2011

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Bioresource Technology 102 (2011) 9579–9585

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Ethanol yields and cell wall properties in divergently bred switchgrass genotypes

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ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 19 July 2011
Accepted 22 July 2011
Available online 29 July 2011

Keywords:
Switchgrass
Divergent breeding
Cell wall
Ethanol
Structure

ABSTRACT

Genetic modification of herbaceous plant cell walls to increase biofuels yields is a primary bioenergy research goal. Using two switchgrass populations developed by divergent breeding for ruminant digestibility, the contributions of several wall-related factors to ethanol yields was evaluated. Field grown low lignin plants significantly out yielded high lignin plants for conversion to ethanol by 39.1% and extraction of xylans by 12%. However, across all plants analyzed, greater than 50% of the variation in ethanol yields was attributable to changes in tissue and cell wall architecture, and responses of stem biomass to dilute-acid pretreatment. Although lignin levels were lower in the most efficiently converted genotypes, no apparent correlation were seen in the lignin monomer G/S ratios. Plants with higher ethanol yields were associated with an apparent decrease in the lignification of the cortical sclerenchyma, and a marked decrease in the granularity of the cell walls following dilute-acid pretreatment.

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1. Introduction

Switchgrass (*Panicum virgatum* L.) is a promising potential lignocellulosic biofuel crop because it can be cultivated on marginal lands in a sustainable manner, and has a wide geographical range (Schmer et al., 2008). To meet the expected biomass requirements for future biorefineries, continued improvement is needed in biomass yields and cell wall properties that will permit more efficient extraction of sugars during biochemical conversion processes such as simultaneous saccharification and fermentation (SSF) (Carroll and Somerville, 2009; McCann and Carpita, 2008; Sarath et al., 2008b). Grasses contain Type II cell walls, which are comprised of cellulose, hemicellulose, lignin and considerable levels of two phenolic (hydroxycinnamic) acids: ferulic (FA) and p-coumaric acid (pCA) (Vogel, 2008). FA content and cross-linking has been shown to negatively affect biomass digestibility in ruminants (Buanafina et al., 2008). Similarly, these acids also might impede enzymatic release of sugars from cell wall polymers for its bioconversion to ethanol (Dien et al., 2006; Vogel, 2008). Lignin is a complex amorphous polymer consisting largely of H-, G-, and S-lignin units, and other aromatic compounds including aldehydes (Boerjan et al., 2003). Lignin deposition occurs during secondary cell wall biogenesis and is especially abundant in sclerenchyma and xylem. Lignin is known to be an antinutritive in forages and a bottleneck for biomass conversion into biofuels (Carroll and Somerville, 2009; Dien et al., 2006; Falls et al., 2011; Grabber et al., 2009; Sarath et al., 2008b).

Studies using brown midrib (bmr) mutants in sorghum and maize with decreased lignin in cell walls demonstrated linear correlations between reduced lignin content and increased sugar and ethanol yields (Dien et al., 2009). In switchgrass, divergent breeding generations for decreased or increased in vitro dry matter digestibility (IVDMD) (Hopkins et al., 1993) resulted in populations with reciprocal direct increase (NE T-1) or decrease in the lignin concentration (NE T3), biomass yields (Casler et al., 2002), as well as significant decreases of stem, sheath, and leaf acid detergent lignin (ADL) concentrations of the NE T3 as compared to the NE T-1 populations (Vogel et al., 2005). Selected plants from these two populations also displayed altered cell wall composition and accessibility to hydrolytic enzymes (Sarath et al., 2008a). These earlier results indicated that lignin content, gross cell wall composition and IVDMD has been altered at the total biomass level, although other details of specific cell wall changes and effects on ethanol yields were not evaluated. Overall, these data indicated that significant genetic modifications had occurred within the NE T-1 and NE T3 switchgrass populations as a consequence of divergent breeding for IVDMD.

Here plants from the NE T-1 (low digestibility–high lignin) and NE T3 (high digestibility–low lignin) populations were evaluated to understand changes in cell wall architecture, composition, and plant anatomy that accompanied differences in ethanol yield via SSF from these plants.

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0960-8524/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.biortech.2011.07.086

2. Methods

2.1. Plant materials

Field-grown switchgrass plants or genotypes from the NE T3 and NE T-1 populations known from previous research to differ for stem and leaf ADL, cell wall concentration, and cell wall digestibility (Sarath et al., 2008a) were selected, clonally subdivided into four clonal pieces or ramets and transplanted into a replicated ($r = 4$) nursery on 1.1 m centers in the spring of 2004. A total of 110 genotypes were transplanted. Six genotypes were selected from each population for intensive analysis, three with low IVDMD and high stem lignin concentration (HL) and three with high IVDMD and low stem lignin concentration (LL) (Table 1). The nursery was managed using best management practices to optimize plant growth and development (Vogel et al., 2002). Tissue from the replicated genotypes was harvested after the plants were well established in July of 2007 at the boot-stage. The harvested material was oven dried at 50 °C, and hand separated to obtain stems free from leaves and sheaths. Only stems were ground and subjected to further analyses. This approach was used to be able to determine how selection based on stem lignin content influenced tissue and conversion properties of this component of plant biomass. Interference from changes in leaf and sheath to stem ratios that could have occurred during the breeding process and could affect conversion in a manner unrelated to lignin content and cell wall architecture was also avoided.

2.2. Composition analysis

Fiber analysis was performed on ground stem materials as described earlier (Sarath et al., 2007).

Cell wall phenolic esters and relative lignin monomer levels following thioacidolysis was analyzed and calculated as described by Palmer et al. (2008).

Total xylans (xylose + arabinose) were determined by HPLC after digesting biomass samples with 2 N trifluroacetic acid (Dien et al., 2006, 2009).

2.3. SSF and ethanol yield analyses

SSF of switchgrass samples was performed as follows: oven-dried switchgrass samples (1.5 g) were placed in 25 mL screw capped glass Pyrex bottles and 8.5 mL of 1.75% (w/v) sulfuric acid was added to each sample. Bottles were placed in a glass Pyrex™ dish containing water to a depth of approximately 1.25 cm (to ensure uniform heating) and autoclaved for 1 h. Bottles were cooled to room temperature before uncapping. Acid was neutralized by adding 1.2 mL sterile 10% $Ca(OH)_2$ followed by 0.55 mL 1 M sodium citrate buffer (pH 4.5) and 1.1 mL of a peptone/yeast extract mix (200 g L^{-1} peptone and 100 g L^{-1} yeast extract). The enzymes cellulase GC200 (Genencor, Palo Alto, CA) 5 FPU/g biomass and 12 U/g biomass cellubiase 188 (Novozyme, Davis, CA) were added to affect cellulose hydrolysis. The hydrolysates were inoculated with *Saccharomyces cerevisiae* D5A. All samples were inoculated with cells that resulted in an optical density at 600 nm (O.D. 600) of 1.0. The inoculum was prepared by concentrating an over-night culture of *S. cerevisiae* in a phosphate saline solution (8.5 g NaCl, 3 g anhydrous KH_2PO_4 and 0.6 g anhydrous Na_2HPO_4 L^{-1}). Bottles were fitted with septa lined caps vented with 22 g needles for CO_2 gassing. Cultures were incubated at 35 °C and mixed at 150 rpm. Sampling for ethanol and released sugars was performed after 72 h (Dien et al., 2006). Initial tests performed with both washed and unwashed solids following dilute acid pretreatment did not reveal any differences in final ethanol yields. All subsequent analyses were done with unwashed solids.

2.4. Microscopy

For microscopic analyses, small segments from the second internode below the peduncle were excised and fixed in a solution containing ethanol: acetic acid (75:25% v/v) for 24 h at ~6 °C, and washed and stored in 75% ethanol:25% water (v/v) at ~6 °C until analyzed. Fixed stem segments were dehydrated in an ethanol series, embedded in paraffin and sectioned at the University of Nebraska-Lincoln, Veterinary Diagnostic Center. Prior to staining, sections were deparaffinized in xylene and passed through a graded ethanol–water series and stained with the FASGA protocol as described by (Mechin et al., 2005). Stained sections were mounted in 10% glycerol and observed by light microscopy using a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) attached to a digital camera (Diagnostics Instruments Inc., Sterling Heights, MI). Digital images were taken at the same exposure and light settings for all sections imaged using digitizing software (SPOT, Diagnostics Instruments Inc., Sterling Heights, MI). Duplicate slides containing sections of stems from every plant were processed at the same time using the same batch of stain to minimize artifacts associated with changes in concentration of any chemicals used during this process.

For scanning electron microscopy (SEM), ground samples of stems before and after dilute-acid pretreatment were used. Samples were mounted onto SEM holders, sputter coated with chromium and viewed on a Hitachi S4700 field emission scanning electron microscope set at 5 kV.

2.5. Statistical analyses

The following linear model was used in the statistical analyses to test for statistical differences among populations and genotypes within populations:

$$Y_{ijkm} = \mu_{...} + \alpha_i + \beta_{j(i)} + \gamma_k + (\alpha\gamma)_{ik} + (\gamma\beta)_{kj(i)} + \varepsilon_{ijkm} \quad (1)$$

where Y_{ijkm} = response variable; α_i = population (1–4, basic lignin grouping); $\beta_{j(i)}$ = genotype (nested within the lignin grouping); γ_k = field replicate (field replicate with laboratory replicates subsumed; 4 total field reps per genotype with two lab (technical) reps per field rep); $(\alpha\gamma)_{ik}$ and $(\gamma\beta)_{kj(i)}$ = interaction effects; ε_{ijkm} = error term.

Table 1
NE Trailblazer switchgrass populations developed by multiple generations of divergent selection for reduced (low) or increased (high in vitro dry matter digestibility (IVDMD) that resulted in concomitant changes in biomass lignin concentrations.

Population	Previous designation and breeding history	Abbreviation
NE Trailblazer C0	EY × FF synthesized base population	
NE Trailblazer C-1	EY × FF low IVDMD C-1, 1 cycle of breeding for reduced IVDMD from C0	T-1
NE Trailblazer C-1 low lignin	Genotypes in NE Trailblazer C-1 population with reduced (low) stem lignin concentration.	LL T-1
NE Trailblazer C-1 high lignin	Genotypes in NE Trailblazer C-1 population with increased (high) stem lignin concentration.	HL T-1
NE Trailblazer C3	EY × FF C3, strain developed by 3 cycles of breeding for increased IVDMD from C0	T3
NE Trailblazer C3 low lignin	Genotypes in NE Trailblazer C3 population with reduced (low) stem lignin concentration	LL T3
NE Trailblazer C3 high lignin	Genotypes in NE Trailblazer C3 population with increased(high) stem lignin concentration	HL T3

The above model [Eq. (1)] represents a three factor, partially nested design. The experimental design was balanced and the model was analyzed as a fixed-effects model. All statistical tests were conducted with a Type I error rate of $\alpha = 0.05$ using PROC GLM in SAS.

3. Results and discussion

3.1. Stem digestibility and ethanol yields

The predicted relationships between the different populations and the potential space occupied by the different subpopulations used in this study are shown in Fig. 1a. Previously, it was shown that the population means for IVDMD in T-1 and T3 populations were significantly different (Vogel et al., 2005), and the genotypes utilized in current study have been shown to possess a strong negative relationship between lignin content and DMD (Sarath et al., 2008a). Thus, plants in the HL T-1 and LL T3 subpopulations represent the high and low lignin extremes of their respective populations and can be predicted to possess different cell wall composition, and potentially ethanol yields. Whereas, the LL T-1 and HL T3 genotypes represent the opposite extremes of their respective populations and, therefore can be expected to have over-lapping lignin content and similar ethanol yields.

The impact of 4 generations of divergent, recurrent breeding for increased IVDMD resulted in a significant ($p \leqslant 0.0001$) 39.1% increase in net mean ethanol yields from stems for the plants in the low lignin subpopulation LL T3 as compared to the plants in the highest lignin subpopulations (64.46 mg ethanol g^{-1} dry matter for LL T3 vs 46.45 mg ethanol g^{-1} dry matter in HL T-1; Fig. 1b). Significant differences ($p \leqslant 0.05$) in ethanol yields were observed between the low and high lignin groups in each population and between the ethanol yields of plants in HL T3 as compared to the plants comprising HL T-1. As observed for ethanol yields (Fig. 1b), plants in the LL T3 subpopulation (Fig. 1c) exhibited significantly higher extraction of xylans upon dilute acid pretreatment (187.0 ± 8.1 mg sugars g^{-1} dry matter) as compared to the plants in the other subpopulations. Again, plants in the HL T-1 subpopulation had the lowest amount of pentoses released (167.1 ± 4.2 mg sugars g^{-1} dry matter), whereas plants in the LL T-1 and HL T3 subpopulations had intermediate levels of sugar released during pretreatment (174.9 ± 2.1 and 173.0 ± 3.5 mg sugars g^{-1} dry matter, respectively). On an average, plants in the low lignin LL T3 subpopulation had approximately 12% greater sugar release from hemicellulose as compared to the plants in the HL T-1 subpopulation (Fig. 1c). These data were consistent with studies in sorghum and more recently with transgenic switchgrass that have shown this inverse relationship between lignin content, saccharification and ethanol yields (Dien et al., 2006, 2009; Fu et al., 2010, 2011; Saathoff et al., 2011). Data shown here likely reflect the variation to be found in switchgrass germplasm present within conventional breeding programs. As an example, Yang et al. (2009) have shown that distinct switchgrass germplasm derived from synthetic, wide-crosses of several lowland accessions displayed differences in ethanol yields. This diversity in plant quality in switchgrass collections can be expected, since switchgrass plants are obligate outcrossers so cultivars developed to date by plant breeders or local ecotype-based cultivars are populations of related heterogeneous individuals.

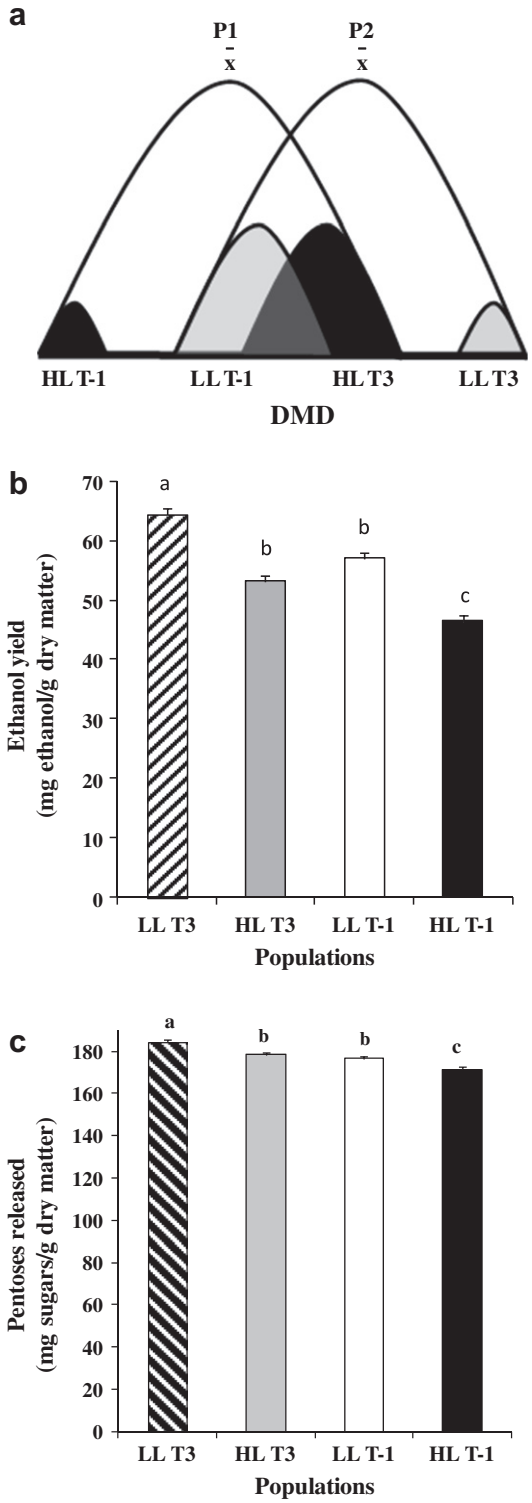

Fig. 1. A plausible distribution of IVDMD, ethanol and pentose yields for plants comprising the T-1 and T3 populations. (a) Potential occupancy of the different subpopulations within the overall distributions for IVDMD is shown (black for T-1 and gray for T-3). (b) Mean ethanol yields for plants within each population and subpopulation. Bars with the same letter were not statistically different (Tukey's HSD, $\alpha = 0.05$). (c) Release of pentoses from hemicellulose. Bars with the same letter were not statistically different (Tukey's HSD, $\alpha = 0.05$). Error bars represent ±95% confidence intervals for the means; ($n = 6$).

3.2. Cell wall factors and sugar release

Although, there was a modest relationship between plants with lower lignin contents and higher ethanol yields, there was substantial variability among the individual plants within a subpopulation (Fig. 2a). Regression analysis using a two-parameter linear model ($R^2 = 0.464$) indicated that less than half of the total variation among the genotypes was due to differences in stem lignin concentration.

The scatter along the regression line (Fig. 2a) indicated the effect of plant-related factors other than lignin on ethanol yield, while the high-leverage points on the ends of the line suggested the overall influence of lignin on ethanol yields. As an example, plants in the LL T3 subpopulation (closed triangles, Fig. 2a) had about the same level of stem lignin (~8% stem lignin) but had over a 50% difference in ethanol yields between the lowest yielding plant (48.6 mg ethanol g^{-1} dry matter) as compared the highest ethanol yielding plant at 72.4 mg ethanol g^{-1} dry matter (Fig. 2a; closed triangles). A mixed effect of lignin content on ethanol yields has been observed for large number of unrelated poplar trees with a range of lignin concentration (Studer et al., 2011). The overall relationships between lignin and ethanol yields in these switchgrass plants were consistent with earlier reports (Carroll and Somerville, 2009; Dien et al., 2009). However, a diversity of other factors appeared to have impacted conversion of biomass to ethanol observed in these closely related switchgrass genotypes (circle, Fig. 2a). Since conversion is a function of biomass characteristics, responses of biomass to pretreatment and subsequent degradability of cellulose to glucose, these data indicate that substantial plasticity in the organization of tissues and cell walls exist in switchgrass.

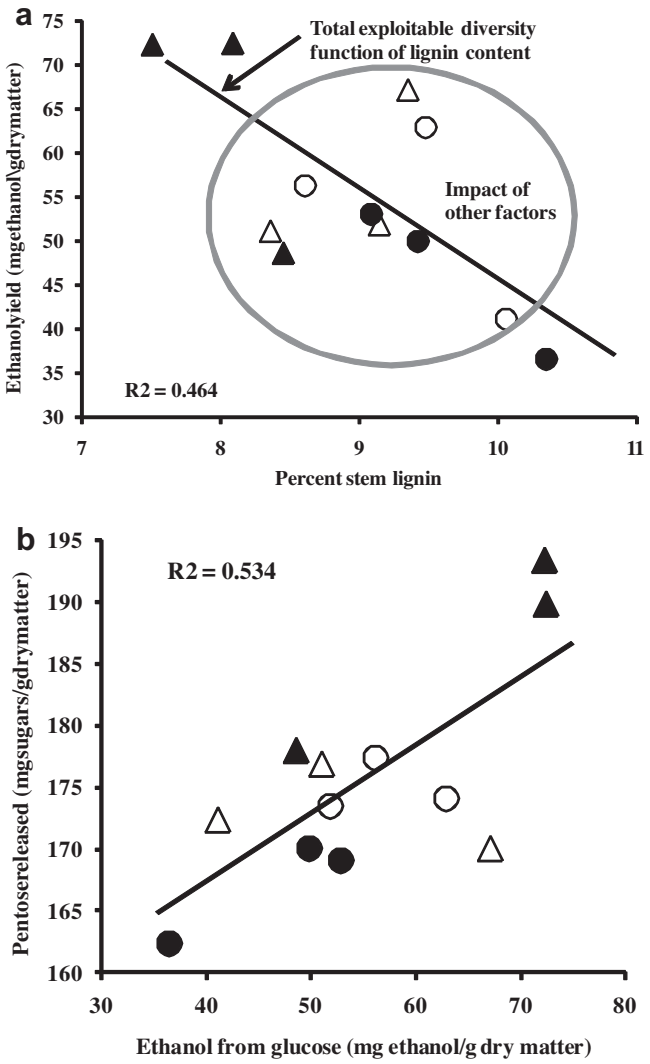

Fig. 2. Relationships between lignin content, ethanol and pentose yields for plants used in this study. (a) Relationship between lignin content and ethanol yields for all plants. The regression line indicates the overall effect of lignin content in stems to ethanol yields, and shows the maximal exploitable diversity that exists for these two parameters within the T-1 and T3 populations. The circle indicates the impact of other factors that impact ethanol yields. (b) Relationship between pentoses released after dilute acid pretreatment and ethanol yields for all plants used in the study. ▲ = LL T3; Δ = HL T3; ○ = LL T-1; ● = HL T-1.

There was a positive correlation (r = 0.73) between pentoses released by dilute acid pretreatment of stems and to ethanol yields from SSF on a dry matter basis (R^2 = 0.534; Fig. 2b). Greater stem cell wall hemicellulose content can be expected to yield greater concentrations of pentoses, and potentially impact cellulose accessibility through concurrent changes in lignin content and cell wall architecture both before and after dilute acid pretreatment (Hallac et al., 2010; Pingali et al., 2010). Results summarized in Figs. 1 and 2 demonstrated that switchgrass plants can be bred for variable ethanol yields per Mg of biomass. Also, the impact of lignin content of stems on ethanol yields was apparently tempered by other plant factors related to cell types and cell wall composition that can modulate conversion (Carroll and Somerville, 2009; Dien et al., 2009; Fu et al., 2011; Studer et al., 2011).

3.3. Changes in cell wall phenolics

There were significantly different levels of pCA, FA, and total wall bound esters detected among the 4 sub-populations (Fig. 3a). Plants in the T-1 population had significantly greater amounts of esterified pCA and total phenolic esters, as compared to the T3 population. FA ester levels were different between the two populations and within each subpopulation, although results were not always statistically significant. Overall, there were greater changes in the pCA ester levels as compared to the FA ester levels among the four subpopulations (Fig. 3a). Increased levels of pCA esters relative to FA esters could indicate subtle changes to cell wall architecture, since FA can crosslink to other wall polymers, and prevent efficient deconstruction of cellulose both in the rumen of cattle (Buanafina et al., 2008) and during biochemical fermentation (Dien et al., 2006).

Across all populations, the relative G-lignin levels were always greater than S-lignin levels (Fig. 3b). Apparent G-lignin levels were highest in the plants in the high lignin subpopulations relative to the plants from the low lignin subpopulations. The HL T-1 plants had a pooled S/G ratio of 0.55 as compared to a ratio of 0.75 for the LL T-1 low lignin subpopulation. In contrast there was limited variation in the S/G ratios for the T3 population, which had values of 0.50 and 0.51 for the LL T3 and HL T3 subpopulations respectively. There is evidence from poplar that lower G/S ratios combined with decreased lignin content improved saccharification (Studer et al., 2011). These authors speculated that the shorter, more linear chains produced by S-lignin could be more prone to removal during pretreatment (Studer et al., 2011). Only data with the T-1 plants are consistent with these findings; there was no obvious relationship between S/G ratios and ethanol yields for plants from the more digestible, higher ethanol T3 plants indicating that selection pressure for greater IVDMD led to several different organizations of switchgrass secondary cell walls. Since multiple processes affect overall IVDMD (Casler and Jung, 2006), and cell wall composition is thought to be controlled by over 750 genes in grasses (Penning et al., 2009) it is likely that switchgrass plants that possess different combination of traits could yield phenotypes that have similar ethanol yields.

The H- and hG-lignin contents in grasses are present in much lower abundances than G and S-lignins, but can provide clues about changes in the availability of monolignol intermediates during lignin deposition (Boerjan et al., 2003; Palmer et al., 2008). For these switchgrass plants, H-lignin content was significantly different between the T3 and T-1 populations, although, H-lignin content was statistically indistinguishable between the LL T3 and HL T3 subpopulations (Fig. 3c). Variation was also observed for relative levels of hG-lignin content in these four switchgrass subpopulations. The level of hG-lignin was greatest in the LL T-1 low lignin

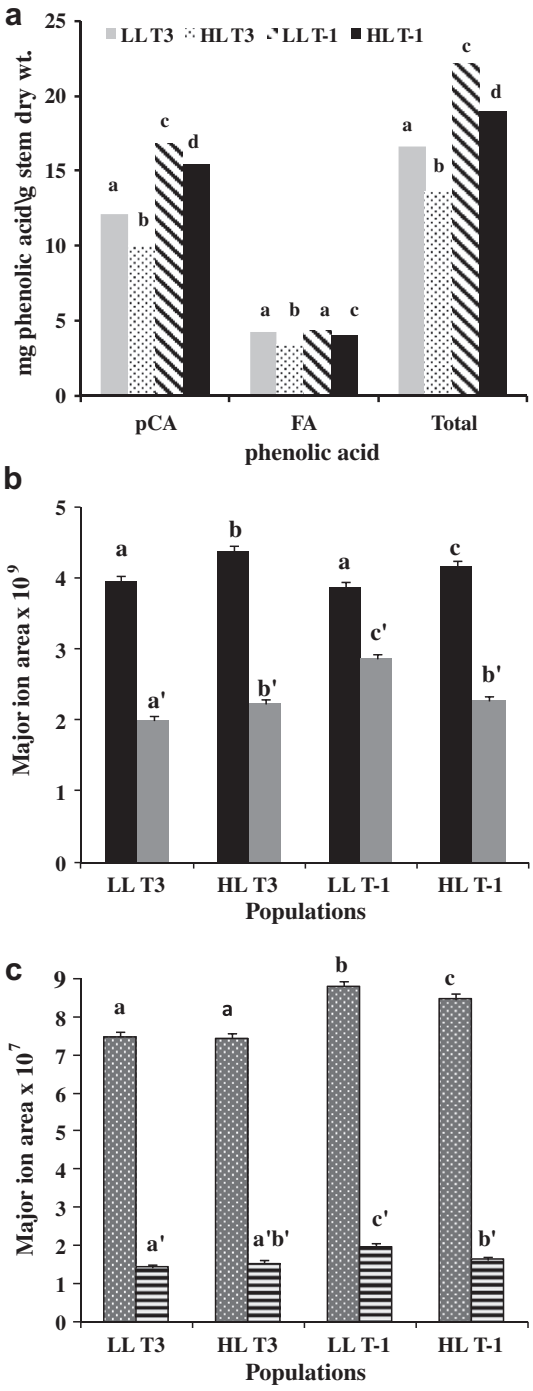

Fig. 3. Phenolic content in stems. (a) Hydroxycinnamic acid esters in stem biomass expressed as mg phenolic acid g^{-1} dry weight. pCA = p-coumaric acid, FA = ferulic acid; Total = total of pCA + FA. Gray bars = LL T3; stippled bars = HL T3; stripped bars = LL T-1; black bars = HL T-1. (b) Relative lignin monomer levels in switchgrass populations. G-lignin (black bars) and S-lignin (gray bars). (c) H-lignin (gray bars) and hG-lignin (stippled bars). The relative amounts of each component was statistically analyzed separately. For all figures, bars with the same letter were not statistically different (Tukey's HSD, $\alpha = 0.05$). Error bars represent ±95% confidence intervals for the means; three aliquots of stem biomass from two field replicates were analyzed for each sample ($n = 6$).

subpopulation (1.97×10^7 area units) and least in the LL T3 subpopulation (1.35×10^7 area units). The levels in the contrasting high lignin subpopulations, HL T-1 and HL T3, were intermediate to these values (Fig. 3c). These variations could indicate differential activity of the biosynthetic processes affecting the availability of monolignols in these plants and thereby affect the lignification

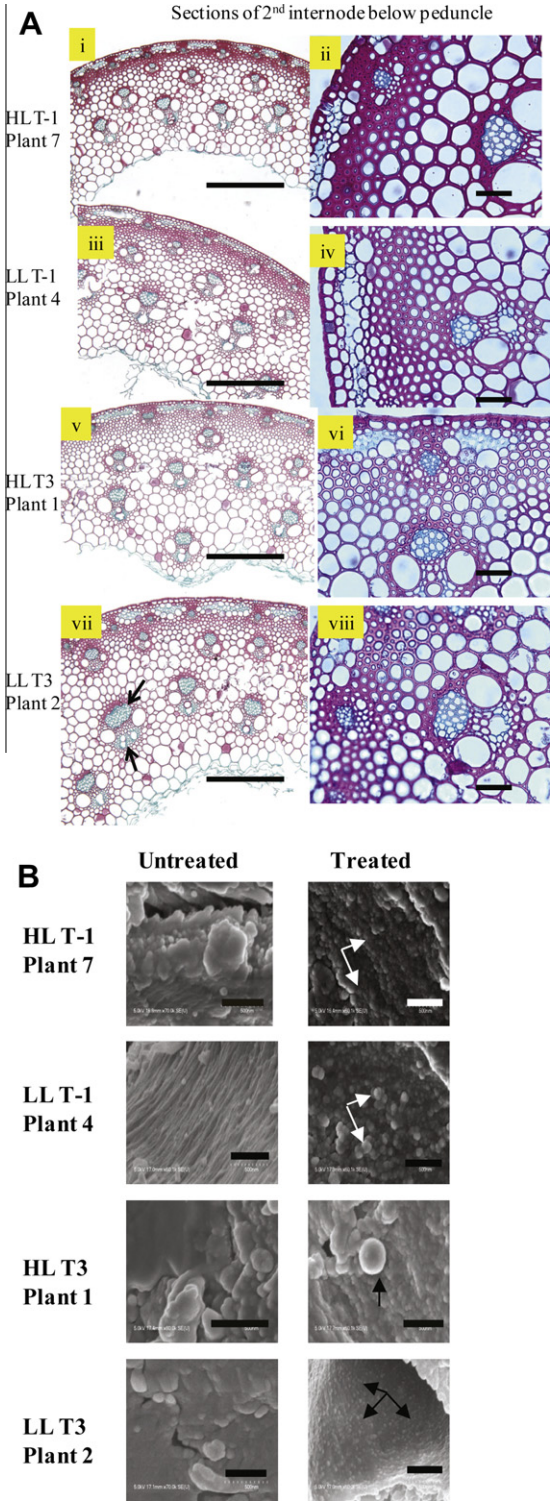

Fig. 4. Microscopy of plant stems and biomass. (a) Light microscopy of stem sections. Subpopulation and plant identity are shown on the left. Panels at low magnification (bar = 1 mm). Panels at higher magnification of cortical region (bar = 200 μm). Lignified tissues are stained various shades of red. The intensity of color is dependent on the extent of lignification. Cell walls with high cellulose content (for example phloem) are stained blue. A vascular bundle with enhanced amount of phloem and xylem parenchyma is shown with arrows in Panel at low magnification for plant 2 of the LL T3 subpopulation. Representative data from two slides per plant, containing sections from internode tissues from different replicates. (b) Field Emission SEM of ground stem tissues from switchgrass plants before (untreated) and after dilute-acid pretreatment (treated). Subpopulation and plant identity are shown on the left. Bars in images are 200 nm. Arrows in images show potential lignin globules.

patterns in tissues. To evaluate potential anatomical changes in stems, a single plant from each subpopulation was selected for examination.

3.4. Impact of breeding on stem anatomy

The FASGA staining protocol (Mechin et al., 2005) stains cell walls containing lignin red and non-lignified cell walls blue. The intensity of red coloration indicates extent of lignification. For a plant from HL T-1, the cortical sclerenchyma and fibers surrounding the vascular bundles were vividly stained and these cells appeared to be relatively uniform in size with thick cell walls, staining of the ground parenchyma cell walls was also more intense (Fig. 4a). Within the LL T-1 subpopulation plants exhibited less intense staining of the sclerenchyma, fiber sheaths and ground parenchyma, although the cortical sclerenchymatous region was several cell layers deep (Fig. 4a). For a plant from the HL T3 high lignin subpopulation, the relative lignin content of the cell walls appeared to be lower based on intensity of staining. The cortical sclerenchyma was distinct, but did not appear to be comprised of as many cell layers when contrasted to a plant from the LL T-1 subpopulation. Cross sections of the second internode from a plant in the LL T3 low lignin subpopulation (Fig. 4a) showed that there were fewer layers in the cortical sclerenchyma and some of the vascular bundles were larger with greater amounts of phloem and xylem parenchyma (arrow in first panel). At a higher magnification, there appeared to be some variation in the staining intensity of the cell walls which suggested uneven lignin deposition in both the cortical sclerenchyma and parenchyma (Fig. 4a). These anatomical data suggested that both tissue type and apparent extent of lignification had been modified as a result of breeding for digestibility. It is likely that these modifications affected the susceptibility of cell walls to dilute acid pretreatment and the subsequent biochemical conversion of cellulose to ethanol. Plants in the T-1 population appeared to have greater amounts of lignified cells with thicker cell walls as compared to the plants in the T3 population.

3.5. Ultrastructural changes in stem biomass

Field emission scanning electron microscopy of untreated and dilute-acid pretreated samples indicated two important features in the cell walls of individual plants: (i) increasing digestibility and or ethanol yields appeared to increase the smoothness of the cell walls, and (ii) upon pretreatment the granularity of wall surfaces was dramatically increased for plants from the T-1 population and for the higher lignin plant in the T3 population (Fig. 4b). In marked contrast, the observed spherical globules were much smaller and relatively well dispersed on wall surfaces in plant 2 of the LL T3 subpopulation (Fig. 4b). It is likely that some if not many of these globules were formed by coalescence of lignin (Donohoe et al., 2008), although this was not directly verified. How these changes physically impacted accessibility of dilute-acid pretreated cellulose fibers to glucanses still remains to be investigated.

3.6. Cell wall composition and accessibility

Composition analyses of the specific plants used in the histological studies demonstrated that the plants with the lowest stem lignin content tended to have the highest concentrations of cell solubles, hemicellulose, and highest G/S lignin monomer ratios, lowest level of cellulose, and the highest ethanol and released pentose yields (Fig. 5). Conversely, plants with the highest lignin content had higher levels of cellulose. Plants in the LL T-1 and HL T3 subpopulations were generally similar in composition (Fig. 5), displayed differences in G/S ratios, but not in pentose or ethanol yields. These compositional differences can be expected to be a

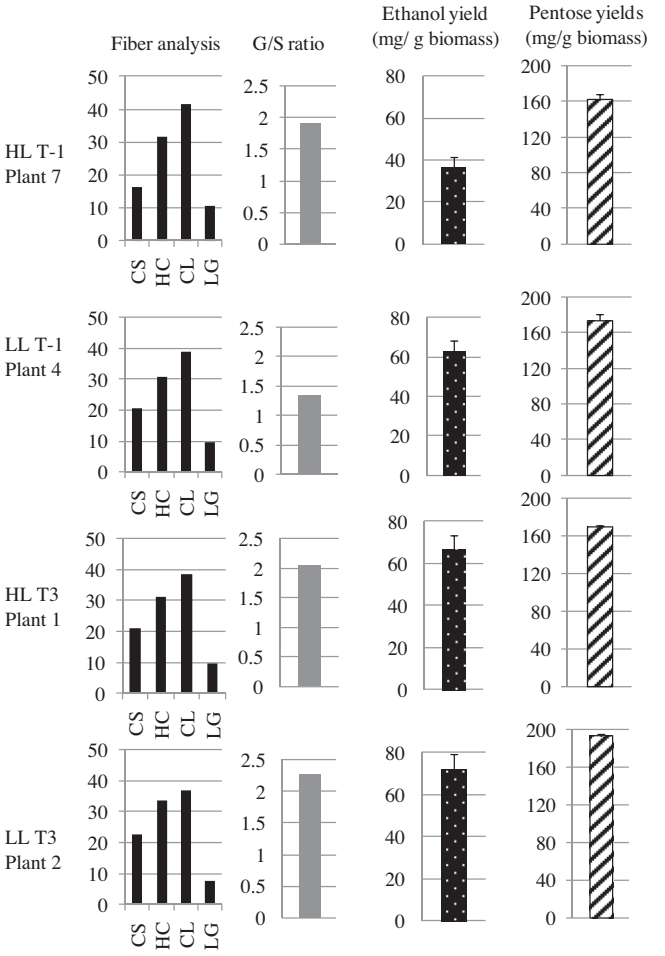

Fig. 5. Cell wall composition, ethanol and pentose yields for selected plants from the different subpopulations. The population and plant identity are on the left. Fiber analysis was performed on ground stems and the components are shown as a percentage. CS = cell solubles, HC = hemicellulose, CL = cellulose, LG = acid digestible lignin. G/S ratio was determined from the relative area of the major ion peaks after thioacidolysis. Ethanol and pentose (xylose + arabinose) yields were calculated as described in the methods.

function of tissue anatomy, cell types and relative accretion of primary and secondary cell walls. For the switchgrass plants analyzed, some of these changes appear to be in the amount and relatively lignification of the sclerenchyma. Lowering lignification of the cortical sclerenchyma to improve digestibility and/or deconstruction has been suggested as a target in tall fescue (Chen et al., 2002) and in switchgrass (Sarath et al., 2008b). More recently, sorghum brown midrib plants carrying both the *bmr*-6 and *bmr*-12 alleles were shown have significantly less lignification of the cortical sclerenchyma, and greater ethanol yields as compared the wild-type plants (Dien et al., 2009; Palmer et al., 2008). These studies indicated that decreasing lignin along with genetic manipulation of cell wall architecture should lead to improved saccharification in herbaceous perennials such as switchgrass.

Recent reports have indeed shown that downregulation of cinnamyl alcohol dehydrogenase (CAD) or caffeic acid-*O*-methyltransferase (COMT) protein levels in switchgrass results in plants with lowered lignin and higher ethanol (saccharification) conversion ratios (Fu et al., 2010, 2011; Saathoff et al., 2011). A number of studies have observed that chemical/physical removal of lignin is insufficient by itself to improve glucose yields (Li et al., 2010; Nlewem and Thrash, 2010; Rollin et al., 2011). Generally, increased glucose yields appear to be tied to greater accessibility of cellulases to individual cellulose fibers (Li et al., 2010; Pingali et al., 2010). Here it is

shown that breeding switchgrass for increased IVDMD (and indirectly ethanol yield), resulted in genotypes with reduced lignin content and variability in other cell wall and tissue properties that together exerted a distinct influence on cellulose digestion. A notable property that was affected appeared to be physical characteristics of the cell wall that positively or negatively impacted cellulose digestibility and conversion to ethanol by SSF. These observations are consistent with recent data published by Donohoe et al. (2011) that evaluated the effect of different pretreatments on switchgrass biomass structure.

4. Conclusions

Genetically related switchgrass plants with comparable lignin content displayed marked differences in plant anatomy, cell wall architecture, and subsequently ethanol yields. The application of emerging genetic tools for example (Okada et al., 2010): to well-characterized populations such as the one studied here should eventually allow for the discovery of genes acting independently or within networks that impact cell wall architecture and composition. These data can then be utilized in breeding programs to develop genotypes with optimized biomass properties and yields.

Acknowledgements

We thank Nathan Palmer, Patricia O'Bryan Steve Masterson, Patrick Callahan and Kevin Grams for excellent technical assistance. This work was supported by the USDA-ARS CRIS project 5440-21000-028-00D. A.J.S. was supported in part by the Office of Science (BER), US Department of Energy Grant No. DE-AI02-09ER64829. The US Department of Agriculture, Agricultural Research Service, is an equal opportunity/affirmative action employer and all agency services are available without discrimination. Mention of commercial products and organizations in this manuscript is solely to provide specific information. It does not constitute endorsement by USDA-ARS over other products and organizations not mentioned.

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