of the WT. The addition of *bmr6* to *bmr12* in the stacked hybrid appeared to attenuate the increased carbohydrates associated with *bmr12*.

DISCUSSION

Previously, it was shown that the negative impact of *bmr6* or *bmr12* on either grain or cellulosic tissue yield could be overcome through heterosis in the AWheatland × RTx430 hybrid background (*27*). *bmr12* grain yield was statistically equivalent to that of the WT, but *bmr6* hybrids exhibited a 10% reduction in grain yield (*27*). In a previous study, lignocellulosic tissue yields were highest for *bmr12*, intermediate for the WT, and lowest for *bmr6* in these near-isogenic hybrids with greater than 20% yield differential between *bmr6* and *bmr12* near-isogenic hybrids (*27*). However, in the present two year study, statistically significant differences in grain yield or lignocellulosic tissue yield were not observed between WT and *bmr6*, *bmr12*, or stacked hybrids. It is important to note the differences between the previous study and the current one; both irrigated and dry-land environments were included in the previous study (*27*). In the current study, only irrigated environments were utilized, which was expected to maximize the yield of all genotypes and reduce potential negative pleiotropic effects associated with water stress. The current study corroborates the previous findings and emphasizes that the yield potentials of *bmr6*, *bmr12*, or stacked hybrids are relatively close to those of the WT yield potentials under sufficient moisture. The stacked near-isogenic hybrid was not included in the previous study, but our previous experience stacking these mutations in near-isogenic inbred backgrounds Atlas, BWheatland, and RTx430 (*32*) led us to expect further yield reductions relative to those of *bmr6* or *bmr12* hybrids. Surprisingly, there was no statistically significant difference in either grain or lignocellulosic tissue yields from stacked lines relative to those of WT, *bmr6*, or *bmr12*, which suggests that the negative effects on yields of combining *bmr6* and *bmr12* were neither additive nor synergistic in this study.

Grain. Differences in grain chemistry were limited to ADF and ADL, with the *bmr12* hybrid having the lowest ADL and *bmr6* having higher ADL and ADF than the WT or *bmr12*, which suggests that the *bmr6* grain has more cell wall material relative to that of other grain components. How a mutation in a CAD results in increasing cell wall deposition in grain remains to be determined. The magnitude of the difference in these grain components was relatively small, but important. Using a standard predictive model (*41*), the net energy available to cattle for gain would be 2% higher for *bmr12* grain compared to that of *bmr6*. Because fiber and lignin in the grain would be expected to be concentrated in the seed coat, it is probable that these differences in predicted energy availability and resulting animal performance would be magnified in distillers grains. Assuming complete conversion of starch (72% of the grain dry matter) and no loss of other cell components, ADL could be concentrated by a multiplier of 3.8. Small differences in ADL could then become quite sizable with *bmr12* distiller grains potentially having 57 g kg^{-1} ADL as compared to 68 g kg^{-1} ADL for *bmr6* distiller grains.

Lignocellulosic Tissue. Differences in lignocellulosic chemistry were striking. Most importantly, these data indicate that it is possible to further reduce lignin content in a high yielding hybrid background by stacking *bmr6* and *bmr12* without a substantial reduction in either grain or lignocellulosic tissue yield (**Table 1**). The stacked hybrid has the potential to further reduce ADL by 33% and 22% relative to that of *bmr6* or *bmr12*, which is strongly negatively correlated with both ruminant animal performance and lignocellulosic ethanol conversion efficiency. Using standard equations to predict theoretical ethanol yields using both hexose and pentose sugars (*26*), we predicted lignocellulosic ethanol yields of 389, 357, 355, and 346 L T^{-1}, respectively, for *bmr12*, WT, *bmr6*, and stacked hybrids.

Similar to previous results, *bmr12* appeared to be superior to *bmr6* in this study. IVDMD and ADL results were rather enigmatic because *bmr12* had the highest IVDMD, but its ADL was comparable to that of *bmr6*. More importantly, ADL was significantly decreased in the stacked hybrid relative to that of *bmr6* or *bmr12*, but this reduction did not translate into a higher IVDMD compared to that of *bmr12* (**Table 3**). These observations cannot be readily explained by how *bmr6* and *bmr12* affect monolignol biosynthesis (**Figure 1**) because *bmr6* plants exhibited reductions in all three lignin subunits (H-, G-, and S-lignin), whereas *bmr12* plants had reduction primarily in S-lignin as analyzed through thioacidolysis in the Wheatland and Tx430 near-isogenic inbred lines (*24*). S-lignin was reduced in *bmr6* stalks to levels approaching *bmr12* in both varieties, and S-lignin subunits were further reduced to nearly undetectable levels in stacked near-isogenic lines in both varieties (*24*). Increased carbohydrate levels and decreased crude protein level in *bmr12* lignocellulosic tissue relative to those of the WT or *bmr6* (**Tables 3** and **4**) could explain the elevated IVDMD observed in *bmr12* relative to that of *bmr6*. In addition, these compositional changes in *bmr12* may also explain the similar IVDMD levels between *bmr12* and the stacked hybrid even though ADL was significantly reduced in the stack hybrid relative to that of *bmr12*. Unlike *bmr6*, the effects of *bmr12* on lignocellulosic tissue appear to go beyond lignin biosynthesis and impact other plant metabolic pathways. Although the reason that a null mutation in COMT would have these broad metabolic effects remains unclear, perhaps the loss of COMT activity would increase the amount of the cofactor SAM available for other metabolic pathways. The lignocellulosic tissue composition of the stacked hybrid was similar to that of the WT or *bmr6*, but these results could be due to the additive or epistatic effects of *bmr6*. Flowering was delayed 4 days in the stacked hybrid similar to that in *bmr12* (**Table 1**). Genetic evidence suggests that delayed flowering in *bmr12* plants may be caused by a closely linked locus rather than the mutation in *bmr12*. Flowering time of plants with an allele of *bmr12*, *bmr18*, was indistinguishable from WT (*42*). Both *bmr12* and *bmr18* contain stop codons in the first exon of the gene, and both are likely null alleles (*28*), suggesting that another gene affecting flowering time is tightly linked to the *bmr12* locus.

Previously, ethanol conversion efficiency was determined for the lignocellulosic tissue from WT, *bmr6*, *bmr12*, and *stacked* near-isogenic lines in the forage sorghum variety Atlas.

The stacked *bmr6 bmr12* near-isogenic line significantly increased actual lignocellulosic ethanol conversion efficiency by up to 56%, 30%, and 39% compared to that of the WT, *bmr6*, and *bmr12* near-isogenic lines, respectively (*26*). Actual conversion was not attempted in the present study. The discrepancy in ranking of our calculated theoretical (above) versus Atlas actual conversion yields is likely due to the fact that only glucose released from washed lignocellulosic tissue after pretreatment was fermented into ethanol in the Dien et al. (*26*) study. In the Atlas study, soluble sugars were absent following the pretreatment and wash, and yeast (*Saccharomyces cerevisiae*) cannot utilize pentoses (xylose and arabinose). The Dien et al. (*26*) study highlights the importance of *bmr* genes in the modification of lignin composition and content and its effect on ethanol yields using current fermentation technologies. The theoretical ethanol yields calculated from compositional data reported in this study highlight the additional effect of *bmr12* on carbohydrate content and composition, and the potential for *bmr12* to further increase ethanol yields as conversion technologies evolve.

Together, these data demonstrated that lignin content of lignocellulosic tissue could be further reduced by stacking *bmr6* and *bmr12* without a substantial yield penalty in the grain sorghum hybrid AWheatland × RTx430. The *bmr12* gene had broader effects on lignocellulosic tissue composition than *bmr6* or stacked genes for reasons that remain unclear. The higher carbohydrate levels and modified lignocellulosic tissue composition associated with *bmr12* could explain the higher IVDMD and higher theoretical ethanol yields associated with *bmr12*. Furthermore, these data demonstrate that the lignin content of grain can be modified by brown midrib mutations with *bmr6* appearing to increase grain lignin and fiber content relative to that of the WT.

ABBREVIATIONS USED

ADF, acid detergent fiber; ADL, acid detergent lignin; bmr, brown midrib; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid *O*-methyltransferase; IVDMD, in vitro dry matter digestibility; SEM, standard error of the mean; stacked, *bmr6 bmr12* double mutant; WT, wild-type.

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