Comparative feedstock analysis in *Setaria viridis* L. as a model for C₄ bioenergy grasses and panicoid crop species

Carloalberto Petti¹, Andrew Shearer¹, Mizuki Tateno¹, Matthew Ruwaya², Sue Nokes², Tom Brutnell³ and Seth DeBolt*¹

¹ Plant Physiology, Department of Horticulture, College of Agriculture, Food and the Environment, University of Kentucky, Lexington, KY, USA
² Department of Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY, USA
³ Enterprise Institute for Renewable Fuels, Donald Danforth Plant Science Center, St. Louis, MO, USA

*Correspondence: Seth DeBolt, Plant Physiology, Department of Horticulture, College of Agriculture, Food and the Environment, University of Kentucky, Room 318 Agricultural Science Center North, Lexington, KY 40546, USA e-mail: sdeb29@uky.edu

INTRODUCTION

As the depth of knowledge ever increasingly grows in the model plant Arabidopsis and the utility of this information proves translatable, there remains a need for additional model plants, to decode and translate traits that are absent in Arabidopsis (Rensink and Buell, 2004). Monocotyledonous taxa (grasses) display both marked phenotypic and metabolic variability. Moreover, they produce the majority of the world food crops and a growing need for biomass for biofuel production is being driven toward perennial grasses. Thus, a notable need exists for a range of model species to ask a great many questions underscoring agronomically important traits. Existing examples of model grasses include Brachypodium distachyon and rice (*Oryza sativa*), which have served as tractable model plants for gene discovery and translational biology (Ouyang et al., 2007; Vogel, 2008). However, there are also important physiological differences among the grasses. A principle difference exists between mechanisms of photosynthesis. Rice and Brachypodium utilize C₃ (carbon fixation) photosynthesis whereas the existing and most promising bioenergy feedstocks including maize (*Zea mays*), sorghum (*Sorghum bicolor*), switchgrass (*Panicum virgatum* L.), or Miscanthus (*Miscanthus × giganteus*) employ C₄ (carbon fixation) photosynthesis whereas the large evolutionary adaptation in plant photosynthesis. Despite the large evolutionary adaptation in plant photosynthesis between C₃ and C₄ metabolism, only minor differences are noted between the C₃ and C₄ plants, in our case focused solely on monocotyledonous (monocots) examples (some dicotyledonous (dicots) plants exist), involve structural (physiologically) and metabolic advances in leaf anatomy and atmospheric carbon during photosynthesis. Despite the large evolutionary adaptation in plant physiology between C₃ and C₄ metabolism, only minor differences are noted in the cell walls, such as suberized bundle sheath cell walls to prevent CO₂ leakage (Langdale, 2011). Little evidence exists that C₃ versus C₄ metabolism is accompanied by any marked shifts in the composition of cell wall polysaccharides comprising lignocellulosic biomass. However, the primary metabolic response(s) to

Second generation feedstocks for bioethanol will likely include a sizable proportion of perennial C₄ grasses, principally in the Panicoideae clade. The Panicoideae contain agronomically important annual grasses including *Zea mays* (maize), *Sorghum bicolor* (L.) Moench (sorghum), and *Saccharum officinarum* L. (sugar cane) as well as promising second generation perennial feedstocks including *Miscanthus × giganteus* and *Panicum virgatum* L. (switchgrass). The underlying complexity of these polyploid grass species is a major limitation for their direct manipulation and thus driving a need for rapidly cycling comparative model. *Setaria viridis* (green millet) is a rapid cycling C₄ panicoid grass with a relatively small and sequenced diploid genome and abundant seed production. Stable, transient, and protoplast transformation technologies have also been developed for *Setaria viridis* making it a potentially excellent model for other C₄ bioenergy grasses. Here, the lignocellulosic feedstock composition, cellulose biosynthesis inhibitor response and saccharification dynamics of *Setaria viridis* are compared with the annual sorghum and maize and the perennial switchgrass bioenergy crops as a baseline study into the applicability for translational research. A genome-wide systematic investigation of the cellulose synthase-A genes was performed identifying eight candidate sequences. Two developmental stages: (a) metabolically active young tissue and (b) metabolically plateaud (mature) material are examined to compare biomass performance metrics.

Keywords: *Setaria*, cell wall, lignocellulose, Panicoideae, biofuel, cellulose synthase
environmental constraints, such as drought (Doust et al., 2009), are notable different in plants with C₃ versus C₄ metabolism and therefore the feedback regulation of carbon through primary metabolism is feasibly influenced. Such shifts could in turn influence cell wall processes. Indeed, in a study by Hibberd and Quick (2002), the spatially discrete evolution of C₄ metabolism around xylem vessels in a C₃ plant was associated with both a pH shift and provision of carbon skeletons into lignin synthesis for rapid incorporation in lignified xylem vessels. Regardless, the development of physiological, compositional, and genetic resources in Setaria as a model organism for lignocellulosic feedstocks should complement resources in Arabidopsis and Brachypodium. Briefly, Setaria, is the weedy counterpart to the grain crop Setaria italica (millet), more commonly known as the green foxtail. Many crop grasses have vast and repetitive genome structure (Vicient et al., 2001; Benne et al., 2007). By contrast, Setaria viridis has a relatively manageable genome and yet displays synergy to the Panicoideae grasses, which include the major biofuel grasses (Li and Brunnell, 2011). Setaria (as referred to from this point onward) displays a life cycle of around 8 weeks, produces abundant seed each generation, is self-compatible and is a diploid providing for genetic tractability (Doust et al., 2009).

As an initial step in the development of Setaria as a model for bioenergy grasses, the cell wall carbohydrate composition of Setaria was compared with other crop species in the Panicoideae at two developmental stages; (a) metabolically active young tissue and (b) metabolically plateaued (mature) material (see Figure 1 for example). Furthermore, employing current genomic data we phylogenetically examine the cellulose synthase-A (CESA) gene family. Metabolically active tissue was examined because such a sampling time is representative of an early season perennial forage production system (Panicoideae clade species such as switchgrass, eastern gamagrass (Tripsacum dactyloides) and Miscanthus × giganteus) and due to being a temporally favorable timeframe to examine phenotypes in a research setting. Secondly, an important comparison is that of mature tissue where genetic manipulations for persistent biomass traits can be compared and potentially translated.

RESULTS AND DISCUSSION

Setaria AS A MODEL FOR CELL WALL SYNTHESIS IN THE PANICOIDEAE: THE IDENTIFICATION OF TWO DEVELOPMENTAL STAGES THAT PRESENT SIMILAR CELL WALL COMPOSITION

Cellulose, neutral sugars, and lignin

It is recognized that the relative abundance of cellulose, lignin, and hemicellulose varies depending on tissue type, age, and environmental/biological condition of the plant tissue (Meinert and Delmer, 1977; Rogers and Campbell, 2004). Cellulose, a natural highly abundant macromolecule made up of glucose 1,4-β-linked is a principle substrate for bioethanol conversion (Hidayat et al., 2012). Further, hemicellulose is also a convertible carbohydrate substrate to alcohol fuels (Saba, 2003). By contrast, the presence of lignin inhibits enzymatic processes (Li et al., 2011; Kim et al., 2011) and it’s down-regulation through genetic approaches has been shown to improve the saccharification efficiency (Chen and Dixon, 2007; Saathoff et al., 2011; Wang et al., 2012). Therefore, we surveyed cellulose, lignin, and neutral sugar composition in Setaria and compared these with other annual and perennial members of the Panicoideae. Cellulose and lignin content of acetyl-insoluble residue (AIR biomass) was quantified. As defined in the methods and materials, we sought two developmental stages for comparative studies. Analysis of cellulose content in immature biomass revealed no significant change (P > 0.05) in relative content among the three annual paniceoid species (Setaria, maize, and sorghum). By contrast, lower cellulose content was measured in switchgrass samples (Figures 2A,B; P < 0.05). There are several plausible explanations for these data. Firstly, switchgrass is a perennial warm season grass whereas the other Panicoideae clade species are annual grasses. The modest variation in cellulose accumulation may reflect the ecological and genetic distances between annual and perennial crops. Alternatively, the capacity to synchronize temporal staging (sampling time) may have been congruent for the annual species, but the perennial switchgrass may display minor developmental variation. In prior analysis of numerous
upland and lowland switchgrass cultivars, modest variability in cellulose content was observed (Stork et al., 2009), and therefore species level variation could also exist. Comparison of cellulose content in the mature (S2) compared with immature (S1) biomass revealed an expected and consistent proportional decrease during development (Figures 2A,B; \( P > 0.05 \)). This proportional decrease in cellulose content may be a target for future transcriptional re-wiring efforts that aim to increase cellulose without significant cost to the fitness of the plant. It was also found that the degree to which the cellulose content was reduced in mature tissue relative to immature tissue varied between species. One-way ANOVA with a post hoc Tukey test was performed across the four species per stage. Convention adopted is small letters for stage 1 (S1) and capital letters for stage 2 (S2). Same letters indicate non-significance (\( P > 0.05 \)) different letters indicate significant differences (\( P < 0.05 \)).
to the metabolically active tissue was greatest in the two highly domesticated species, maize and sorghum.

The majority of lignin was measured as insoluble lignin. Lignin content increased significantly between the first and second sampling in both aerial (Figure 2C) and root tissue (Figure 2D). Increased lignification during development was consistent with diversification of the primary cell wall into lignified secondary cell walls in maturing stems, leaves, and roots (Redfearn and Nelson, 2003; Parrish and Fike, 2003; Stork et al., 2009). Soluble lignin although representing a minor proportion of cell wall biomass was measured and found to be less predictable (Figures 2E,F).

For example, in aerial tissue, soluble content did not significantly change in Setaria or maize (P > 0.05) and significantly decreased in sorghum and switchgrass between S1 and S2 (P < 0.05). Collectively, data highlight a contiguous developmental program during the deposition patterns for cellulose and lignin. These data broadly support the utility of Setaria to provide model organism characteristics to above-mentioned crop species, taking note of potential differences when comparing annual and perennial grasses.

Biomass was examined using X-ray diffraction (XRD) to infer a relative crystallinity index (RCI; Segal et al., 1959). The RCI is an empirical measure of the relative proportion of the crystalline versus non-crystalline (amorphous) biomass components, and is not a measure of the crystallinity of cellulose (Anderson et al., 2003). All samples fell in a comparable range of 59–69%, consistent with prior studies (Harris and DelBolt, 2008). Interestingly, there was a general trend of increased RCI from S1 to S2 (Table 1). Lignification during secondary cell wall synthesis would increase the amorphous signal at 2-theta (2θ) 18° and subsequently influence the RCI determination. Of further note, a large peak consistent with calcium oxalate was evident in all samples at 2θ 26.6° and was more pronounced in S2 (data not shown).

To further understand how the cell wall was changing over time, we measured neutral sugars in S1 (Figures 3A,B) and S2 (Figures 3C,D). Neutral sugars collectively comprise the building blocks for hemicellulose biosynthesis in the plant cell walls (Bauer et al., 1973) such as xyloglucans, xylans, mannans and glucuronomannans, and β-(1 → 3, 1 → 4)-glucans (Scheller and Ulvskov, 2010). These polysaccharides make up a third class of cell wall biomass component, in addition to cellulose and lignin and comprise arabinose, mannose, rhamnose, galactose, fucose, xylose, and soluble glucose. In S1 aboveground biomass, rhamnose was more abundant in the switchgrass biomass relative to annual species (Figure 3A). By contrast, arabinose, galactose, fucose, glucose, and xylose did not display notable shifts in relative abundance (% AIR or mg g⁻¹). For stage S2, some variability in xylose, glucose, and rhamnose content was observed among genotypes, but this was also most pronounced in switchgrass (Figure 3). Mannose and galactose were not abundantly represented in our analysis. These can be explained by (glucurono)arabinoxylans being the predominant component of hemicelluloses in the grasses, while xyloglucans dominate in the cell walls of dicotyledons (including gymnosperms; Scheller and Ulvskov, 2010). The proportional shifts between species herein were minor and no pronounced shifts in total neutral sugars between S1 and S2 were observed. These data support that the increased RCI in S2 (Table 1) may be consistent with increased order or abundance of crystalline cellulose within the cell wall rather than a proportional shift in hemicellulose or lignin. The use of hemicellulosic neutral sugars as a feedstock for enzymatic conversion to biofuel requires different degradation enzymes than cellulose (Bahr, 2003). The xylose and arabinose sugar backbones represented the most abundant non-glucose sugars available for bioconversion (Figure 3). Alternatively, recent innovations show that integrated thermochemical deconstruction of cellulosic and hemicellulosic fractions of lignocellulosic biomass can be simultaneously converted to levulinic acid and furfural, respectively (Alonso et al., 2013), and would not require separate bioprocesses. Ultimately, thermochemical deconstruction of lignocellulosic biomass to biofuel may negate modest compositional shifts, but technical hurdles remain in this maturing technology.

Cellulose biosynthesis inhibitor response studies

A chemical inhibitor can be a useful research tool, particularly when combined with genomic tools and model system resources (Robert et al., 2009; Toth and van der Hoorn, 2010). Cellulose biosynthesis inhibitors (CBI) have been effectively used with the dicotyledonous model system Arabidopsis thaliana to identify mutations in CESAs (Scheible et al., 2001; Despres et al., 2002), some of which have led to beneficial deconstruction characteristics of cellulose (Harris et al., 2012). CBIs have also been used to learn important information about the delivery mechanisms of cellulose biosynthetic machinery (Cowell et al., 2009; Gutierrez et al., 2009), which will ultimately be important to regulate cellulose synthesis in plants.

In plants, directional cell growth is facilitated by a rigid, yet extensible cell wall, which acts to collectively constrain internal turgor pressure. Cellulose forms the central load-bearing component of this wall and therefore inhibiting cellulose biosynthesis causes radially swollen cells in seedlings providing a robust visual phenotype drug activity. A panel of CBIs was tested for potency herein, due to their possible use in future studies on biomass traits. These CBIs fell into three different classes of inhibition mechanism in Arabidopsis (summarized in Brabham and DelBolt, 2013), clearance of CESAs (isoniben (N-[1-ethyl-1-methylpropyl]-1,2-oxazol-5-yl-2,6-dimethoxybenzamide), stopping CESAs (2,6-dichlorobenzonitrile – DCB) and co-targeting cortical microtubules and CESAs (morlin

Table 1 | Relative crystallinity index (RCI) determined for specified spatial and temporal regions of each species.

| Species | Aboveground – RCI (%) | Belowground – RCI (%) |
|---------|-----------------------|-----------------------|
|         | Stage 1 | Stage 2 | Stage 1 | Stage 2 |
| Setaria  | 575 ± 1.2 | 566 ± 2.3 | 520 ± 1.9 | 600 ± 3.2 |
| Sorghum  | 64.2 ± 3.6 | 62.0 ± 4.5 | 54.7 ± 2.0 | 61.1 ± 2.3 |
| Maize    | 62.5 ± 2.8 | 61.2 ± 1.8 | 54.2 ± 2.0 | 63.5 ± 3.7 |
| Switchgrass | 66.8 ± 3.8 | 68.1 ± 3.6 | 52.4 ± 2.0 | 60.0 ± 2.5 |

*Values reported are the average of n = 3 ± SE

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FIGURE 3 | Neutral sugar monosaccharide analysis for aboveground material. The relative abundance of each sugar was compared to a true standard and determined for Setaria, maize, sorghum, and switchgrass at S1 (A, B) and S2 (C, D). n = 3 biological replicates. Error bars represent the standard error from the mean. One-way ANOVA with a post hoc Tukey test was performed across the four species per stage. Convention adopted is small letters for stage 1 (S1) and capital letters for stage 2 (S2). Same letters indicate non-significance (P > 0.05), different letters indicate significant differences (P < 0.05).

FIGURE 4. The LD50 dose rates for isoxaben, morlin, and DCB in Arabidopsis are 4 nM, 500 nM, and 5 μM (Heim et al., 1989; DeBolt et al., 2007a,b). Results showed that isoxaben failed to induce radial LD50 root length suppression until 50–100 nM (Figure 4), which represented a 15- to 20-fold reduction in activity. Morlin also displayed a 10-fold reduction in activity, with an LD50 of approximately 50 μM. Based on these data, DCB alone remained a highly potent CBI inhibitor for the monocot with dose rate similar to those observed in Arabidopsis. The lower degree of CBI potency observed for isoxaben and morlin, but not for DCB, requires future exploration. Indeed, 14C-isoxaben studies from Heim et al. (1993) suggest that isoxaben uptake is not blocked at the cuticle in monocotyledons. Further, the action of DCB does not support a non-specific biochemical drug tolerance such as more abundant cytochrome P450 enzymes (Morant et al., 2003). Hence, future chemical genetic studies employing mutagenesis populations to map based clone resistance loci to CBIs are needed. Such studies will be aided by a genetic model such as Setaria.

Phylogenetic analysis of CESA genes

In order to ask whether similarities in cellulose biosynthesis were reflected by similar organization of the CESA gene family, we performed a simple phylogenetic analysis of annotated genes. To aid in providing confidence in the bioinformatic analysis, two separate phylogenetic trees were established using distinct protein prediction compared with genomic sequence. Homology-based associations were made between CESA identified in the genomic data derived from A. thaliana (AT), Setaria italica (SI), Z. mays (ZM), Sorghum bicolor (SB), and P. vulgare (PV). Firstly, the MUSCLE multiple alignment tool (Edgar, 2004) was utilized followed by the PhyML program (Guindon et al., 2010) to create the phylogenetic tree based on the translational predictions (protein sequences) of the putative CESAs. GARLI was utilized in parallel to produce a phylogenetic tree based on genomic sequence information. When grouping CESA genes or gene products within the broad CESA phylogenetic tree, it was necessary to visualize groups (sub-clades) of putative CESAs within the tree, and therefore they were referred to based on the closest homology.
FIGURE 4 | Comparing response to cellulose biosynthesis inhibitors (CBIs) in Setaria and sorghum. Response to 2,6-dichlorobenzonitrile (DCB), morlin (7-ethoxy-4-methyl chromen-2-one), and isoxaben (N-[3-(1-ethyl-1-methylpropyl)-1,2-oxazol-5-yl]-2,6-dimethoxybenzamide) from switchgrass (A) and sorghum (B) was examined during early seedling growth using in-plate assays on sterile Murashige and Skoog (MS) agar media. Average root length was measured in ≥ 10 plants and error bars represent standard error from the mean.
to the established Arabidopsis CESA nomenclature of Richmond (2000). The phylogenetic tree inferred by protein sequences (Figure 5) clustered in five classes with the Arabidopsis CESA1, 3, 7, 6-like, and 4. It was perhaps noteworthy that the Arabidopsis CESA6-like clade, which also comprised proteins similar to Arabidopsis CESA2, 5, and 9 (Persson et al., 2007) was the most highly represented cluster. By contrast, the Poaceae homologs with Arabidopsis secondary cell wall CESA6 were least common, which only Z. mays and Setaria containing putative members of the CESA4-associated clade. With the exception of the Arabidopsis CESA4 clade-comprising of only two putative CESA members, the remaining clades contain at least one putative CESA member from each of the five species investigated. Using genomic data to assemble and predict phylogenetic relationships (Figure 5), the putative CESAs divide readily into four distinct clades, clustering around Arabidopsis CESA1 (primary cell wall; Arioli et al., 1998), CESA3 (primary cell wall; Scheible et al., 2001), CESA4/7/8 (vascular secondary cell wall; Taylor et al., 2003), and CESA6-like (CESA2, 5, and 9 included in primary and seed coat secondary cell wall; Stork et al., 2010; Mendu et al., 2011; Sullivan et al., 2011). It was found that at least one putative CESA member from each of the five species was represented within each sub-clade. However, in the heterotrimeric model for cellulose synthase complex (CSC) formation in the primary and secondary cell wall (Delmer and Amor, 1995; Tanaka et al., 2003), the lack of at least three distinct CESAs within the CESA4, 7, and 8 sub-clade suggests either divergence among heterotrimeric associations or an example of large incompletions within the genome sequencing for all of the Poaceae. In comparing the two phylogenetic trees, there were no marked differences in the CESAs that comprised a particular sub-clade. For example, in both phylogenetic trees the Arabidopsis CESA6-like CESAs 2, 5, 6-like, and 9 cluster closely with each other and were broadly represented. The discrepancy in the number of clades between the two trees can be attributed to the fact that within the protein sequence phylogenetic tree, the Arabidopsis CESA4 clade and CESA7 clade are separated while they are combined into one clade within the genetic sequence phylogenetic tree. Ultimately, targeted reverse genetics are urgently
DEGRADABILITY

Saccharification efficiency was measured for the four species as a criterion for utilizing lignocellulose biomass to produce alcohol-based biofuels. Several different tests were performed to determine the dynamics and cross species consistency of digestibility. Firstly, we examined the pseudo-apparent kinetics of digestibility in semi-purified cellulose (Harris et al., 2009) from the mature stem (S2) of each panicoid (Figure 6). Results showed a consistent rate of conversion of cellulose to fermentable sugars, with sorghum displaying a notable increase in digestibility.

We subsequently used a cellulose digestion via Clostridium thermocellum and measured the output of carbon from raw unprocessed biomass. During the saccharification reaction two main products are generated, glucose and cellobiose. Additionally, C. thermocellum is capable of metabolizing glucose and cellobiose, and acetate, lactate and ethanol are products of this metabolism. It is possible to estimate the total amount of carbon released from the biomass as the result of the simultaneous saccharification and fermentation process by the inclusion of all the products, thus accounting for the total carbon signature of the converted biomass. Time course fermentation (from 0 to 240 h) released an increased amount of carbon as time progressed (Figure 7A). For both stages, we observed a similar increase in the temporal series; although, as expected for stage 2, a lesser amount of total carbon was released (Figure 7A).

Three noteworthy trends were observed, (1) Setaria displayed the second highest rate in stage 1 and the highest total carbon conversion for the root material. (2) The highest rates of total carbon release was, for both stages, observed in switchgrass, and (3) the values determined for maize and sorghum were intermediate. The most interesting aspect, when single fermentation products were taken into account was related to the production of acetate whereby the highest release was associated with the fermentation of the root biomass independently of the species considered although, it was most obvious at the 240 h time point than the other time assayed (Figure 7B). This was the only case where the root biomass conversion products were higher than the conversion products from the shoot biomass.

CONCLUSION

Several studies highlight Setaria as a model organism for the panicoid (see Li and Brutnell, 2011), complementing the existing resources for C3 grasses via rice and Brachypodium. In this study, we examined the baseline composition, CBI response, and CESA gene family in Setaria. Data indicated that commonly used CBIs for dicot studies, such as isoxaben as less effective for Setaria, whereas DCM displays similar LD50 responses. Eight members of the CESA gene family were identified for functional genomic characterization. The use of two growth stages demonstrated the potential translational role of Setaria as a model species for lignocellulosic panicoid crops. To summarize, aboveground Setaria biomass was similar to the comparative (non-model) species.

MATERIALS AND METHODS

PLANT GROWTH CONDITION AND MATERIAL COLLECTION

Four grasses species were considered and include maize-hybrid 684GENSS, sorghum var. Della, Panicum virgatum switchgrass var. Alamo and Setaria. Seeds were sown on soil less media (MetroMix 360, SunGro Industries Bellevue, WA, USA). Plants were grown and maintained in a temperature-controlled glasshouse at 24°C, on a 16 h:8 h light-dark cycle with additional supplemental light if natural light was absent. MetroMix 360 media was maintained at field water conditions and fertilized with 3 g of osmocote (The Scotts Company, Marysville, OH, USA) integrated into the media when planting. A physiological staging was found to be most appropriate, as opposed to temporal staging due to inherent variation in reproductive cycle. Stage 1 was defined by four fully developed leaves (not including the cotyledon) and evidence of culm elongation and stage 2 (mature) determined by the onset of apical inflorescence protrusion. Plant specimen were divided into aboveground and belowground material. Each sub-sample was air dried for two days at 26°C then oven dried at 45°C for a further 7 days. Approximately 10 samples were pooled for each replicate. Four biological replicates of randomly harvested material were sampled per species and per developmental stage. Each biological replicate was made of pooled samples per species and per stage. After oven drying, shoot and root material was ground using 1 mm fragments (Arthur H. Thomas Co Scientific, Philadelphia, PA, USA) and used for further analytical determinations detailed below.

CELL WALL CARBOHYDRATE COMPOSITIONAL STUDIES

Cellulose content for all samples was measured colorimetrically (Uydegraaf, 1969). Neutral and acidic sugar composition was determined by high performance liquid chromatography (HPLC,
FIGURE 7 | Total carbon (A) and lactate (B) production obtained through Clostridium thermocellum system during biomass deconstruction.

In (A), biological degradation of the four grasses was completed through Clostridium thermocellum system. The saccharification efficiency was determined for stage 1 and 2 (S1, S2) and for aboveground and belowground material for *Setaria viridis* (SV); *Z. mays* (ZM); *Sorghum bicolor* (SB), and *P. vulgare* (PV). In (B), the graph displays the acetate production for stage 1 and 2 and for three time points (24, 120, and 240 h) representing the initial stage, the middle and the final stage of the process. The convention employed in the graph was: name of species plus S (S denotes shoot) for the aboveground and R (R denotes root) for the belowground material.

Mendu et al., 2011). In brief, samples were homogenized using a grinder (Arthur H. Thomas Co Scientific, Philadelphia, PA, USA) equipped with a 1-mm sieve. Twenty-five milligrams plant material were destarched as by the specification in National Renewable Energy Laboratory (NREL), LAP-004 (1996) guidelines by incubating the samples in 1 ml 70% ethanol overnight at 65°C, washed twice with 1 ml 70% ethanol for 1 h and once with 1 ml acetone for 5 min. The samples were dried overnight at 30°C with shakings.

Cellulose content was determined by weighing out 5 mg of dry biomass extract in quadruplicate and boiled in acetic-nitric reagent.
Acid-soluble lignin measurement

Acid-soluble lignin was measured using NREL LAP-004 (1996). Briefly, the autoclaved solution was allowed to cool to room temperature before withdrawing 1 ml of the hydrolysate without disturbing any solid particles in the solution. A 4% solution of sulfuric acid was used as a reference blank. To accommodate the absorbance range (0.2-0.7) of the spectrophotometer at 240 nm, both the hydrolysate and reference 4% sulfuric acid solution were diluted 1:30 with deionized water. The absorbance of the hydrolysate at 240 nm using a 1-cm light path cuvette was used to calculate the acid-soluble lignin.

Acid-insoluble lignin measurement

Porcelain filter crucibles containing a glass microfiber filter (Whatman 934-AH) were placed in a furnace at 575 °C for 4 h. The crucibles were removed from the furnace and placed into a desiccator to cool for a minimum of 1 h before being weighed. The autoclaved samples were vacuum filtered through the crucibles. The crucibles and their contents were dried in an oven at 105 °C overnight before transferring into a desiccator to cool. The weight of the crucibles were recorded and placed in a furnace at 575 °C for 4 h before reducing the temperature to 105 °C overnight. The crucibles were removed and placed into a desiccator to cool and weighed.

Enzyme kinetic of semi-purified cellulose

Crude cell walls were prepared from total biomass of each species (stage 2) as by published protocol (Reiter et al., 1993) and it was scaled up in order to meet the requirements for enzyme kinetics. Cellulose which was purified according to the method by Updegraff (1969) was homogenized using an Arthur H. Thomas Co Scientific grinder (Philadelphia, PA, USA) equipped with a 0.5-mm sieve. A cocktail of cellulase and cellobiase (Sigma, USA), equivalent to 2 FPU (filter paper unit), were used to digest an increased amount of cellulose substrates (2, 5, 7.5, 10, 12.5, 15, 17.5, and 20 mg) in a 30-mM citrate buffer (pH 4.8) at 50 °C for 2 h. Prior to glucose determination, the enzymes were heat-inactivated by boiling the samples for 5 min at 100 °C. Glucose quantification was done by using a Yellow Springs Instruments (YSI) glucose analyzer. The inability to exactly calculate the number of catalytic ends in the complex mixture of cell wall biomass allowed only for the calculation of relative estimation, expressed as relative kinetics (relative V_{max} and K_{m}). The glucose values were converted in mmol min$^{-1}$ unit protein$^{-1}$ and used to determine the apparent kinetics values using the program GRAPHPAD PRISM-4 (Graphpad, La Jolla, CA, USA).

Cellulose biosynthesis inhibitor studies

The effect of three CBI, isoxaben (1-[3-(1-ethyl-1-methylpropyl)-1,2-oxazol-5-yl]-2,6-dimethoxybenzamide; Dow Chemical Company, Midland, MI, USA), DCB (Sigma Aldrich, St Louis, MO, USA), and morlin (7-ethoxy-4-methyl, coumarin Sigma Aldrich, St Louis, MO, USA) were employed to evaluate the plant responses to increased concentration on an in plate assay. Due to the large-sized maize seeds and the poor germination rate of switchgrass
post-germination. Drugs were evaluated on the root length and determined 8 days later.

Inoculum preparation and chemicals
Clostridium thermocellum ATCC 27405 was provided by Michael Flythe (USDA, Lexington, KY, USA) and was maintained in our laboratory, as described by Johnson et al. (1981). Chemically defined thermophile medium was prepared as described by Uhamagadda et al. (2010). A bacterial culture was grown for 24 h in Balch tubes (pH 6.7) at 63°C containing cellulose strips (Whatman Grade 1 filter paper) suspended in 8.5 ml basal medium. This initial culture was used to inoculate 54 ml of cellulose fiber in 100-ml sealed serum vials (Bellco Biotechnology, Vineland, NJ, USA) under CO2 atmosphere. After 18 h of growth, the secondary culture was diluted with fresh basal media (substrate free) to prepare the standard inoculum stock (final optical density of 0.162 OD 600; ~0.078 g dry cells l−1).

Biomass microbial cultures and sampling
One milliliter of prepared inoculum culture was inserted by syringe into a test tube containing 8.5 ml of thermophile medium and 0.5 g plant material. One hundred and twenty-five microliters of prepared inoculum culture was inserted by syringe into a test tube containing 8.5 ml of thermophile medium and 0.5 g of plant material to achieve a 5% w/w bacterial suspension.

X-ray diffraction analysis
Finely ground biomass samples were then contained in a custom-built sample holder of pressed boric acid. In brief, plant material was placed into a mold, containing a sleeve and hand pressed with a solid metal plug forming a disk shape. The sleeve and plug were removed and a boric acid (Fischer, Madison, WI, USA) base was then formed by pouring the boric acid over the bottom and sides of the sample and applying 40,000 psi of pressure to the 40 mm × 40 mm mold using a Carver Autopellet Press (Wabash, IN, USA). Samples were pressed to create an even horizontal surface. A Bruker AXS D8 Diffractometer (Bruker-AXS USA, Madison, WI, USA) was used for wide angle XRD with Cu Kα radiation generated at 30 mA and 40 kV. The experiments were carried out using Bragg-Brentano geometries (symmetrical reflection). Diffractograms were collected between 5° and 35° (for samples with little baseline drift), with 0.02° resolution and 2-s exposure time interval for each step. Sample rotation to redirect the X-ray beam diffraction site was achieved per replicate. The data analysis was carried out using the calculation for RCI of: RCI = Ibg2 − Imin/IBG2 × 100, where Ibg2 is the maximum peak height above baseline at approximately 22.5° and Imin is the minimum peak height above the baseline at ~18°. For assessment of experimental accuracy, the pressed samples were examined using reflective geometries at 22.5° 2-theta with the sample scanned rotationally (360°) and in an arc (90°) to obtain an intensity/spatial orientation plot of a sample for which the RCI had already been established. The range of reflective intensities was then used to estimate the accuracy of the RCI determination using a 95% cutoff across the plot range. Diffractograms were collected in Diffrac-Plus-XRD Commander software (Bruker-AXS, Karlsruhe, Germany) and minimally processed (baseline identification, noise correction, 3D display, and cropping of RCI signature region) using the EVA and TextEval (Bruker-AXS Karlsruhe, Germany) software.

Phylogenetic analysis
The sequences of the A. thaliana cellulose synthase genes were obtained from the TAIR database3 and were utilized as the initial reference in order to obtain the putative CESA genes of the grasses. The cDNA sequences of the ten cellulose synthase genes of A. thaliana were employed to conduct the BLASTX (Altschul et al., 1997) search within the Setaria italica, Sorghum bicolor, Panicum virgatum, and Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI4. Due to the very few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI. Due to the few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI. Due to the very few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI. Due to the few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI. Due to the very few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI. Due to the few number of putative CESA genes for Panicum virgatum, Z. mays genomes. BLASTX searches were conducted using the phytozome database provided by JGI.

3http://www.arabidopsis.org
4http://phytozome.net

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analyzed using PhyML (v.3.0; Guindon et al., 2010). The advanced setting within PhyML conducts a sequence alignment using MUSCLE (v.3.7; Edgar, 2004), then automatically utilizes the alignment to create the phylogenetic tree. A total of 500 bootstrap replicates were conducted under the WAG (Whelan And Goldman) protein substitution model (Whelan and Goldman, 2001).

In addition to this tree, a new tree was constructed using the DNA sequences of the 36 putative CESA genes as well as the 10 Arabidopsis CESA genes as reference. The sequences were obtained from the Phytozome database and the TAIR database, respectively. The sequences were submitted to the Guidance web server (Penn et al., 2010) and aligned with MAFFT (Katoh et al., 2005). Guidance is a program that both aligns sequences and assesses confidence in each position in the alignment, in order to objectively identify and remove regions of ambiguous alignment prior to phylogenetic analysis. Any column in the alignment that had a confidence score of less than 95% was removed by Guidance.

The sequences were submitted to the Phylogeny Analysis and Statistical Inference Tree (GBL) and the United States Department of Agriculture (DOE) were conducted, both using the default settings.}

The resulting maximum likelihood tree was then visualized via FIGTREE1.

STATISTICAL ANALYSIS
Statistical analysis consisting of one-way analysis of variance (ANOVA) with a post hoc Tukey test was performed by using PRISM (Graphpad, La Jolla, USA). The outcomes are listed in letters on the corresponding graphs. Same letter signify non-significance (P > 0.05) whereas different letters indicate significance (P < 0.05).

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REFERENCES
Alonso, M. D., Wetstein, S. G., Muller, M. A., Garber, E. I., and DiNapoli, J. A. (2013). Integrated conversion of hemi- and cellulose from lignocellulosic biomass. Energy Environ. Sci. 6, 76–80. doi: 10.1039/C2EE23487F
Ahmed, S. I., Madden, T. L., Schaf- fer, A. A., Zhang, J., Zhang, Z., Miller, W., and (1997). Capped BLAST and PI-BLAST: a new gen- eration of protein database search programs. Nucleic Acids Res. 25, 3389–3402. doi: 10.1093/nar/25.17. 3389
Anderson, S., Serima, B., Pfeiffer, T., Saranpää, P., and Pouwen, E. (2005). Crystalinity of wood and the size of cellulose crytals in Norway spruce (Picea abies). J. Wood Sci. 51, 533–537. doi: 10.1007/s10086-003-0154-8
Andrus, T., Peng, L., Botner, A. S., Burn, J., Wittke, W., Huth, W., and (1990). Molecular analysis of cellulose biogenesis in Arabidopsis. Science 279, 717–720. doi: 10.1126/ science.279.5351.717
Bauert, W. D., Talmage, K. W., Keegstra, K., and Akoima, C. (1975). The structure of plant cell walls II: The hemi-cellulose of the walls of sunflower cultured immature cells. Plant Physiol. 55, 174–187. doi: 10.1104/pp.55.1.174
Bennetzen, J. L. (2007). Patterns in grass genome evolution. Curr. Opin. Plant Biol. 10, 176–181. doi: 10.1016/j.pbi.2007.01.010
Bradham, C., and Dedhia, S. (2013). Chemical genetics to examine cellulose biosynthesis. Front. Plant Sci. 3:99. doi: 10.3389/fpls.2012.00099
Brennan, T., Wang, L., Swartwood, K., Goldsmith, A., Jackson, D., Zhu, X.-G., and (2010). Satetie unveils a model for Ca+ Pla nphotynase. Plant Cell 22, 2537–2544. doi: 10.1105/tpc.109.105703
D’Alonzo, D. F., and Amor, Y. (1995). Cellulose biosynthesis. Plant Cell 7, 985–1006.
Doppen, T., Vernet, S., Jastrzebski, M., Reiter, S., Denes, T., A., et al. (2003). Resistance against herbivore-induced cellulase defi- ciency caused by distinct mutations in same cellulase synthase isofor. CESA6. Plant Physiol. 128, 482–490. doi: 10.1104/pp.010822
Dhamaldal, V. S., Nokes, S. E., Steed, H. J., and Hyre, M. D. (2010). Investigation of the metabolic inhibition observed in solid substrate cultivation of Gluconobacter thermodenitrificans on cellulose. Bioresour. Technol. 101, 6038–6044. doi: 10.1016/j.biortech.2010.02.097
Doust, A. N., Kellogg, E. A., Devos, K. M., and Bennetzen, J. L. (2009). Zonal motif: a sequence-driven grass model system. Plant Physiol. 148, 137–141. doi: 10.1104/ pp.108.123627
Downe, R., and Beffley, J. D. (1994). Dormancy in white spruce (Picea glauca (Moench.) Voss) seeds is imposed by tissue surrounding the embryo. Seed Sci. Res. 4, 9–15. doi: 10.1017/s0960258500002944
Edgar, R. C. (2004). MUSCLE: a multi-ple sequence alignment method with reduced time and space complex-ity. BMC Bioinformatics 5:113. doi: 10.1186/1471-2105-5-113
Guindon, S., Dufrairé, J.-F., Lafort, V., AmiMarina, M., Hovfuk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML. Syst Biol. 59, 307–321. doi: 10.1093/sysbio/sys057
Gutierrez, R., Lindholm, J. J., Paredos, A. R., Emoto, A. M. C., and Ehrlund, D. W. (2009). Arabidopsis cortical microtubule position cellul-lose synthase delivery to the plasma membrane. BMC Biol. 7, 3389–3402. doi: 10.1186/bi0186
Harris, D., and DeBolt, S. (2006). Genetic modification in cellulase-synthase reduces crystallinity and improves biochemical con-version to fermentable sugar. Gbi: Chem. Bio. Bioenergy 1, 51–61. doi: 10.1111/j.1754-1094.2006.00100.x
Harris, D. M., Corbin, K., Wang, T., Gutierrez, R., Bertoia, A. L., Potts, C., et al. (2012). Cellulose microfibril crystallinity is reduced by mutating C-terminal transmem-brane region residues CESA5R536V and CESA3R542C of cellulase syn- thase. Proc. Natl Acad. Sci. U.S.A. 109, 2608–2613. doi: 10.1073/ pnas.1202531109
Heinl, D. R., Bröder, L. A., Jons, I., Schneegurt, M. A., and Larrinua, I.

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Heim, D. R., Roberts, J. L., Pike, P. D., and Larrman, I. M. (1999). Mutation of a locus of Arabidopsis thaliana confers resistance to the herbicide isoxaben. Plant Physiol. 119, 136–140. doi: 10.1104/pp.119.2.136

Hillman, J. M., and Quick, P. W. (2002). Characteristics of C4 photosynthesis in stems and petioles of C3 growing plants. Nature 415, 451–454. doi: 10.1038/415451a

Hidayat, B., Belley, C., Johannsen, K., and Thiyagarajan, G. (2012). Cellulose is not just cellulose: a review of dilucinations as reactive sites in the enzymic hydrolysis of cellulose microfibrils. Cellulose 19, 1405–1409. doi: 10.1007/s10570-012-9740-2

Johnson, E. A., Mada, A., and Domair, A. L. (1981). Chemically defined minimal medium for growth of the anaerobic, cellulolytic -thermophile (Cystalium thermocellum Appl. Environ. Microbiol. 41, 1060–1062.

Kath, K., Kima, K., Eoh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33, 51–58. doi: 10.1093/nar/gkj130

Kim, Y., Mosier, N., Ladisch, M., et al. (2005). Microbial conversion of a locus of tolerance in M. (1993). Mechanism of isoxaben resistant barley. J. Exp. Bot. 44, 1185–1189. doi: 10.1093/jxb/191361598

Kuehne, B., Smith, K., and Medina, J. R. (2007). The TIGR rice genome annotation resource: improvements and new features. Nucleic Acid Res. 35, 2003–2008. doi: 10.1093/nar/gkm979

Kurihara, R., Robertson, S., Raikhel, N. V., and Freeling, M. (2009). Regional examination of large biological sequence alignments. Trends Plant Sci. 14, 723–727. doi: 10.1016/j.tplants.2009.04.003

Kudla, J. (2003). The biology and agronomy of switchgrass for biofuels. Crop. Rev. Plant. Physiol. 24, 425–490. doi: 10.1104/pp.102.058947

Kudla, J. A. (2011). C4 cytos: past, present, and future in C4 photosynthesis. Plant Cell 23, 1879–1892. doi: 10.1105/tpc.111.088820

Kümer, L., and Buell, C. (2004). Comparative study on enzymatic digestibility of switchgrass varieties and harvests processed by leading pretreatment technologies. Biotechnol. Biofuels 7, 11089–11096. doi: 10.1186/1757-1626-7-8

Lee, S., Noh, S., and Han, M. (2009). The genetic control of lignin deposition in stems and petioles of C3 flowering plants. Nature 459, 451–454. doi: 10.1038/nature08041

Lefebvre, A., and Higuera, C. M. (1959). Analysis of Large Biological Sequence alignments Confidence score capturing robustness to guide tree uncertainty. Mol. Biol. Evol. 27, 1779–1787. doi: 10.1093/molbev/msr088

Li, X., Ximenes, E., Kim, Y., Slininger, J. (1993). Mechanism of isoxaben resistance in barley. J. Exp. Bot. 44, 1185–1189. doi: 10.1093/jxb/191361598

M., Meilan, R., Ladisch, M., et al. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 104, 15366–15371. doi: 10.1073/pnas.0706592104

Penncross M., Oula, G. (2008). C4 photosynthesis and plant adaptation. Ann. Bot. 101, 105–110. doi: 10.1093/aob/mcm153

Penncross M., and Buell, C. (2004). Comparative study on enzymatic digestibility of switchgrass varieties and harvests processed by leading pretreatment technologies. Biotechnol. Biofuels 7, 11089–11096. doi: 10.1186/1757-1626-7-8

Rogers, L., and Campbell, M. (2004). Mechanisms of lignin deposiation during plant growth and development. New Phytol. 164, 12–30. doi: 10.1111/j.0006-3553.2004.01010.x

Storck, J., Harris, D., Griffiths, J., and Schulze, H. V., et al. (2011). Plant Physiol. 153, 580–589. doi: 10.1104/pp.111.179069

Wang, X., Gowik, U., Tang, H., Bowker, S., Grover, J., and Packer, A. (2008). Comparative genomic analysis of switchgrass phytology evolution in grasses. Genome Biol. 10, 198. doi: 10.1186/gb-2009-10-6-198

Wang, Z., Li, R., Xu, J., Martina, J., Hartfeld, E., Cui, Q., et al. (2012). Sodium hydroxide pretreatment of genetically modified switchgrass for improved enzymatic release of sugars. Biotechnol. Biofuels 5, 3670. doi: 10.1186/2046-1816-5-3670

Uehleke, R., and van der Hooren, B. (2010). Emerging principles in plant chemical genetics. Trends Plant Sci. 15, 81–86. doi: 10.1016/j.tplants.2009.11.005

Updegraff, D. M. (1969). Semimicro determination of cellulose in biological materials. Anal. Chem. 41, 420–424. doi: 10.1021/ac6026400

Updegraff, D. M. (1969). Semimicro determination of cellulose in biological materials. Anal. Chem. 41, 420–424. doi: 10.1021/ac6026400
Datasets Under the Maximum Likelihood Criterion. Austin: University of Texas.

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