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Lignin Properties and Cell Wall Response to Deconstruction by Alkaline Pretreatment and Enzymatic Hydrolysis in Brown Midrib Sorghums

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ABSTRACT

Lignin has an adverse impact on the deconstruction of plant cell wall biopolymers in biorefining processes and its reduction and/or alteration during biosynthesis is one target for decreasing plant cell wall recalcitrance. In this work, the impact of two brown midrib mutations (*bmr*6 and *bmr*12) in two sorghum background lines (the commercial hybrid Atlas and near-isogenic BTx623) on lignin properties and the plants' response to cell wall deconstruction to monomeric sugars via alkaline pretreatment and enzymatic hydrolysis is investigated with the goal of assessing how differences in lignin content and properties impact the plant's response to pretreatment. We identify that both *bmr* sorghum lines show significantly lower abundance of water-extractable sugars (glucose, sucrose, and fructose) and alkali-saponifiable *p*-coumarate. Furthermore, both these properties exhibited identical trends across both background lines. Next, both untreated and mild alkali-pretreated *bmr* sorghums were shown to exhibit higher glucose hydrolysis yields following enzymatic hydrolysis than the control lines. Following pretreatment, the Atlas *bmr* sorghums exhibited more lignin solubilization and the solubilized lignin was of lower molar mass than the background control line suggesting that differences in the lignin response to pretreatment resulted these differences. Finally, significant differences were observed in the lignin content, lignin monomer distribution, and inter-unit linkages in the Atlas *bmr* line relative to the control line with key differences including lower syringyl monomer

content in both *bmr* lines, higher relative abundance of β-O-4 linkages in the *bmr6* line, and the presence of 5-hydroxy guaiacyl monomers and benzodioxane (α-O-5/β-O-4) linkages in the *bmr12* line.

Key words: brown midrib, sorghum, lignin, alkaline pretreatment

1. INTRODUCTION

Global demand for energy, including liquid transportation fuels, are projected to increase substantially over the next 30 years even in scenarios with slow economic growth and high petroleum prices (Sendich et al., 2019). While the additional greenhouse gas emissions from increasing energy use will be substantial, the inclusion of renewable carbon feedstocks such as plant biomass provides the opportunity to address the dilemma of how to produce more energy with less carbon emissions (BP, 2019). While first-generation biofuels are currently produced from the starch contained in corn grain, sucrose from sugarcane, and lipids in oil seed crops, the structural polymers contained within the cell walls of plants (cellulose, hemicelluloses, and lignin) have enormous potential for the production of petroleum-displacing biobased fuels, chemicals, and materials. A recent study projects that more than 1.3 billion dry tons of lignocellulosic biomass could be produced annually in United States from agriculture and forestry for the production of biofuels and bio-based chemicals (Langholtz et al., 2016).

Technologies for the utilization of structural biopolymers within the cell walls of plants include a "deconstruction" step such as a pretreatment coupled to an enzymatic hydrolysis process to yield cellulosic sugars followed by a subsequent conversion of the sugars to a biofuel or chemical product (Ong et al., 2014). As a feedstock for renewable fuels and chemicals, feedstocks such as sugarcane or sorghum have the potential to yield water-extractable sugars (sucrose, glucose, fructose, and starch) from their stems, structural biopolymers (cellulose and hemicelluloses) from the lignocellulose, as well as grain-derived starch, oils, and protein. Approaches for biorefining of both cellulosic and extractable stem-accumulated sugars include the use of conventional sugar extraction technologies currently employed for sucrose extraction from sugarcane followed by deconstruction of the lignocellulose or, alternatively, hybrid extraction and pretreatment processes for both pools of sugars (Williams et al., 2019).

Lignins can comprise a significant fraction of the cell wall mass (Ralph et al., 2004a) and are well-known to contribute to the recalcitrance of the cell wall to digestion by ruminants (Jung and

Allen, 1995) as well as impacting their susceptibility to "deconstruction" to utilizable sugars and lignin-derived aromatics that can be used to produce bio-based fuels and chemicals (Himmel et al., 2007; Li et al., 2015). In addition to the total content of lignin, the relative abundance of monomer subunits and linkages between these monomers can also impact the response to a conversion process. Engineering or breeding of plants with altered lignin content (Fu et al., 2011), differences in the relative abundance of monomer types (Chen and Dixon, 2007), increasing the abundance of ether linkages (Huntley et al., 2003; Li et al., 2018b), or introduction of novel linkages (Bhalla et al., 2018) are strategies for improving ruminant digestibility or the susceptibility to a plant cell wall deconstruction process.

Relevant to the present work, the grasses exhibiting *brown midrib* (*bmr*) mutations are one example of plants with reduced cell wall recalcitrance due to altered lignins (Barrière et al., 2004). The *bmr* mutations were first identified in maize in 1924 as imparting improved cell wall digestibility by ruminants and have since been identified in several other C4 grasses (Barrière and Argillier, 1993; Cherney et al., 1990; Christensen and Rasmussen, 2019; Porter et al., 1978). The phenotype for improved ruminant digestibility in *bmr* plants is due to alterations in the level of expression of key genes involved in lignin biosynthesis and result in significant differences in the total lignin content, the type and abundance of aromatic monomers within the lignin polymer, and the type and abundance of interunit linkages between monomers (Barrière et al., 2004; Sattler et al., 2010a). Two key *bmr* mutations in sorghum include *bmr6* (equivalent to *bm1* in maize) and *bmr12* (*bm3* in maize) (Oliver et al., 2005). Prior work in sorghum has identified lower dry matter yields (kg/ha), lower lignin content, and higher *in vitro* digestibility by rumen microbial communities for *bmr6* and *bmr12* in a number of background lines (Oliver et al., 2005; Pedersen et al., 2008; Porter et al., 1978). These mutations correspond to deficiencies in cinnamyl alcohol dehydrogenase (CAD) activity for *bmr6* and decreased caffeate *O*-methyltransferase (COMT) activity in *bmr12* (Sattler et al., 2010b). Both these mutations result in reduced lignin contents and incorporation of "non-canonical" monolignols. Specifically, the CAD deficiency results in incorporation of the hydroxycinnamyl aldehyde monomers into the polymer (Kim et al., 2003) while COMT deficiency results in incorporation of 5-hydroxyconiferyl alcohol monomers into the lignin polymer (Ralph et al., 2001b), which can result in an increased frequency of benzodioxane linkages (Ralph et al., 2001a).

In addition to their use as forage for ruminants, there has been interest in utilizing *bmr* plants as feedstocks for biorefining processes due to the lower lignin content that facilitates deconstruction and conversion. As examples, our prior work found that relative to a commercial hybrid, the inbred *bm1* and *bm3* maize lines exhibited lower lignin content, improved delignification, and higher enzymatic hydrolysis yields when subjected to alkaline and alkaline-oxidative pretreatments (Li et al., 2012). Other work has found that *bmr* sorghums generally exhibit higher monomeric sugar yields when subjected to enzymatic hydrolysis by cellulases as well as enzymatic hydrolysis following pretreatments such as dilute acid, dilute NaOH, and dilute ammonia (Dien et al., 2009; Godin et al., 2016; Guragain et al., 2017; Saballos et al., 2008). The two classes *bmr* sorghums (*bmr6* and *bmr12*) selected for the present work were chosen since these feedstocks are considered to exhibit promising agronomic and processing phenotypes for applications in cellulosic biofuels (Yang et al., 2021; Silva and Vermerris, 2020) as well materials applications such as cellulose nanofibers (Pennells et al., 2021). These applications utilize the carbohydrate fraction of the biomass and require pretreatment or delignification. Since the *bmr* sorghums are more susceptible to delignification and have altered lignin properties a key component of the present work is to both characterize the impact of these differences in lignin on processing (delignification and enzymatic hydrolysis) and on the resulting properties of the recovered lignins.

In the present work, we investigate the differences in the composition and response to deconstruction of *bmr* sorghums (*bmr6* and *bmr12*) relative to the wild-type controls for two background lines (the commercial hybrid Atlas and isogenic BTx623) and relate differences in characterized properties to processing responses. Specifically, we first perform detailed characterization with respect to cell wall composition, water-extractable sugars, and *p*-coumarate and ferulate content. Next, the processing of these sorghum lines via dilute NaOH pretreatment and enzymatic hydrolysis was assessed and differences in hydrolysis yields, lignin solubilization, and molar mass of the recovered lignins were assessed. Finally, differences in "native" and alkali-extracted lignin properties were characterized by NMR and related to differences in processing behavior.

2. MATERIALS AND METHODS

2.1 Biomass

Sorghum (*Sorghum bicolor* (L.) Moench) exhibiting the *bmr6* and *bmr12* mutations as well as controls in the commercial hybrid Atlas (Pedersen et al., 2008) and the isogenic BTx623 (Pedersen et al., 2006) lines were employed in this work. Sorghum was grown at the Texas A&M University Farm (College Station, Texas) and, following harvest, were air-dried to ~5% moisture. Prior to analysis or processing, grain was separated from the rest of the biomass and the biomass was subjected to knife milling (Wiley Mini Cutting Mill, Thomas Scientific, Swedesboro, NJ, USA) using a 20-mesh (0.841 mm) screen.

2.2 Composition of Extractives, Cell Wall Biopolymers, and Hydroxycinnamates

The determination of water-extractable sugar content of the six sorghum lines was done according to our previous work with minor modifications (Li et al., 2018a). Initially, a known quantity of air-dried, Wiley-milled sorghum sample was mixed with deionized water at 10% solids (wt/vol) at room temperature in 50-mL centrifuge tubes and manually mixed for 1 minute followed by mixing by vortexing at 1100 rpm for 10 s (Vortex Mixer, Fisher Scientific, Waltham, MA, USA). Thereafter, the mixture was subjected to mixing by rocking (Intelli-Mixer™ RM-2L) at 30 °C for 1 h followed by storage at 4 °C for 48 h. The liquid fraction was separated, and water-extractable sugars were quantified using an Agilent HPLC 1260 series equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) using aqueous solution of 5 mM sulfuric acid solution as a mobile phase (0.6 mL/min). The water-extracted sorghum was thoroughly washed using DI water and dried at room temperature for 48 h for subsequent processing by pretreatment and enzymatic hydrolysis. Extractions and analysis were performed in triplicate for each sample.

Water-extracted and alkali-pretreated sorghum samples as well as biopolymers recovered from pretreatment liquors were next subjected to two-stage acid hydrolysis using NREL/TP 510-42618 (Sluiter et al., 2012) to determine the composition of structural polysaccharides and Klason lignin using ~100 mg dried sample. Ash content and extractive removal of water extractable sugars samples and recoverable biopolymers samples were done using NREL/ TP-510-42622 (Sluiter et al., 2008a) and NREL/TP-510-42619 (Sluiter et al., 2008b), respectively. The quantification of structural sugars was done using the same HPLC system that was used for water-extractable sugars analysis. The xylan contents presented are the sum of xylan, galactan,

and mannan as these sugars coelute with the method. Composition analysis was performed in triplicate for each sample.

For determination of *p*-coumaric acid (*p*CA) and ferulic acid (FA), triplicate samples of extractives-free sorghum samples (250 mg) were placed in Teflon-lined stainless steel synthesis reactors with 12.5 mL of 3 M NaOH. The reactors were immersed in an oil bath heated to 180 °C for 2 h and subjected to stirring at 280 rpm. After cooling, alkaline liquor was adjusted to below pH 2 using 150 µL of 96.7% H_2SO_4 and centrifuged to remove the precipitate. Hydroxycinnamic acids in the liquor were quantified by HPLC as reported in our prior work (Saulnier et al., 2020).

2.3 Alkaline Pretreatment

Alkaline pretreatment of the water-extracted sorghum samples was performed in a 100 mL stainless steel Amar reactor (Amar Equipments Ltd, Mumbai, India). Biomass was pretreated at a 100 mg NaOH/g biomass loading at a 10% solids (wt/vol) loading, at a temperature of either 90 or 120 ℃ (plus a 25 min heat-up time and 20 min cool-down time for 120 ℃), for 1 h with 500 rpm stirring. The stirring speed was maintained at 100 rpm during heating and cooling. After completion of reaction, solid and liquid fractions were separated by filtration. The solid fraction was thoroughly washed with DI water and stored at 4 °C prior to enzymatic hydrolysis. The quantification of "mass extracted" was calculated by using the difference between initial biomass and recovered biomass on a dry basis after alkali pre-extraction. The liquid fraction was acidified to pH ~2 using 72% sulfuric acid to precipitate biopolymers. Following the acidification, the solution was held at room temperature for five minutes, filtered (Whatman Grade 42 filter paper), and dried.

2.4 Enzymatic Hydrolysis

Enzymatic hydrolysis of alkali-pretreated and water extractives-free untreated sorghum samples was performed in triplicate using Cellic CTec2 (Novozymes A/S, Bagsværd, Denmark) cellulase cocktail. The protein content of the cellulase was determined to be 87 mg/mL with the Bradford method (Sigma-Aldrich) using BSA as a protein standard. Hydrolysis was performed at an enzyme loading of 25 mg protein/g of glucan (based on untreated or pretreated glucan content) at 8% solids (wt/vol) in 15 mL disposable centrifuge tubes in 50 mM Na-citrate buffer (pH 5.0) and 40 µg/mL tetracycline. Hydrolysis was performed at 50 ℃ for 72 h in an incubator

containing a mixer (Intelli-Mixer™ RM-2L). Following the hydrolysis, the mixture was centrifuged (4500 rpm for 15 min) and the liquid was collected and analyzed for glucose and xylose using the same HPLC system as used for water-extractable sugars analysis (Singh et al., 2019). The recovered unhydrolyzed sample was thoroughly washed with DI water, centrifuged and dried at room temperature for 72 h.

2.5 Isolation of enzymatic mild acidolysis lignin (EMAL)

Lignin was isolated from the Atlas sorghum lines to assess the properties of "native" lignin and for comparison with alkali-extracted and recovered lignins. Isolation of EMALs was performed following a previously described method with minor modifications (Guerra et al., 2006; Wu and Argyropoulos, 2003). Wiley-milled samples were subjected to two-stage removal of water-soluble sugars (*i.e.*, sucrose, glucose, fructose, and starch) and ethanol-soluble extractives using Soxhlet extraction according to NREL/TP-510-42619 (Sluiter et al., 2008b). Extractives-free biomass was subjected to ball-milling (Mixer mill MM400, Retsch GmbH, Haan, Germany) at 30 MHz for 24 cycles of 10 minutes of ball-milling and 5 minutes of cooling on ice. Ball-milled samples were subjected to enzymatic hydrolysis using Cellic CTec2 (Novozymes A/S, Bagsværd, Denmark) at an enzyme loading of 50 mg protein/g glucan at 8% solids (mass biomass/volume liquid phase). The hydrolysis was performed in 50 mL centrifuge tubes containing a 50 mM Na-citrate buffer (pH = 5.0) with 40 µg/mL tetracycline to prevent bacterial growth and subjected continuous mixing by rocking (Intelli-Mixer™ RM-2L, NJ, USA) in an incubator at 50 ℃ for 72. Following hydrolysis, the samples were centrifuged at 4500 rpm for 15 min and the liquid was decanted. The remaining solid was washed two times using 100 mL acidic water (pH ~2), and centrifuged at 4500 rpm for 15 min. The hydrolyzed recovered solids were lyophilized and subjected to mild acidolysis using a solution of acidic dioxane:water (85:15 vol/vol) mixture. Dried enzymatic lignin (~1.5 g) was suspended in 20 mL acidified dioxane:water mixture containing 10 mM HCl. The reaction mixture was refluxed in an oil bath for one hour followed by filtration with the filtrate further washed with 100 mL dioxane-water mixture followed by filtration and a final cycle of washing with fresh 25 mL solvent. The combined filtrate was neutralized using sodium bicarbonate and subjected to solvent removal using rotatory evaporation at 25 ℃ until the solution became highly viscous. This viscous solution was added to 300 mL acidic water (pH ~ 2) and the precipitated lignin was separated by

centrifugation (4500 rpm) for 10 min and the recovered lignin was washed with acidic water (pH ~ 2) two more times and subjected to lyophilization.

2.6 Lignin Characterization

The weight average molecular weight $\overline{M}_W$, number average molecular weight $\overline{M}_N$ and polydispersity index ($\overline{M}_W/\overline{M}_N$) of recoverable biopolymers derived from water-extracted sorghum samples were characterized using a gel-permeation chromatography (GPC). GPC analysis was performed using an Agilent HPLC 1260 series, attached with a Waters Styragel HR 4 (7.8 × 300 mm, Milford, MA, USA) column (Part No. WAT044225). THF (HPLC grade) was used as a mobile phase with a flow rate of 0.5 mL/ min at 40 °C. UV detector was set at 280 nm. A variety different molecular weights of Readycal polystyrene standards (Fluka Analytical) in the range of 250−70,000 Da was applied to calibrate the column. Prior to GPC analysis, recovered biopolymers sample was acetylated by using our previous report with slight modifications (Singh et al., 2019). Approximately 15 mg recoverable biopolymers sample was weighed, and 3.75 mL anhydrous pyridine was added. This mixture was stirred for 20 min at room temperature and following, 0.38 mL acetic anhydride was added. The resulting mixture was stirred at room temperature for 20 h. Thereafter, the unreacted pyridine and acetic anhydride solvents was evaporated using a rotatory evaporator. The rotatory dried mixture was subjected for vacuum dried to remove the remaining solvents at room temperature for 10 min. In acetylated dried sample, 2 mL THF was added and sonicated to dissolve at room temperature for 10 min. The sonicated reaction mixture was filtered using a 0.2 µm syringe filter (PTFE membrane) two times. The diluted THF soluble acetylated biopolymers sample was subjected for GPC analysis.

The 2D $^{1}H-^{13}C$ correlation heteronuclear single quantum coherence (2D HSQC) NMR spectra of recoverable biopolymers were recorded at a Bruker NMR 600 MHz instrument using 150.90 and 600.13 MHz respectively. Approximately 100 mg sample and 2 mg chromium (III) acetylacetonate were weighed and added in a 1.5 mL HPLC vial. Dimethyl sulfoxide-d_6 (DMSO-d_6 700 µL) was added to vial. The solution was sonicated for 10 min at room temperature to solubilize the biopolymers and chromium (III) acetylacetonate in DMSO-d_6. Soluble fractions were added to an NMR tube for acquisition of 2D HSQC spectra using the current pulse program hsqcetgpsisp2.2. Semiquantitative relative abundance of the tricin (T), 5-hydroxy guaiacyl (hG), *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties were taken from the aromatic

region of the spectra and β-O-4, β-β, β-5, and benzodioxane (α-O-5, β-O-4) linkages were determined using a Bruker Topspin 4.0.6 software with peaks assigned based on the literature (Ralph et al., 2004b; Singh et al., 2019). 2D ^{1}H-^{13}C HSQC spectra of recoverable biopolymers are shown in **Supplemental Figure S1** and **S2**, respectively, and the approximate relative abundance of each monomer or interunit linkage was quantitated as the sum of identified and annotated peak areas per C-H bond.

3. RESULTS AND DISCUSSION

3.1 Characterization of Cell Wall Composition and Extractives in Untreated Sorghum

For the first part of this work, characterization of untreated control and *bmr* sorghum lines were performed to determine how differences in the content of cell wall structural biopolymers, extractable sugars, and esterified *p*-coumarate (*p*CA) and ferulate may impact biomass processability and the biofuel yield potential. Key results are presented in **Figure 1** and show significant differences in extractable sugars, Klason lignin, and hydroxycinnamates (*p*CA and ferulate) across the six sorghum lines. Interestingly, these three properties (total extractable sugars, Klason lignin, and hydroxycinnamates) show identical trends for the control, *bmr6*, and *bmr12* lines for the commercial hybrid lines (Atlas) and the isogenic line (BTx623). Specifically, when these properties are compared between background lines, strong significant correlations can be observed (**Supplemental Fig. S3**) for total sugars ($R^2 = 0.997$), Klason lignin ($R^2 = 0.997$), *p*CA content ($R^2 = 0.9997$), and ferulate content ($R^2 = 0.991$) providing compelling evidence that these three properties are strong phenotypic consequences of the *bmr6* and *bmr12* mutations.

For the first property, the water-extractable sugars glucose, fructose, and sucrose are significantly lower in the *bmr* sorghum lines than in the wild-type (**Fig. 1A**) based on a comparison with the control and range from 231 mg in *bmr6* line to 340 mg extractable sugar per g cell wall in the control line for the Atlas sorghum and 68 mg (*bmr6*) to 123 mg/g (control) for the BTx623 sorghum. The total sugar content in the Atlas lines are at levels comparable to our previous findings for sorghum hybrid TX08001 and sweet sorghum cultivar Della stems (Li et al., 2018a). However, these results are contradictory to other prior work that found a higher content of water-extractable sugars in Atlas *bmr6* and *bmr12* relative to a control (Guragain et al., 2017). Atlas is a sweet sorghum hybrid that is well-known as a silage, forage, and fodder

sorghum exhibiting high stem sugar accumulation (Wilsie et al., 1941). During storage, drying, and water extraction, the sucrose may be hydrolyzed to glucose and fructose and may be responsible for the higher content of monosaccharides relative to sucrose. Starch was not quantified but may also represent a non-trivial contribution to the total content of water-extractable sugars (McKinley et al., 2018). Together water-extractable sugars represent a readily available source of renewable carbon and can be converted directly through bioprocessing to products such ethanol. As examples of extraction strategies, water-extractable sugars derived from sorghum could be employed with first generation hybrid technologies such as diffuser or tandem milling technologies to produce cellulosic bioethanol (Eggleston et al., 2013; Masarin et al., 2013). While the increased digestibility of brown midrib sorghum is a positive for forage and biorefining applications, the 40-45% lower content of extractable sugars coupled with the known lower biomass yields of brown midrib sorghum (Sattler et al., 2010a), represents a challenge.

Differences in the total Klason lignin content on an extractives-free basis are presented in **Figure 1B**, with complete compositional profiles of both untreated and pretreated biomass presented in **Supplemental Figure S4**. The results show slightly lower lignin contents in the *bmr* mutants in both background lines although only the *bmr12* in the BTx623 line is statistically lower than the control ($p \leq 0.05$). Lower lignin content in Atlas *bmr* sorghums has been shown in prior studies (Dien et al., 2009; Godin et al., 2016; Oliver et al., 2005). Differences in the lignin content in *bmr* mutants as well as contributions from differences in the structure and properties of these lignins have been well-established and are known to contribute to their reduced recalcitrance to digestion by ruminants (Barrière et al., 2004) and processing for cellulosic biofuels (Li et al., 2012).

Esterified hydroxycinnamate (*p*CA and ferulate) contents are significantly different in all three *bmr* mutants tested and exhibit similar trends across both background lines (**Fig. 1C**). Grass lignins are known to contain *p*CA esterified to primarily syringyl monomers at the γ position of the aliphatic region of lignin (Ralph, 2010) as well as to arabinoxylans and its content is known to be a complex function of tissue type, development stage, and genotype (Crowe et al., 2017). Our prior work has shown that *p*CA content in untreated diverse maize lines can be inversely correlated to enzymatic hydrolysis yields of glucose from cellulose (Li et al., 2015). In the present work, all the *bmr* lines exhibited substantially lower (37-67%) *p*CA contents than the control lines (**Fig. 1C**). Prior literature has shown lower *p*CA contents in barley (Daly et al.,

2019), maize (Piquemal et al., 2002), and sugarcane (Jung et al., 2013) with impaired expression of COMT (corresponding to *bmr*12 in sorghum). This same trend of lower *p*CA content in *bmr*6 relative to the *bmr12* has also been shown in two other genetic backgrounds for sorghum (Palmer et al., 2008). In grasses such as sorghum, ferulates are involved in ester and ether crosslinks within lignins and arabinoxylans (de O. Buanafina, 2009) and can play a significant role in the cell wall recalcitrance of these plants. As an example, prior work has demonstrated negative correlations between ferulate content in rye grass and *in vitro* dry matter digestibility (Iiyama, 2001). Conversely, positive correlations between ferulate content and enzymatic hydrolysis yields have been observed in diverse maize lines following alkaline pretreatment (Li et al., 2015), which target the hydrolysis of ferulate cross-links and results in lignin and xylan solubilization. In the present work, relative to the control the alkali-saponifiable ferulate is slightly lower in the *bmr*6 lines and slightly higher in the *bmr*12 lines (**Fig. 1C**). Interestingly, these same trends of lower ferulate content in *bmr* lines were identified for these mutations in two other background sorghum lines ("Wheatland" and RTx430) (Palmer et al., 2008).

3.2 Biomass Response to Pretreatment and Enzymatic Hydrolysis

In the next part of this work, the biomass was subjected to alkaline pretreatments to assess how the pretreatment impacted the cell wall composition and biomass recalcitrance to enzymatic deconstruction and, ultimately, how differences in the lignin properties could be linked to these outcomes. First, the biomass was subjected to mild alkaline pretreatment at 90 °C (Atlas background lines only) and 120 °C (both backgrounds) using 100 mg NaOH/g biomass alkali loadings for 1 hour. Increasing alkali loading, temperature, and time will increase delignification and enzymatic hydrolysis yields up to a certain point (Singh et al., 2019) although there are additional economic costs associated with this. However, the goal of the pretreatment was to screen the various biomass feedstocks under consistent conditions to observe the response. Thus, the pretreatment conditions were selected as a screening for cell wall recalcitrance as in our prior work (Crowe et al., 2017; Li et al., 2015) rather than as an optimal pretreatment with respect to criteria such as hydrolysis yields or process economics. Furthermore, the 90 °C condition was used for the Atlas sorghum lines as an additional lower severity pretreatment screening condition to better observe differences in cell wall recalcitrance since the 120 °C pretreatments resulted in glucose hydrolysis approaching the theoretical maximum for all sorghum lines.

Solubilization and removal of lignin is one of the primary outcomes of alkaline pretreatments resulting in the improvement of cell wall polysaccharide accessibility to cellulolytic enzymes. The extent of lignin removal was determined based on composition analysis and mass yields following pretreatment. The extent of lignin solubilization can yield insights into how differences in the lignins' structure impact the plant cell wall's response to pretreatment and how the pretreated/delignified biomass responds to enzymatic hydrolysis. The results show that in the Atlas background, all the *bmr* lines resulted in higher lignin removal for both pretreatment levels relative to the control (**Fig. 2A**). This indicates that in addition to having less lignin, the lignins in the *bmr* lines may also be more susceptible to removal/solubilization under the conditions used in this work. However, the results for lignin removal for the *bmr* mutations in the BTx623 background did not show the same trends (**Fig. 2A**). A similar result was observed, where minimal differences in lignin solubilization were observed in *bmr* maize lines subjected to alkaline pretreatments relative to the control lines (Li et al., 2012). Additionally, increasing pretreatment temperature from 90 °C to 120 °C significantly increased the amount of lignin solubilized in the Atlas sorghum lines (**Fig. 2A**). This result is the expected outcome as it is well-established that the rate of delignification increases with increasing temperature during alkaline pulping or pretreatment according to the Arrhenius equation (Bajpai, 2018).

Following pretreatment, the solubilized lignin and xylan in the pretreatment liquor were recovered by precipitation following acidification. The xylan content of the recovered lignins is presented in **Supplemental Figure S5**, while the molar masses of these lignins are presented in **Fig. 2B**. The pretreatment-solubilized lignins show the general trend of lower molar masses in the *bmr* lines relative to the control in both backgrounds (**Fig. 2B**). One interpretation of these results is that the lignins from the *bmr* lines underwent more depolymerization, resulting in more solubilization (**Fig. 2A**) and lower molar masses (**Fig. 2B**).

The enzymatic hydrolysis yields for untreated and pretreated sorghum were next assessed. The results show that the *bmr* sorghums exhibited statistically higher glucose enzymatic hydrolysis yields relative to the control (**Fig. 3**), while xylose hydrolysis remain low as the enzyme cocktail was not optimized for xylose hydrolysis (**Supplemental Fig. S6**). The property of improved ruminant digestibility, *in vitro* digestibility or cellulose hydrolysis yields is well-established for brown midrib mutants of maize, sorghum, and pearl millet (Porter et al., 1978; Sattler et al., 2010a). For *bmr* lines subjected to pretreatment, prior work demonstrated higher

glucose hydrolysis yields in alkali-pretreated *bmr*6 and *bmr*12 mutants in Atlas, Kansas Collier, and Early Hegari backgrounds (Guragain et al., 2017) relative to controls, *bmr*6, *bmr*12, and the stacked *bmr*6,12 mutants in "Wheatland" sorghum subjected to dilute acid pretreatment (Godin et al., 2016), and for these same mutations in Atlas sorghum subjected to dilute aqueous ammonia pretreatment (Dien et al., 2009). For the pretreated biomass in the present work, only Atlas *bmr*12 at 90 °C and the BTx623 *bmr*6 pretreated at 120 °C exhibited significantly higher enzymatic hydrolysis yields than the control lines (**Fig. 3**) under the same treatment conditions (i.e., untreated, 90 °C, or 120 °C). Potential reasons that more significant differences were not observed could be that the pretreatments were already harsh enough to achieve >80% glucose hydrolysis yields and that small differences in cell wall recalcitrance were not observable.

3.3 Properties in Native- and Pretreatment-Solubilized Lignins

As outlined in the **Materials and Methods**, "native" lignins were isolated following the procedure of Guerra et al. (2006) to generate a relatively unaltered lignin for the purpose of identifying pretreatment-induced changes to the lignin. Specifically, the properties of "native" and alkali-solubilized lignins were next characterized for the control, *bmr*6, and *bmr*12 lines in the Atlas background in order to assess how differences in lignin properties could be correlated to differences in their response to pretreatment and how pretreatment impacted the lignin properties. Specifically, ^{1}H-^{13}C HSQC NMR is used to assess the relative abundance of monomers types (**Fig. 4A**) and the relative abundance of linkages between subunits (**Fig. 4B**) for both the "native" lignins and the pretreatment-solubilized (120 °C alkaline pretreatment) lignins. Relative to the other lines, it is expected that the COMT deficiency in the *bmr12* sorghum lines will have both lower syringyl (S) monomer contents and an increased abundance of benzodioxane linkages due to the incorporation of 5-hydroxyconiferyl alcohol monomers into the lignin polymer (Ralph et al., 2001a) to yield 5-hydroxy guaiacyl (hG) monomers. The results for both the syringyl content (**Fig. 4A**) and the benzodioxane linkages (**Fig. 4B**) for the *bmr12* line follow these expected trends. The flavone tricin can participate in radical coupling reactions with monolignols and has been proposed to act as nucleation sites for lignification (Lan et al., 2015). Prior work has shown decreased incorporation of tricin into lignin in *bmr12* sorghum relative to a wild-type control as COMT is also thought to methylate tricin precursors (Eudes et al., 2017). In the present work, this finding is confirmed with the relative abundance of tricin

substantially lower in lignins recovered from the *bmr12* relative to the control (**Fig. 4A**). A final observation is that the alkali-extracted and recovered lignins exhibit similar properties (relative abundance of monomers and linkages) to the "native" lignins, indicating that the alkali-solubilized lignin is not substantially modified.

As previously discussed, biorefining of sorghum as a feedstock represents opportunities for utilization of extractable sugars and cell wall polysaccharides for applications including fuels, chemicals, and materials. In addition to the carbohydrate fraction in these feedstocks, the lignins recovered from the liquors of alkaline-pretreated grasses and specifically sorghums and *bmr* sorghums may have a number of potential promising applications for value-added co-products. As examples, *p*CA content in lignins recovered from grasses may be a useful property in phenol-formaldehyde resins (Saulnier et al., 2020), while two adjacent aromatic hydroxyl functional groups resulting from 5-hydroxy guaiacyl monomers at the end of a chain may show promise as sites for glycidylation and incorporation into a wide range of polymeric resins (Zhao and Abu-Omar, 2017). Additionally, a wide range of possibilities exist for utilization in polyurethanes (Alinejad et al., 2019).

3.4 Correlation of Properties

Enzymatic hydrolysis yields were next correlated with lignin properties to highlight potential causal relationships between lignin properties and processing outcomes (**Fig. 5**). The first key finding is that the total lignin content both before and after pretreatment exhibit strong correlations to the glucose hydrolysis yields (**Fig. 5A**). Notably, this trend is strong in the untreated and pretreated results for the control and the two mutations (*bmr6* and *bmr12*) for each of the two background lines (Atlas and BTx623). This relationship between total lignin content and enzymatic hydrolysis yields in both untreated and pretreated lignocellulosic biomass is well-established in a number of studies for both untreated (Jung et al., 1997) and pretreated (Li et al., 2015) grasses. Another observation is that the initial *p*CA content of the biomass is correlated to the enzymatic hydrolysis yields following pretreatment (**Fig. 4B**). This correlation has been observed in our prior work with untreated maize (Li et al., 2015), although in the present work it's not possible to deconvolute the impacts of total lignin content and *p*CA content as the more highly digestible *bmr* lines exhibiting substantially lower *p*CA contents (**Fig. 1C**) also exhibit low lignin contents (**Fig. 1B**). Finally, a negative correlation can be observed that the solubilized

lignin weight average molar mass ($\overline{M}_W$) and glucose hydrolysis yields. As discussed previously, it can be hypothesized that the lignin present in the *bmr* lines was more susceptible to both depolymerization and solubilization resulting in more digestible cell walls following pretreatment.

4. CONCLUSIONS

Lignin abundance and structure both provide significant contributions to the recalcitrance of plant cells to deconstruction and understanding the relationships between lignin abundance, structure, and cell wall recalcitrance is critical to developing plants with phenotypes optimized for a conversion process. In this work, these relationships were explored in *bmr* sorghums corresponding to deficiencies in the lignin synthesis pathway enzymes CAD (*bmr6*) and COMT (*bmr12*) that result in lower lignin contents and increased incorporation of "non-canonical" monomers. Results from this work demonstrated significant differences in the lignin properties, extractability of the lignins during alkaline pretreatment, and the response to enzymatic hydrolysis in both untreated and pretreated sorghum. Specifically, we identified lower lignin contents, lower water-extractable sugars, lower saponifiable *p*CA, and generally higher enzymatic hydrolysis yields in all the *bmr* lines. Furthermore, in the Atlas lines we showed that, relative to the control, the *bmr6* sorghum exhibited higher β-O-4 contents, while the *bmr12* sorghum contained 5-hydroxy guaiacyl monomers and a significant fraction of the benzodioxane di-ethers. Both these differences contribute to higher ether-linked monomers and were hypothesized to contribute to both improved lignin removal during alkaline pretreatment and higher enzymatic hydrolysis yields following pretreatment.

5. ABBREVIATIONS

bmr: brown midrib; CAD: cinnamyl alcohol dehydrogenase; COMT: caffeate *O*-methyltransferase; FA: ferulate; HSQC: heteronuclear single quantum coherence; *p*CA: *p*-coumarate.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version.

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FIGURES

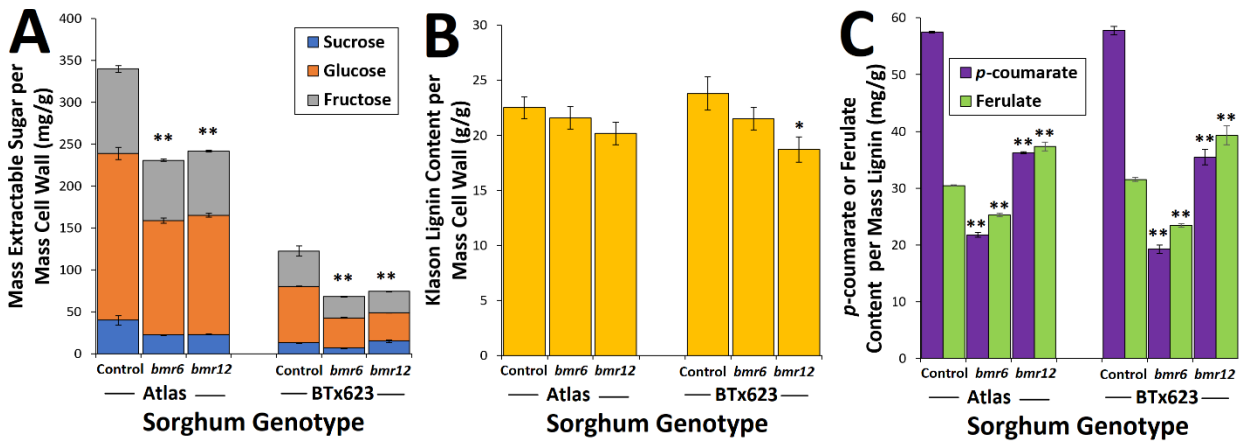


Figure 1. Summary of differences in **(A)** extractable sugars, **(B)** lignin content, and (**C**) *p*-coumarate content. * $p \leq 0.05$; ** $p \leq 0.01$ difference from the control line based on a Student's t-test.

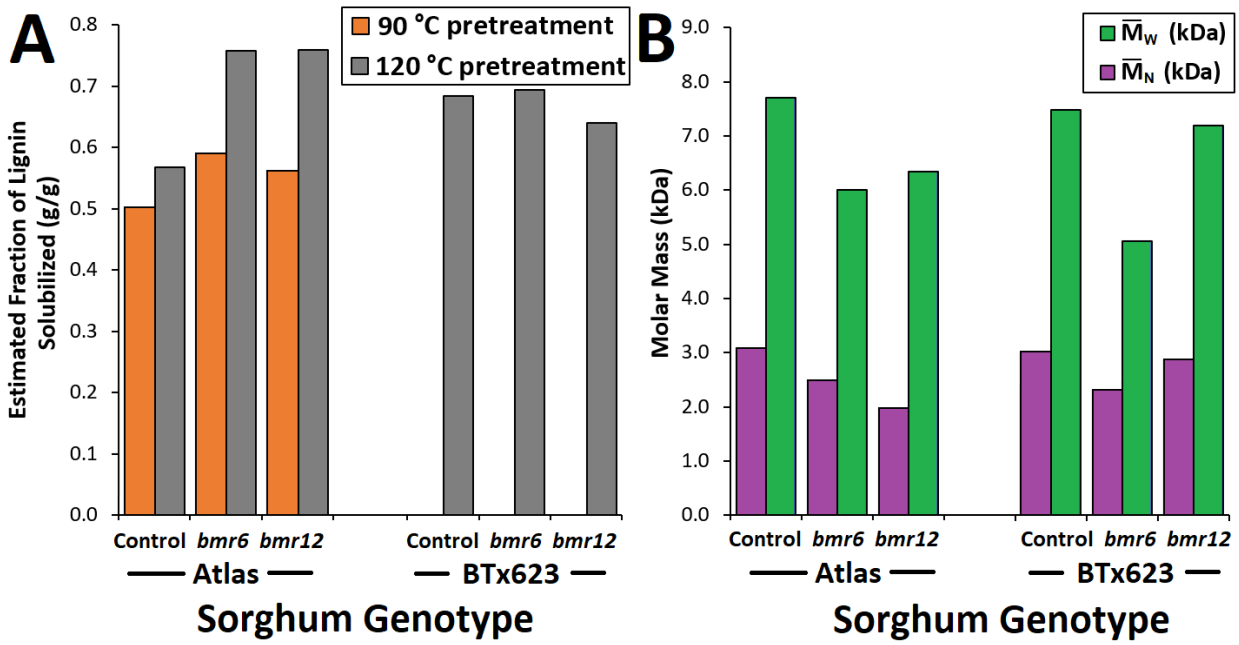
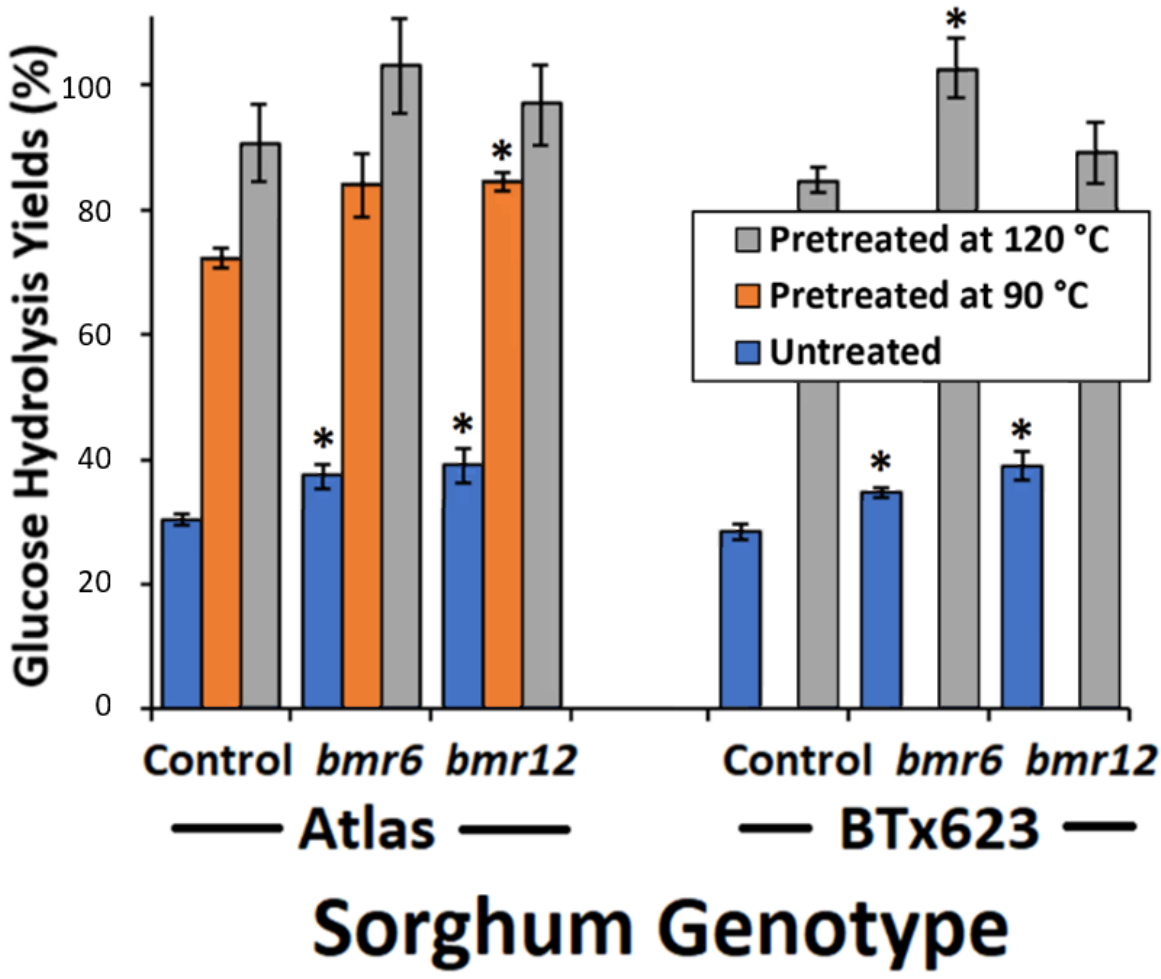


Figure 2. Impact of pretreatment on **(A)** lignin extraction and **(B)** molar mass of the pretreatment-solubilized lignins from 120 °C alkaline pretreatment.



662

663 **Figure 3**. Impact of pretreatment on enzymatic hydrolysis yields. * $p \leq 0.05$ difference in the

664 control line based on a Student's t-test..

665

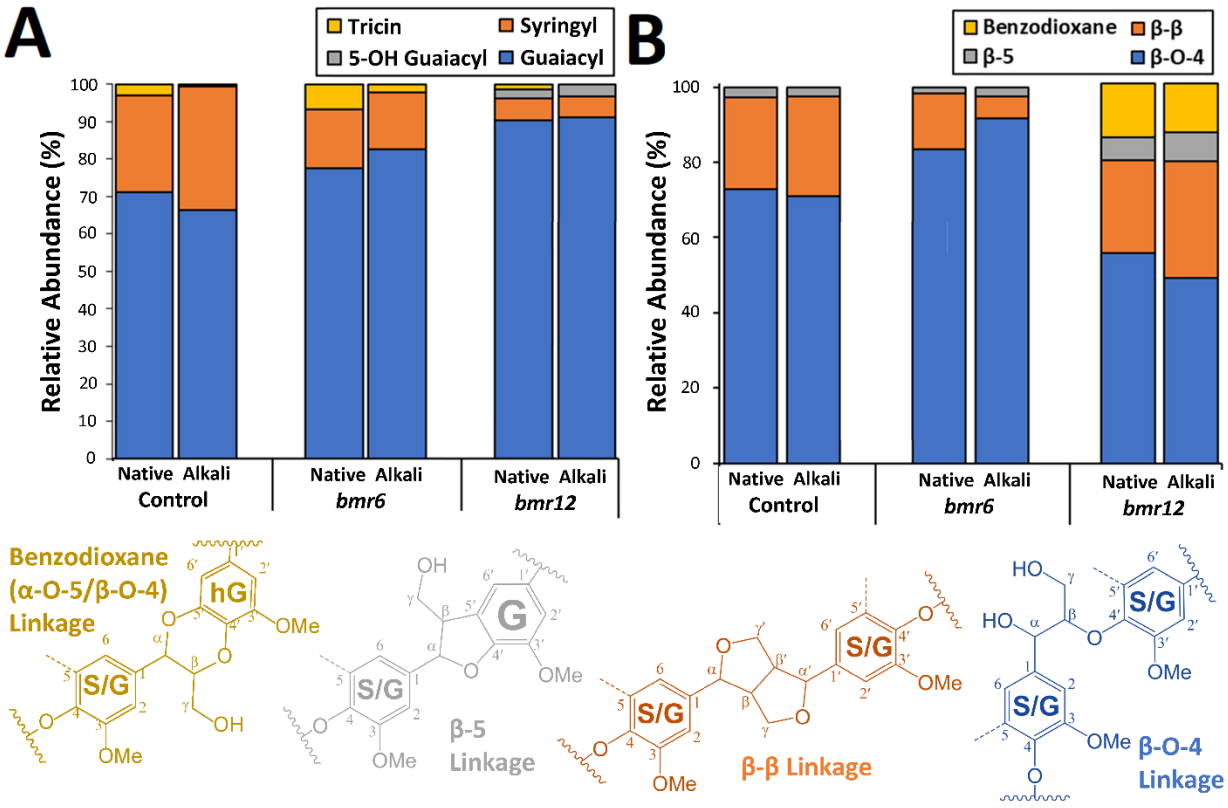
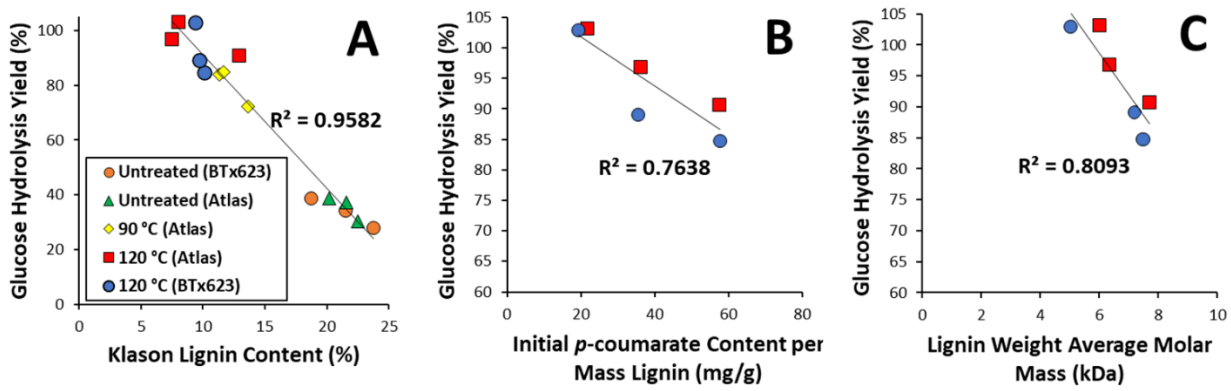


Figure 4. Lignin properties of untreated and treated Atlas sorghum from HSQC NMR characterization showing **(A)** relative abundance of monomer subunits and **(B)** relative abundance of interunit linkages.



672

673 **Figure 5**. Correlations between glucose enzymatic hydrolysis yields and (**A**) lignin content, (**B**)
674 initial *p*-coumarate content, and (**C**) weight average molar mass of the alkali-extracted and
675 recovered lignins from pretreatment at 120 °C.