The Center for Bioenergy Innovation (CBI)

Performance Metric for FY20: Report on genomic science-based advances and testing of new plant feedstocks for bioenergy purposes.

Q2 Metric: Report on the development of switchgrass variants as a dedicated bioenergy crop.

March 2020

1. Introduction

Unlocking the secret to recalcitrance, or the resistance of a plant to release sugar from its cell walls, was a primary goal of the BioEnergy Science Center project (BESC, 2007-17). In addition, the goals of the Center for Bioenergy Innovation project (CBI, 2017-present) are to increase the yield and sustainability of plant feedstocks as well as to enable valuable bio-based fuels and products, made from the cell wall polymers. *Populus* and switchgrass were chosen as the primary feedstocks to study cell-wall-related genetic modifications that could impact biomass properties. Both are high yield perennials and recognized as potential domestic feedstocks.

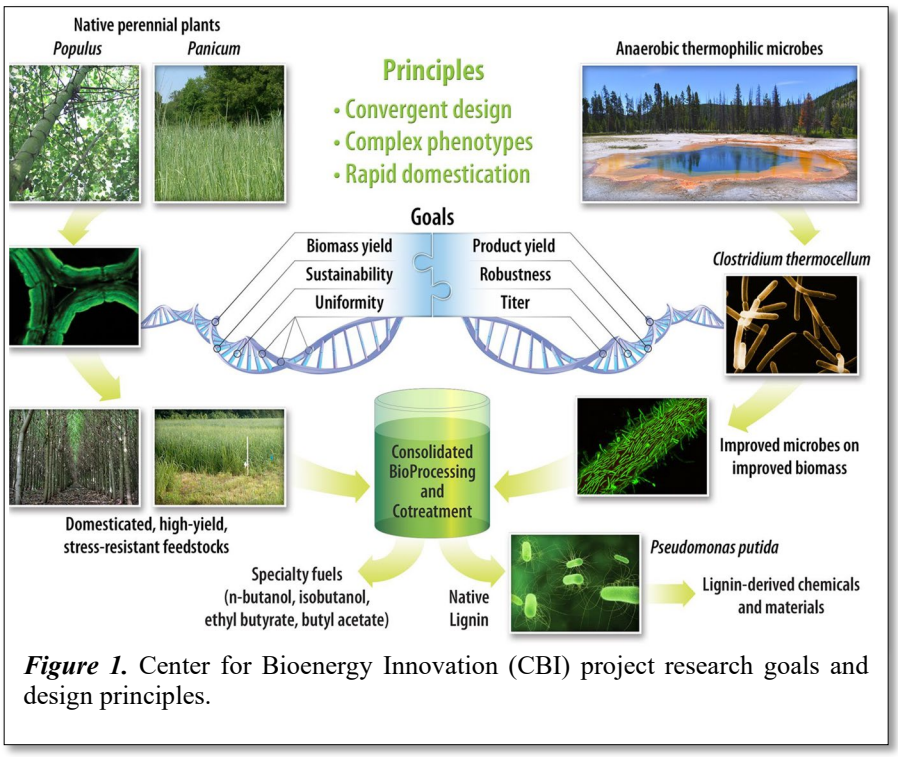


Figure 1. Center for Bioenergy Innovation (CBI) project research goals and design principles.

Through our DOE Bioenergy Research Center projects, BESC and CBI, we have sought and continue to seek to create major advances in accelerating the domestication of these two recognized bioprocessing feedstocks, switchgrass and poplar (Figure 1).

Switchgrass, *Panicum virgatum*, is a perennial, fast growing, warm season grass, which is widely adapted to native environments indigenous to North America, making switchgrass well suited to deployment in target marginal environments. From a breeding point of view, switchgrass is largely an obligate outcrossers, meaning that self-pollination is not feasible with this species. A range of resources are available for research with switchgrass, including association (GWAS) and quantitative trait loci (QTL) mapping populations, genetic tools and an assembled and annotated reference genome.

Here we summarize our research towards the development of switchgrass variants as a dedicated bioenergy crop. Transgenic approaches, as well as QTL approaches based on structured pedigrees and GWAS approaches based on random collections of unrelated genotypes, were employed to gain insights in cell wall chemistry, biomass productivity and sustainability of switchgrass. Optimizing the genetic transformation systems has occurred over a 10-year period. Outcomes and conclusions from these investments are presented in chronological order starting with production of switchgrass variants using conventional transgenic approaches, followed by most recent CRISPR based genome editing results, and concluding with the identification of high-performing genotypes and significantly associated genetic loci from natural or breeding populations.

Highlighted Results

2. Generation of switchgrass variants using transgenic approaches

Development of efficient switchgrass transformation methods. Traditionally low efficiency of switchgrass transformation had been an issue in the scientific community at the start of this project. Over the initial 5 years, we increased the overall efficiency of the switchgrass transformation from initially 5% up to 90% and decreased the turnaround time to less than four months. This development was key in accelerating improvements of switchgrass as a dedicated bioenergy crop. Inter-Institutional research partnerships (Noble Foundation, University of Tennessee-Knoxville (UTK) and University of Georgia-Athens (UGA)) developed a high-throughput switchgrass transformation system that enabled a switchgrass transformation pipeline producing over 120 gene constructs (Mann et al., 2012; Nageswara et al., 2013; Nelson et al. 2018). The switchgrass genetic transformation system was optimized by using inflorescence-derived calli for *Agrobacterium* infection. The developed methods to generate transgenics are restricted to a given genetic background (Figure 2). Overall, transformable embryonic or seed-derived calli from Alamo or Alamo-derived clones (ST1, ST2, SA1, NFCX1) were used for the stable

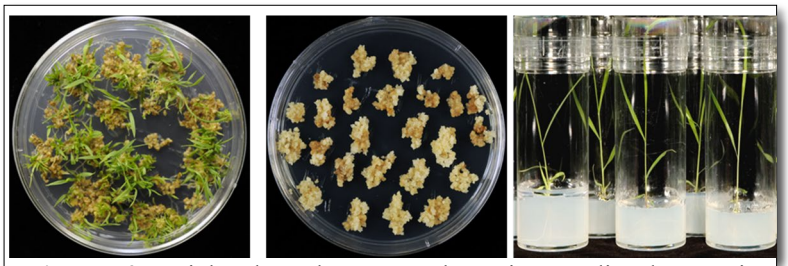


Figure 2. High throughput Agrobacterium-mediated genetic transformation of switchgrass.

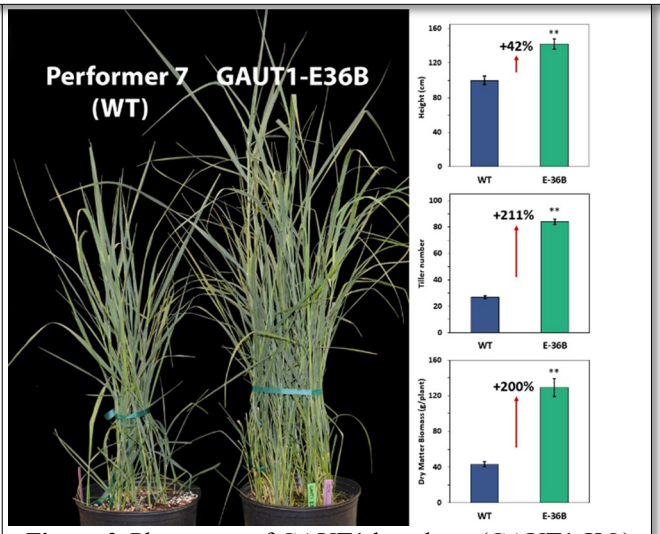


Figure 3. Phenotype of GAUT1 knockout (GAUT1-KO) in switchgrass. Image showing a wild-type (genotype Performer 7) plant on the left and a GAUT1-KO (GAUT1-E36B) on the right. The GAUT1-KO plant displays significantly increased (top) height, (middle) tiller number and (bottom) dry matter biomass.

agrobacterium-mediated transformation pipeline of the project (Nelson et al. 2018). In addition, an extensive Gateway-compatible vector set was optimized for under- and overexpression (OE) of target genes in switchgrass. This promoter has been used broadly across labs for functional analysis of candidate genes impacting recalcitrance (Mann et al., 2012). Finally, the switchgrass *ubi1* promoter was identified as a strong promoter for expressing transgenes, which has subsequently used in a wide variety of other plant species.

Switchgrass genome editing technologies. Recent efforts in rapid targeted genetics include development of a CRISPR-Cas9 system for switchgrass. CRISPR-KO efficiency was evaluated in switchgrass using three different Cas9 proteins (i.e., SpCas9, SaCas9 and St1Cas9) and two Cpf1/Cas12a proteins (i.e., AsCpf1 and Mb3Cpf1) at 27°C and 37°C to add flexibility and improve efficiency to the CRISPR-CASA9 system. As proof-of-principle, we recapitulated the GAUT4/GAUT1-KD phenotype using CRISPR-Cas9 in switchgrass which had been previously developed using conventional agrobacterium-mediated RNAi technologies (Figure 3). Transient expression of embryogenesis genes to develop a more genotype-independent transformation method is currently under investigation.

In all, 128 switchgrass genes were selected for transgenic construct generation in the project. Data on gene construct cloning, expression quantification and phenotypic effects are summarized in a peer-reviewed report in 2018 (Nelson et al. 2018). The successes and lessons learned in building a multi-institutional transformation pipeline for switchgrass is summarized in the report (Nelson et al. 2018). Here, we present some highlights of salient phenotypes found in the switchgrass transgene lines developed through using our pipeline. (Attached is a link to published summary table of all tested phenotypes reported by Nelson et al. 2018: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-017-0991-x/tables/1). Some of the selected "TOP" lines and their putative functions are listed in Table 1. Characterization of some of these lines are reported below.

Table 1. Reduced recalcitrance switchgrass TOP Lines: selected, grown in greenhouse and field.

***P. virgatum* gene**	**Function (putative)**
PvGAUT4-KD	pectin synthesis
PvCCR1-KD	lignin synthesis
PvFPGS1-KD	carbon metabolism
PvMYB4-OE	transcription factor, lignin
PvCOMT-KD	lignin synthesis
PvmicroRNA156-OE	growth and development
PvSGAMP15_14	natural variant
PvSGAMP15_05	natural variant
PvEFR/SHINE TF-OE	transcription factor
PvGAUT1-KD	pectin synthesis
PvIRX10-KD	hemicellulose synthesis
PvGA2Ox-OE	hormone metabolism

TOP performing lines (TOP lines; Nelson et al. 2018) from a minimum of 10 independent transformation events, with multiple regenerated shoots per event, were selected following reverse transcriptase polymerase chain reaction (RT-PCR) screenings of independent lines generated from these constructs. These selected lines also met the criteria of stable transgene expression, intended modulation of gene expression, positive impact on one or more of the desirable biomass properties (higher biomass production, higher cellulose, lower lignin, higher sugar release efficiency and conversion efficiency to fuels based on standardized trait analysis methods (see Figure 4 for the selection protocol and criteria).

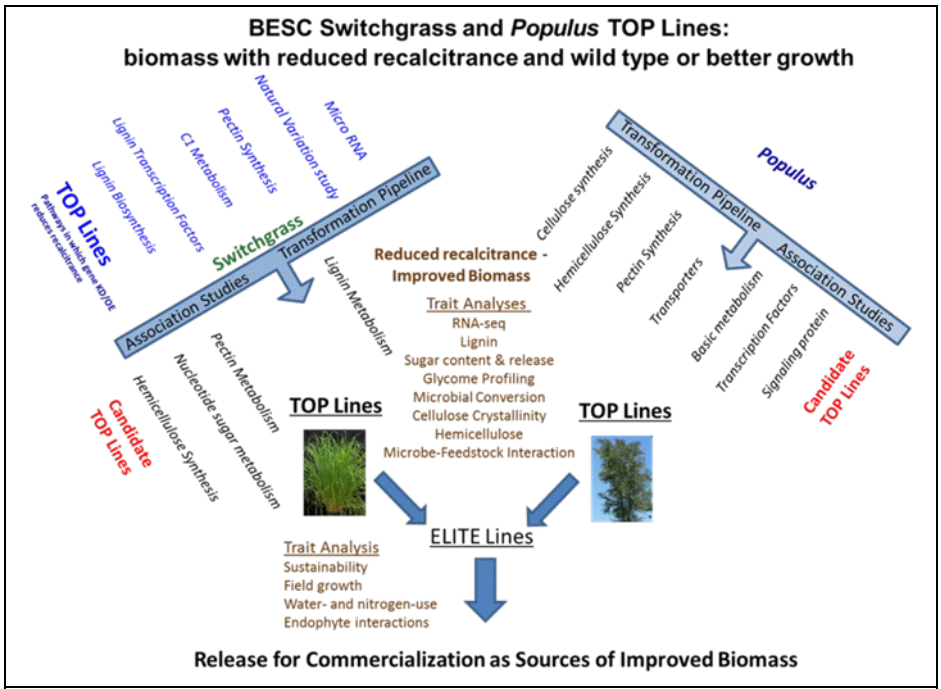


Figure 4. Overview of selection of TOP performing switchgrass lines identified via natural variation and transgenesis.

In summary, we improved the underlying tools for switchgrass by both improved transformation efficiency (to 90%) and time for production of transformed plants (to under four months). This enabled the development of a coordinated transformation pipeline to produce more than 128 modified switchgrass lines.

3. Transgenic line characterization for improved biomass productivity, sustainability, wall chemistry and conversion properties in greenhouse and field

Characterization platforms for plant cell wall chemistry and recalcitrance. Development of new higher-throughput methods or characterization platforms was undertaken through the larger project effort in order to enable detailed characterizations of the chemical, structural and physical

properties of biomass and how these properties influenced breakdown by enzymes and thermophilic microorganisms (Figure 5).

One challenge was to analyze thousands of variable plant samples for composition and digestibility. Over the initial 5 years we developed a method to complete pretreatment and subsequent enzymatic digestion in a stainless steel 96-well plate. Here we were able to decrease the standard sample size from 5 grams to 4 milligrams and consequently enable our ability to analyze more than 10,000 samples per year using robotic assisted sample handling techniques. Combined with high-throughput rapid small-scale methods to measure composition, we were able to analyze tens of thousands of naturally variant or genetically engineered plants. By measuring thousands of samples, we were able to learn of the immense variability of the diverse natural and transgenic variants of switchgrass for composition and digestibility. These results laid the foundation for using conventional breeding and selection to identify better plant parental lines.

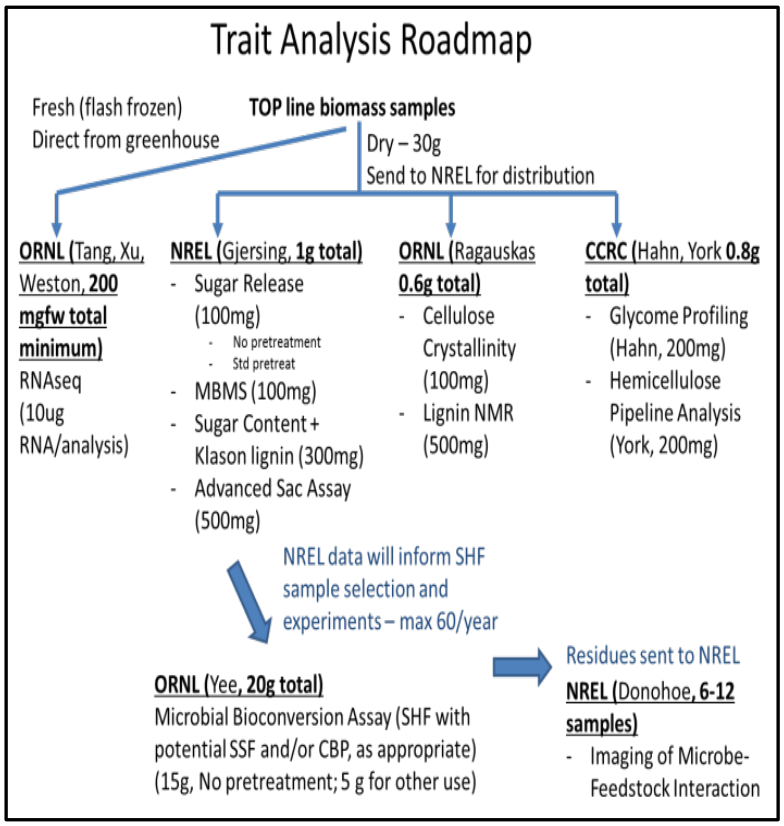


Figure 5. Flowchart showing TOP Lines analyses and amounts of Populus and switchgrass tissues required. Taken from the BESC TOP Line Analysis Booklet.

Deeper characterization of the plant cell wall structure was also performed using high-resolution techniques including gas chromatography (GC) and high-performance liquid chromatography (HPLC) mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and molecular modeling to gain insights into the mechanisms of biomass recalcitrance. In this effort we discovered that enzyme accessibility (i.e., the characterization of pores which allow microbes or enzymes to get to the biomass surface) significantly and positively influenced sugar release in natural variants and transgenics alike. In addition, we definitively determined that plant cell polymers, i.e., cellulose, hemicellulose, pectin and lignin, have critical chemical linkages and physical entanglements that prevent accessibility and that these properties could be measured and modified in plants. For example, the changes in the composition of lignin (a polyaromatic component of biomass which acts as a “glue”) and in the chemical bonds formed within lignin (e.g., syringyl to guaiacyl (S/G ratio)) had significant effect on biomass recalcitrance and sugar release.

In situ characterization approaches also revealed that some pretreatments cause lignin re-precipitation as aggregates, which initially increases the accessibility to biological attack of the carbohydrate surface by pulling the lignin into solution and away from most of the surface. However, aggregates subsequently formed and were deposited on the cellulose surface, ultimately block solubilization. We also discovered that pectin, a relatively minor component in bulk biomass, has a disproportionate effect on recalcitrance – likely due to its complex linkages to the other cell wall polymers, as well as possible entanglements onto itself and other wall polymers. Using imaging techniques, based on atomic force microscopy (Raman spectroscopy) and mass spectrometry, that we optimized we were able to increase the visualization of the chemical nature of pretreated and deconstructed biomass surface at the submicron level.

Greenhouse evaluation of transgenic switchgrass lines modified in expression of cell wall biosynthesis pathway genes. Among the first observations in transgenic switchgrass plant evaluation was that a single gene in lignin biosynthetic pathway, *COMT*, reduces recalcitrance with no apparent growth penalties. Moreover, biomass from these *COMT* knockdown lines resulted in increase in ethanol production by over 30 percent, which translated to a reduction in needed severity of pre-treatment and a concominant reduction in biofuel processing costs by at least 20% with 300-400% lower enzyme costs (Fu et al. 2011, Figure 6). Finally, among analysis of additional lignin biosynthesis pathway genes, saccharification efficiency after dilute acid pre-treatment was shown to increase with biomass obtained from *Pv4CL1* and *PvCAD*.

Figure 6. Genetic manipulation of lignin via knockdown of COMT gene expression reduces recalcitrance and improves ethanol production from switchgrass

Recently, the transformation pipeline work has also showed that a novel gene, the folylpolyglutamate synthetase (*FPGS*), is a new target for reducing recalcitrance in switchgrass. FPGS is involved in the generation of folate polyglutamate, the preferred folate cofactor for enzymes involved in C1 metabolism for formation of methyl units required for the synthesis of lignin. Inflorescence stems of Arabidopsis *FPGS1* mutants had shown reduced lignin content (Figure 7A) and a higher saccharification efficiency. *PvFPGS1* down-

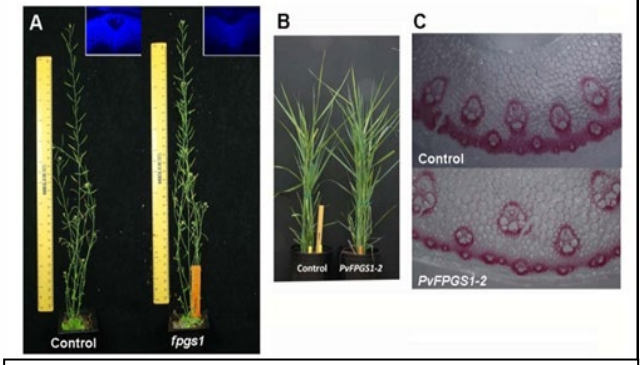


Figure7. Down regulation of FPGS in Arabidopsis (A) and switchgrass (B, C) results in lower lignin.

regulated lines have normal growth, lower lignin content, higher biomass digestibility and improved saccharification efficiency. Switchgrass RNAi lines showed reduced lignin with no adverse effects on biomass (Figure 7B, C).

GAUT4 is a putative pectin biosynthetic homogalacturonan galacturonosyltransferase (Atmodjo et al., 2013) and is one of multiple pectin-associated genes whose modified expression was shown to result in reduced recalcitrance and enhanced growth. Research related to the mechanisms by which knockdown of a pectin biosynthetic gene causes these phenotypes is ongoing; however, the discovery of the plant cell wall proteoglycan, arabinoxylan-pectin-arabinogalactan protein 1 (APAP1), provided a potential mechanism whereby reduction in a pectin domain could affect the tethering of hemicellulose in the wall (Tan et al., 2013). GAUT4 knockdown switchgrass line was identified with 15% increase in total sugar release, 322% increase in tiller number, and 89% greater dry weight per plant compared to wild-type and vector controls, as well as an increased ethanol yield of 36% mg/g cellulose and 51% mg/g dry biomass compared to wild-type. These phenotypic observations made using switchgrass were found to be consistent with the decreased recalcitrance and/or increased growth of GAUT4-KD in *Populus*, rice and foxtail millet. A potential role for pectin and arabinogalactan proteins in the fermentation of plant biomass by microbes was also suggested by the inter-institutional collaborative project on the solubilization of switchgrass by *C. bescii* (Kataeva et al., 2013).

Apart from lignin and pectin synthesis pathway genes (*PvGAUT4*-KD and *PvGAUT1*-KD), the transformation pipeline was used to evaluate genes from cellulose biosynthesis (*PvCesA4* and *PvCesa6* RNAi lines), hemicellulose biosynthesis (*PvIRX10*-KD) and hormone metabolism (*PvGA2O*x-OE) pathways as well. Transgenic switchgrass *PvCesA6*-overexpressing lines had reduced lignin content, increased sugar release efficiency, but reduced overall biomass productivity (Mazarei et al. 2018).

Greenhouse evaluation of transgenic switchgrass lines modified in expression of transcriptional regulators or metabolism genes. Next, characterization of greenhouse-grown switchgrass plants over-expressing PvMYB4 transcriptional repressor of lignification revealed new features of the chemistry of plants with strongly reduced recalcitrance, including alteration in wall-bound coumarate-to-ferulate ratio and fucosylation of cell wall polysaccharides (Shen et al., 2013). Overexpression (OE) of *PvMYB4* gene (lignin/phenylpropanoid pathway repressor), was found to result in a remarkable three-fold increase of sugar release efficiency and a 2.6-fold increase in ethanol production by simultaneous saccharification and fermentation (SSF) without pre-treatment. These transgenic materials have been extensively characterized for cell wall properties by our inter-institutional partners. The studies suggest that wall-bound phenolics, as well as lignin content and lignin molecular weight, are major contributors to the reduced cell wall recalcitrance in switchgrass.

Greenhouse observations also confirmed that transgenic knock-down of the switchgrass ortholog of a WRKY transcription factor acts as a negative regulator of secondary cell wall formation in pith tissues (Wang et al., 2010) and was found to result in increased secondary wall formation in switchgrass stems. However, transgenic lines with the greatest change in transgene expression

also had reduced tiller length and stem diameter. Among other works, modification of microRNA156 (PvmicroRNA156-OE) expression using transgenic means showed that microRNA156 is important to plant growth, conversion, and biomass yield due to increased tiller number (Fu et al., 2012). This overexpressing miR156 genotype is was found to be non-flowering in the greenhouse, a trait is beneficial for transgene containment in outcrossing species like switchgrass.

Contribution to understanding of cell wall synthesis and mechanisms via transgenic switchgrass showing that multiple pathways impact cell wall digestibility in switchgrass. Exciting advances in the biochemistry of switchgrass cell wall polymer synthesis have been complemented by functional studies recording the impacts of modification of the different cell wall polymers on recalcitrance in switchgrass. Throughout the length of the BRC work our studies have highlighted the importance of lignin in cell wall recalcitrance. The cell walls of grasses differ from those of dicots primarily through the possession of much higher levels of wall associated coumarate and ferulate. Interestingly, recalcitrance was reduced in some field grown MYB4 over-expressing plants in which lignin levels were normal, thus, the reduced recalcitrance of the material is therefore likely a result of alterations in wall-associated phenolics (Shen et al., 2013). The greenhouse-based, single transgenes results were highlighted above. However, lignin is not the only polymer affecting recalcitrance. Transgenic switchgrass down regulated in the expression of GAUT4, GAUT1, GAUT 7, GAUT14 (pectin pathway, Figure 5), or IRX10 (xylan xylosyltransferase) all showed enhanced cell wall sugar release, often with significantly increased biomass production. These observations have led us to begin stacking transgenes for the simultaneous modification of lignin, pectin and hemicellulose (xylan) in switchgrass, based on the premise that modification of multiple "recalcitrance pathways" will have additive or possibly synergistic effects on reducing recalcitrance. Multiple independent

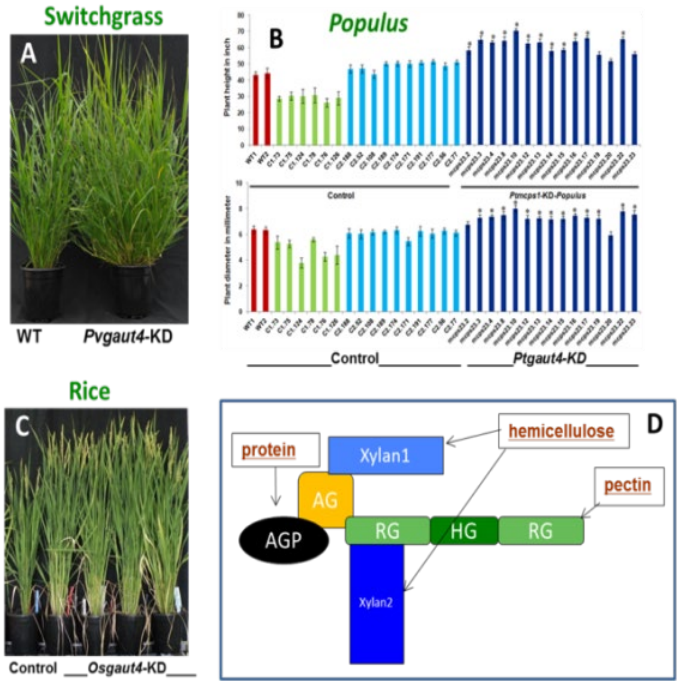


Figure 8. Phenotype of GAUT4-KD in transgenic switchgrass, Populus and rice, and schematic of novel proteoglycan cell wall structure, APAP1. **(A)** PvGAUT4-KD switchgrass lines had greater plant height, number of tillers, and dry weight than controls and 24% more glucose release than controls. **(B)** PtFAUT4-KD Populus lines had greater plant height, increased stem diameter and increased glucose release (4-7%). **(C)** OsGAUT4-KD rice lines had greater plant height, increased stem diameter and increased dry weight biomass than controls. **(D)** Schematic showing structure of novel cell wall proteoglycan structure APAP1.

stacked lines generated in the second five years of the project, though our corporate partner Ceres, are now moving into the analytical pipeline. This work also relates to recent findings, arising from plant and conversion science collaborations among several BRCs, indicating that reduction in lignin levels facilitates growth on plant biomass by *Caldicellulosiruptor* mutants lacking pectinase genes. These results clearly indicate that there are yet to be discovered interactions between lignin and pectin in the secondary cell wall that significantly impact recalcitrance.

Pectin is the most complex wall polysaccharide and increasing evidence suggests it has roles in primary and secondary wall formation and in recalcitrance (Li et al. 2019; Mohnen et al. 2019). Significantly, knockdown (KD) of GAUT4 also yielded transgenic switchgrass with an increased sugar release and increased growth (Figure 8A). Moreover, KD expression of GAUT4 also resulted in increased sugar release and increased growth in *Populus* and rice, suggesting that discoveries in switchgrass will have conserved function in both grass and dicot species in regard to recalcitrance and growth.

Based on characterization of transgenic switchgrass, along with modified *Populus* and Arabidopsis transgenic lines, a hypothesis was developed that GAUT4 synthesizes a pectin or matrix polysaccharide biosynthetic glycan domain in APAP1 or in an APAP1-like proteoglycan. APAP1 (arabinoxylan-pectin-arabinogalactan-protein 1) is a novel cell wall proteoglycan that we recently identified (Tan et al. 2012) (Figure 8D), and its existence indicates that at least some matrix hemicellulosic and pectic polysaccharides are present in plant cell walls covalently linked to arabinogalactan-proteins. These results indicate a role for the APAP1 proteoglycan in plant wall architecture/function and underscore the importance of determining the mechanism by which GAUT4-KD expression affects recalcitrance and APAP1 affects biomass structure.

4. Enabling capabilities for improved switchgrass.

Sustainability studies: Enhancing biomass productivity under marginal (nutrient-, water- and pathogen-based stress) conditions. Selection of superior switchgrass genotypes is as much a factor of its biomass properties as it is a factor of its productivity under abiotic and biotic stressors. Therefore, there is considerable past and current work on identifying switchgrass variants with higher water-use efficiency, nitrogen-use efficiency and pathogen resistance for use in: 1) genomic selection programs, 2) discovery and testing of key genes and mechanisms underlying these important sustainability traits and 3) characterization and deployment of beneficial plant microbes to increase the yield stability of plants under low-input and environmentally challenging agricultural conditions. Progress in nutrient cycling and microbiome management are listed below.

Nutrient recycling in switchgrass. Recycling of nutrients from aboveground biomass to the root system during annual senescence is an effective strategy for conserving limited resources in the plant-soil system, and in the sustainability of switchgrass. PvNAC1 and PvNAC2 cDNAs encode senescence-induced putative transcriptional regulators of senescence. OE of PvNAC2 in switchgrass increased aboveground biomass and transcript levels of key nitrogen metabolism genes in leaves and nitrate and ammonium transporter genes in roots (Yang et al., 2015). These results hold promise for improving nutrient-use efficiency in switchgrass and other plants.

Furthermore, nitrogen concentration in tillers in field-grown control and two COMT-RNAi reduced recalcitrance transgenic lines at pre- and post-senescence stage, showed that reducing recalcitrance through down-regulation of COMT has no negative influence on nitrogen remobilization in field-grown switchgrass.

Managed microbiomes. We have shown that infection of switchgrass roots by the mycorrhizal fungus *Sebacina vermifera* imparts large biomass gains under normal and stressed conditions (Ghimire and Craven, 2011). Towards this end, a bentonite clay particle delivery system was developed for mass production and dissemination of *S. vermifera* (new strain from new collection) for large-scale field trials. This was evaluated in low lignin caffeic acid *O*-methyltransferase [(COMT) down regulated] switchgrass lines in the greenhouse (Figure 10). *S. vermifera* colonization enhanced plant biomass in both wild-type (WT) and transgenic switchgrass. These results suggest that *S. vermifera* can be packaged and effectively delivered to a target host plant. The presence of *S. vermifera* has little or no impact on cell wall composition.

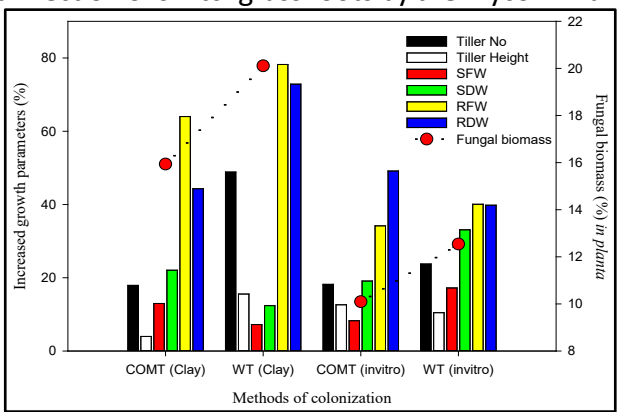
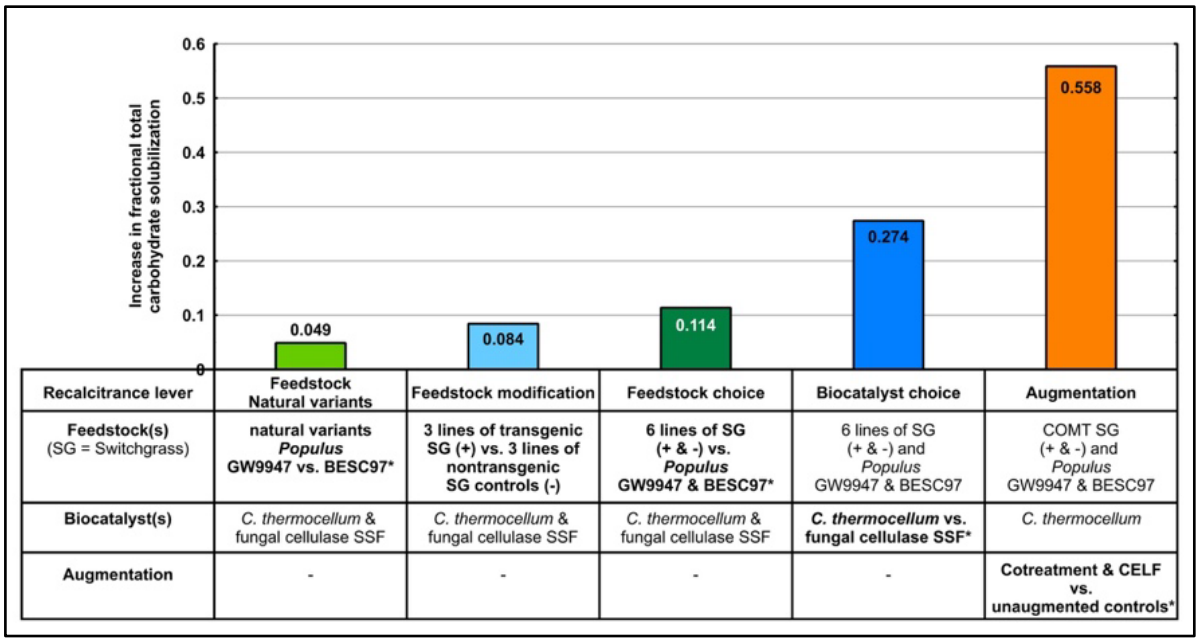


Figure 10. Increased biomass (%) in low lignin (COMT down regulated) and WT switchgrass lines due to S. vermifera colonization 90 dpi. Bars indicate % increase of respective tissue compared to un-inoculated control. Line indicates fungal biomass in planta determined from the ratio of fungus to plant genomic DNA as measured by qRT-PCR (method Charlton et al. 2012). SFW: shoot fresh weight; SW: shoot

Feedstock value enhancement via synergistic improvements in conversion strategies

Enhanced feedstock performance with a new biocatalyst type addition. A comparative investigation involved combinations of three biocatalysts (i.e., naturally occurring micro-organisms like *Clostridium*), three transgenic switchgrass plant lines, two *Populus* natural variants and two non-biological processes to enhance the biological attack using either a mechanical cotreatment or cosolvent-enhanced lignocellulosic fractionation (CELF) pretreatment was undertaken in years 9-10 of the project (Figure 11). Results of this study provide important strategic guidance in overcoming the recalcitrance barrier, proving that solubilization of plant cell walls can be enhanced by non-biological augmentation, the choice of biocatalyst, the choice of plant feedstocks, genetic engineering of plants, and choosing less recalcitrant natural variants. The magnitude of enhancement, however, differs notably under the tested condition, with the largest impacts seen for augmentation and the choice of biocatalyst. This marked the first known study to systematically compare the combined impacts of feedstock and biocatalyst choice, feedstock modification, and non-biological augmentation on plant cell wall deconstruction. While these combined approaches need to be proven at scale, preliminary economic assessments

indicate the potential for nearly eight-fold improvements in the return on investment in a cellulosic biorefinery.



Recalcitrance lever	Feedstock Natural variants	Feedstock modification	Feedstock choice	Biocatalyst choice	Augmentation
Feedstock(s) (SG = Switchgrass)	**natural variants *Populus* GW9947 vs. BESC97***	**3 lines of transgenic SG (+) vs. 3 lines of nontransgenic SG controls (-)**	**6 lines of SG (+ & -) vs. *Populus* GW9947 & BESC97***	6 lines of SG (+ & -) and *Populus* GW9947 & BESC97	COMT SG (+ & -) and *Populus* GW9947 & BESC97
Biocatalyst(s)	*C. thermocellum* & fungal cellulase SSF	*C. thermocellum* & fungal cellulase SSF	*C. thermocellum* & fungal cellulase SSF	***C. thermocellum* vs. fungal cellulase SSF***	*C. thermocellum*
Augmentation	-	-	-	-	**Cotreatment & CELF vs. unaugmented controls***

Figure 11. Relative impact of five recalcitrance levers on total carbohydrate solubilization. The increase in total carbohydrate solubilization for each lever in bold is calculated with other levers as indicated.

5. Concluding Remarks

BESC and CBI have generated a new collection of switchgrass lines with improved biomass properties. These include both targeted (transgenesis through the switchgrass transformation pipeline (TP)) and non-targeted (natural variation) approaches. The collective efforts towards accelerating development of elite switchgrass lines, resulted in creation of significant resources for the community, including establishment of a high-efficiency switchgrass transformation system and vectors, pipeline to streamline gene nomination, cloning, transformation, plant growth and standardized, and in many cases high-throughput, approaches to characterizing chemical and physical properties of feedstocks as they relate to high conversion efficiency. In all, 128 switchgrass genes were selected for transgenic construct generation in the project. Data on gene construct cloning, expression quantification and phenotypic effects are summarized in a peer-reviewed report in 2018 (Nelson et al. 2018). The successes and lessons learned in building a multi-institutional transformation pipeline for switchgrass have been disseminated to the community. We showed that multiple plant genes control cell wall recalcitrance, and that manipulation of these genes can yield lower recalcitrance perennial bio-feedstocks. This included increasing our understanding of the cell wall structure and biosynthetic pathways for lignin, xylan, cellulose and surprisingly pectin and their resultant effects on recalcitrance.

Greenhouse studies were conducted for a limited number of switchgrass lines with reduced recalcitrance arising from both directed transgenics and natural variants. Field trails are reported in a subsequent report. A collective "TOP Line" experimental design protocol was utilized [Nelson 2017] with multiple phenotypic characterization assays. These included, for example, sugar release, sugar and lignin composition, ethanol production, crystallinity. A key discovery was the ability to achieve both lower recalcitrance and higher biomass simultaneously in certain lines.

In summary, significant advances were made in understanding, manipulating, and managing switchgrass biomass and plant cell wall traits and their relation to conversion efficiency. Researchers showed that multiple plant genes control cell wall recalcitrance, and that manipulation of these genes can yield lower recalcitrance perennial bio-feedstocks. This included increased understanding of the cell wall structure and biosynthetic pathways for lignin, xylan, cellulose and surprisingly pectin and their resultant effects on recalcitrance.

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