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Scott E. Sattler
USDA-ARS, Scott.Sattler@ars.usda.gov

Deanna L. Funnell-Harris
USDA-ARS, Deanna.Funnell-Harris@ars.usda.gov

Jeffrey F. Pedersen
University of Nebraska-Lincoln, jpedersen1@unl.edu

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

SCOTT E. SATTLER, DEANNA L. FUNNELL-HARRIS, and JEFFREY F. PEDERSEN

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. 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. In addition, sweet sorghum varieties have the potential to be utilized in place of sugar cane in ethanol fermentation. For the next generation of biofuels, research is being directed toward the conversion of lignocellulosic biomass into biofuels, 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. Traditionally, sorghum grain is used in foods and beverages for humans and in feed for livestock, companion animals, and poultry. Sorghum lignocellulose in various forms (grain stover, forage, silage, and sudangrass) is used as feed for cattle and other ruminant animals.

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. Cellulose microfibrils to each other is a hemicellulose network, which in sorghum is mainly composed of glucuronoxarabinohexans. Lignin, a heterogeneous polymer derived from aromatic amino acid precursors, cross-links these polysaccharides, rigidifying and reinforcing the cell wall structure. 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.

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. Brown midrib forage from maize, sorghum, and pearl millet [Pennisetum glaucum (L.) Leek] has a decreased amount of lignin and increased digestibility. In maize, there are at least five brown midrib (bmr1−5) loci, and in sorghum, there are at least four brown midrib (bmr2, 6, 12, and 19) loci. Bmr6 has been shown to encode a cinnamyl alcohol dehydrogenase (CAD), which catalyzes the last step in the synthesis of lignin subunits (monolignols) prior to the polymerization of lignin. Bmr6 reduces cinnamaldehyde (coniferyl, coumaryl, and sinapyl aldehyde) to their corresponding cinnamyl alcohols.
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 5-adenosylmethionine (SAM) to the 5-hydroxy group of 5-hydroxy-coniferyl aldehydes to alcohols in the final step of monolignol biosynthesis. Bmr12 impairs COMT activity, and bmr6 impairs CAD activity.

In maize bmr3 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 silt loam clay; fine, smectic, mesic Typargidoll). 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 thresh. 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., Fairport, 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 glucoronoarabinoyxans 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 glucoronoarabinoyxylolysis.

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
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 ≤ 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 had significantly higher IVDMD levels than the WT. Likewise, sugars liberated by dilute acid treatment from cellulose were comparable for all of the hybrids except for 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.

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. ADL 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 indicate 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 1. Effects of Brown Midrib (bmr) Genes on Grain and Lignocellulose Traits in the Grain Sorghum Hybrid AWheatland × RTx430

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>bmr6</th>
<th>bmr12</th>
<th>stacked</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>days to 50% anthesis</td>
<td>67 a</td>
<td>68 a</td>
<td>71 b</td>
<td>71 b</td>
<td>1</td>
</tr>
<tr>
<td>height (cm)</td>
<td>133 a</td>
<td>126 b</td>
<td>148 c</td>
<td>136 d</td>
<td>2</td>
</tr>
<tr>
<td>grain yield (T ha⁻¹)</td>
<td>8.1</td>
<td>7.7</td>
<td>8.0</td>
<td>7.7</td>
<td>0.2</td>
</tr>
<tr>
<td>lignocellulose yield (T ha⁻¹)</td>
<td>5.3</td>
<td>5.0</td>
<td>5.5</td>
<td>5.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>bmr6</th>
<th>bmr12</th>
<th>stacked</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF (g kg⁻¹)</td>
<td>44 b</td>
<td>48 a</td>
<td>42 b</td>
<td>45 ab</td>
<td>3</td>
</tr>
<tr>
<td>ADL (g kg⁻¹)</td>
<td>16 bc</td>
<td>18 a</td>
<td>15 c</td>
<td>17 ab</td>
<td>1</td>
</tr>
<tr>
<td>fat (g kg⁻¹)</td>
<td>31</td>
<td>30</td>
<td>32</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>starch (g kg⁻¹)</td>
<td>720</td>
<td>718</td>
<td>717</td>
<td>712</td>
<td>8</td>
</tr>
<tr>
<td>crude protein (g kg⁻¹)</td>
<td>113</td>
<td>115</td>
<td>117</td>
<td>118</td>
<td>3</td>
</tr>
<tr>
<td>12-h IVDMD (g kg⁻¹)</td>
<td>183</td>
<td>176</td>
<td>172</td>
<td>176</td>
<td>9</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>bmr6</th>
<th>bmr12</th>
<th>stacked</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF (g kg⁻¹)</td>
<td>399 a</td>
<td>379 c</td>
<td>392 ab</td>
<td>386 bc</td>
<td>6</td>
</tr>
<tr>
<td>ADL (g kg⁻¹)</td>
<td>41 a</td>
<td>31 b</td>
<td>27 b</td>
<td>21 c</td>
<td>3</td>
</tr>
<tr>
<td>crude protein (g kg⁻¹)</td>
<td>81 a</td>
<td>79 a</td>
<td>73 b</td>
<td>79 a</td>
<td>5</td>
</tr>
<tr>
<td>IVDMD (g kg⁻¹)</td>
<td>566 c</td>
<td>596 b</td>
<td>642 a</td>
<td>639 a</td>
<td>22</td>
</tr>
</tbody>
</table>

*Means in rows with differing letters differ at P = 0.05 using an F-protected LSD.
hybrid appeared to attenuate the increased carbohydrates associated with bmr12 highest for bmr6, the differential between bmr6 and bmr12 in ground (27%) led us to expect further yield reductions relative to those of the WT yield potentials under sufficient moisture. 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, B'Wheatland, and RTx430 (22) 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 cellulosic 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 51 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. IVDMC and ADL results were rather enigmatic because bmr12 had the highest IVDMC, 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 IVDMC 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 IVDMC observed in bmr12 relative to that of bmr6. In addition, these compositional changes in bmr12 may also explain the similar IVDMC 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 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.

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

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>bmr6</th>
<th>bmr12</th>
<th>stacked</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>12.7</td>
<td>11.4</td>
<td>15.4</td>
<td>13.0</td>
<td>4.0</td>
</tr>
<tr>
<td>fructose</td>
<td>26.4</td>
<td>25.3</td>
<td>28.2</td>
<td>27.3</td>
<td>5.6</td>
</tr>
<tr>
<td>sucrose</td>
<td>56.3</td>
<td>58.5</td>
<td>64.9</td>
<td>59.3</td>
<td>7.1</td>
</tr>
<tr>
<td>arabinose</td>
<td>15.3</td>
<td>16.0</td>
<td>19.6</td>
<td>17.1</td>
<td>2.4</td>
</tr>
<tr>
<td>xylose</td>
<td>175.8</td>
<td>179.8</td>
<td>192.0</td>
<td>174.0</td>
<td>11.3</td>
</tr>
<tr>
<td>glucose</td>
<td>87.7</td>
<td>91.2</td>
<td>81.7</td>
<td>83.7</td>
<td>10.8</td>
</tr>
<tr>
<td>conc. acid</td>
<td>172.5</td>
<td>163.3</td>
<td>196.5</td>
<td>161.0</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*Means in rows with differing letters differ at *P* = 0.05 using an F-protected LSD.*
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 (28). 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 (xylene 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 AWeatland × RTx430. The bmr2 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 IVMD 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; IVMD, in vitro dry matter digestibility; SEM, standard error of the mean; stacked, bmr6 bmr12 double mutant; WT, wild-type.

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