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Ethanol yields and cell wall properties in divergently bred switchgrass genotypes

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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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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