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.

Q4 Metric: Describe the latest results of field studies of modified switchgrass variants as dedicated bioenergy crops.

September 2020

1. Introduction

Switchgrass (*Panicum virgatum* L.) is a native perennial C4 grass that is considered as a leading candidate for a dedicated lignocellulosic biofuel feedstock owing to its high biomass production, wide adaptation to various geographic and temperate climates, and low agronomic input requirements (van der Weijde et al., 2013). One of the major problems with lignocellulosic crops is the resistance of the cell wall to chemical, microbial, or enzymatic deconstruction into fermentable sugars, a phenomena known as cell wall recalcitrance (Himmel and Bayer, 2009). Reducing the recalcitrance was a primary goal of the BioEnergy Science Center (BESC) project (BESC, 2007–2017). One approach to reduce recalcitrance is by genetically modifying cell wall characteristics in order to develop transgenic feedstocks that are more amenable to enzymatic or microbial hydrolysis (Nelson et al., 2017; Brandon and Scheller, 2020). Several studies involving the transgenic plants were conducted in greenhouses under tightly controlled environmental conditions and the actively growing green tissues were analyzed, instead of the end-of-season senesced tissue, which is most often used for biofuel production (Fu et al., 2011a,b, 2012; Xu et al., 2011; Shen et al., 2012, 2013; Liu et al., 2018; Mazarei et al., 2018). Experiments conducted under such conditions cannot predict how transgenic plants might perform in the field. Normal plant growth and development could potentially be exacerbated under field conditions, where plants are constantly subjected to a variety of biotic and abiotic stresses. Consistently, further studies have shown that several transgenic lines with normal plant growth and development under greenhouse conditions could not survive or had substantial biomass loss under field conditions (Baxter et al., 2015; Baxter et al., 2018; Mazarei et al., 2020). Therefore, field experiments are especially important for modified plants to better predict agronomic performance across multiple growing seasons.

Furthermore, successful establishment and subsequent sustainability of bioenergy feedstocks are key factors for biofuel production (Stewart and Cromey, 2011). An essential component of field experiments is to ensure that plant defenses are not compromised by their genetic modifications. Of particular significance is the rust disease identified as potentially damaging to switchgrass fields (Uppalapati et al. 2013). Increase productivity (i.e., yield) and sustainability of plant feedstocks in bioenergy crops are the main goals of the Center for Bioenergy Innovation (CBI) project (CBI, 2017–present). These goals further emphasize the importance of performing field studies for

improvement of biofuel yield without negative consequences to biomass production or sustainability.

Highlighted Results

2. Field evaluation of switchgrass lines

Up to now, a variety of BESC/CBI switchgrass field experiments have been conducted to evaluate diverse switchgrass lines, including those involving transgenic materials, natural variants, and genomewide association study (GWAS) and F_2 populations across multiple growing seasons and multiple sites. Field trials of transgenic switchgrass were performed according to USDA APHIS BRS release-into-the-environment permits. The field analyses were performed on senesced plant material harvested at the end of the growing season, compared with the young, green tissue that was typically used in the greenhouse experiments. Following the field establishments, a number of traits for agronomic performance, such as plant height, plant width, tiller number, and dry weight biomass yield, were measured. For cell wall characterization, lignin content and lignin syringyl-to-guaiacyl monomer ratio (S/G) were determined. For biorefinery performance, sugar release efficiency and ethanol yield have also been determined. Disease susceptibility, with emphasis on progression of rust infection, was determined as well.

Field experiments of transgenic switchgrass lines

Examples are field-grown transgenic switchgrass COMT-KD (i.e., KD=knockdown) and MYB4-OE (i.e., OE=overexpression) lines with involvement in lignin biosynthesis in plants. One of the main causes of recalcitrance is the presence of lignin in the plant cell wall limiting the accessibility of cellulose to enzymatic deconstruction (i.e., saccharification) for efficient conversion into biofuels (Chen and Dixon, 2007; David and Ragauskas, 2010).

Analyses with fully established field-grown transgenic switchgrass COMT-KD lines (Baxter et al., 2014, 2016) showed that transgenic lines had equivalent biomass yield relative to the wild-type and empty vector control lines. Yet, sugar release was improved by up to 34% and ethanol yield by up to 28% in the transgenic lines relative to controls (Figure 1). In general, analyses with field-grown plants produced results that were similar to those observed in previous greenhouse studies with these plants (Fu et al., 2011a). Another important finding was that transgenic plants were not more susceptible to rust infection. The results of this study suggest that lignin down-regulation in switchgrass can confer real-world improvements in biofuel yield without negative consequences to biomass yield or disease susceptibility.
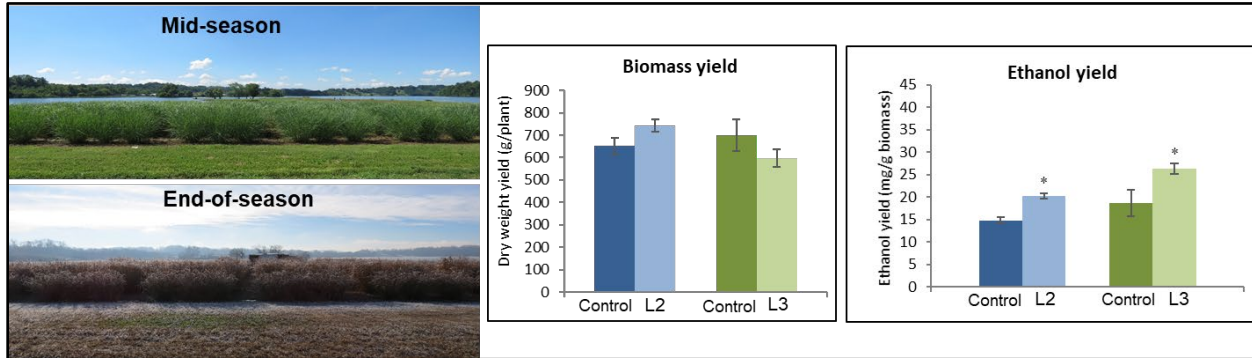
Figure 1. The 2013 field trials of reduced recalcitrance COMT-KD switchgrass planting at the UTK (Knoxville, TN) field site. Bars with asterisk are significantly different from controls at $p \leq 0.05$ calculated using *t*-tests for pairwise comparison with SAS software.

In addition, transgenic switchgrass MYB4-OE lines were also shown to have significant lignin reduction with increased sugar release and biofuel production under greenhouse conditions (Shen et al., 2012, 2013). Analyses with fully established field-grown transgenic switchgrass MYB4-OE lines (Baxter et al., 2015) showed that one transgenic line (L8) had substantial gains in both biomass (63% more) and biofuel yield (32% more) relative to controls (Figure 2). These gains represent a doubling of biofuel production per hectare. In contrast to this transgenic line, which had relatively low ectopic overexpression of the transgene, five of the eight transgenic lines planted did not survive the first field winter. The dead plants were all high-overexpressing lines that performed well in the greenhouse studies. Finally, rust susceptibility was not compromised in any transgenic lines over the field experiments. These results highlight the importance of field studies for "sorting" through various independent transgenic lines.
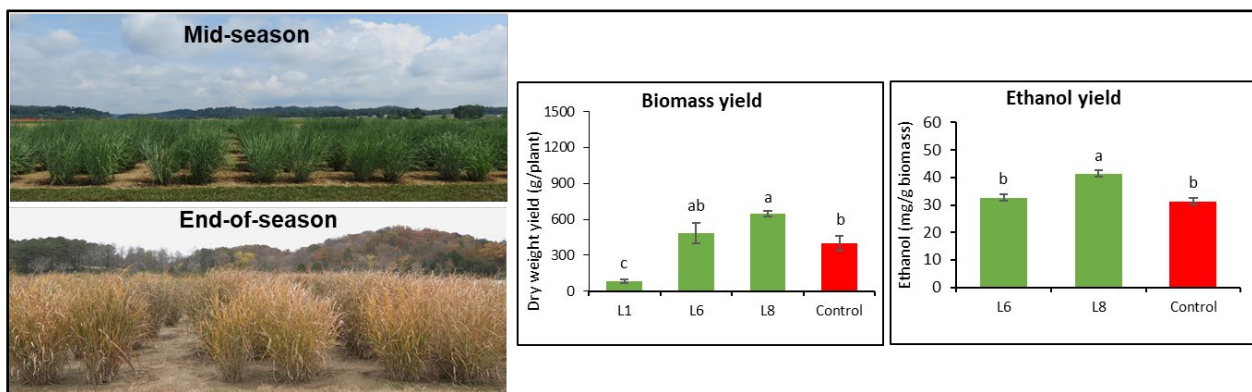

Figure 2. The 2014 field trials of reduced recalcitrance MYB4-OE switchgrass planting at the UTK (Knoxville, TN) field site. Bars represented by different letters are significantly different at $p \leq 0.05$ as tested by LSD method with SAS software.

Another example is transgenic switchgrass microRNA156-OE lines, shown to have dramatic effects on plant growth and development under greenhouse conditions (Fu et al., 2012). Analyses with fully established field-grown transgenic switchgrass microRNA156-OE lines (Baxter et al., 2018) showed that one low expressing transgenic line (T35) consistently produced more biomass (25%–56%) than the control across three growing seasons, which translated to the production of 30% more biofuel per plant during the final season (Figure 3). Three other transgenic lines produced less biomass than the control by the final season, and the two lines with moderate expression levels also exhibited altered rust disease susceptibilities. Results of this field study emphasize the importance of performing multiyear field studies for plants with altered regulatory transgenes that target plant growth and development. Transgenic plants with modifications to transcriptional regulators, which impact such a broad range of genes, will likely require more extensive field evaluations than those with single-gene modifications to evaluate the stability of the desired growth phenotypes.
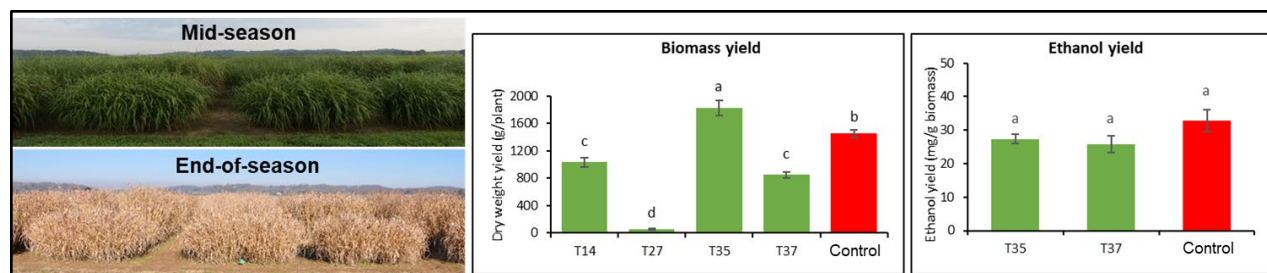

Figure 3. The 2015 field trials of increased growth microRNA156-OE switchgrass planting at the UTK (Knoxville, TN) field site. Bars represented by different letters are significantly different at *p*≤0.05 as tested by LSD method with SAS software.

Pectin is a polymer found in the primary and secondary cell walls of all plants. Down regulation of pectin is expected to decrease plant recalcitrance. Relative to pectin biosynthesis in plant cell walls, transgenic switchgrass GAUT4-KD lines with reduced cell wall recalcitrance and increased growth (Biswal et al., 2018) were tested in the field. The field-grown GAUT4-KD lines showed higher sugar release efficiency (Year-3 average 483%) and ethanol yield (Year-3 average 517%) per plant. Both traits were maintained in a 3-year field trial of GAUT4-KD switchgrass, with up to sevenfold increased saccharification and ethanol production and six-fold increased biomass yield compared with control plants (Figure 4).
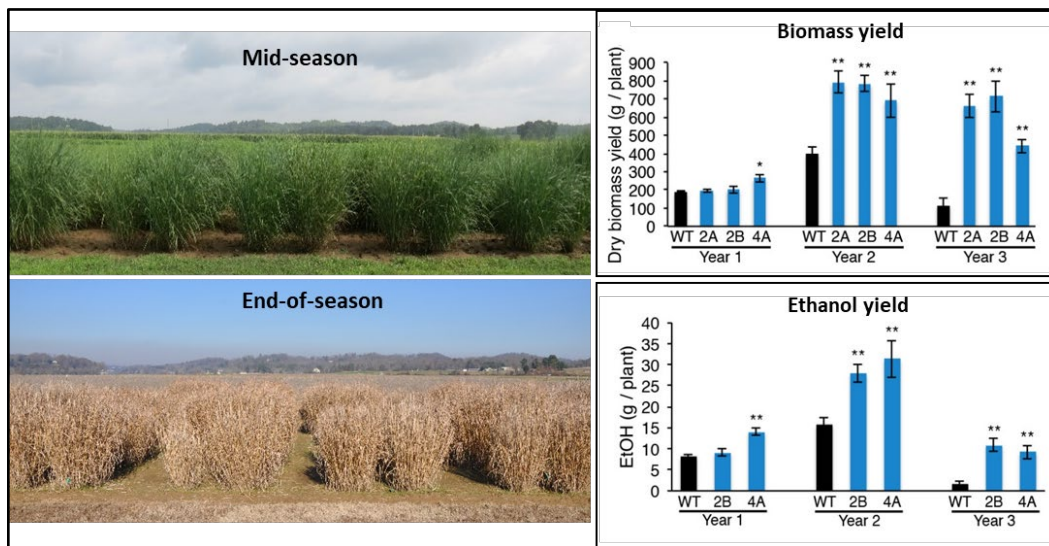
Figure 4. The 2015 field trials of reduced recalcitrance GAUT4-KD switchgrass planting at the UTK (Knoxville, TN) field site. Bars with asterisk are significantly different from controls at $p \leq 0.01$ as calculated using *t*-tests for pairwise comparison with SAS software. WT: wild-type control.

One-carbon (C1) metabolism genes, crucial for the methylation of lignin and hemicelluloses in plant cell wall, is another class of genes of interest to BESC and CBI. Analyses with fully established field-grown transgenic switchgrass FPGS1-KD lines, a 1-carbon metabolism gene (Mazarei et al., 2020), showed a substantial reduction in growth traits and biomass yield in the transgenic lines, which correlated highly with the high reduction in transgene expression. Transgenic lines with the low or moderate reduction of transgene expression had equivalent biomass yield relative to the control lines. Yet, one transgenic line (T10) produced up to 18% more ethanol over the field experiments while maintaining congruent growth and biomass as controls (Figure 5). Moreover, the severity of rust disease among transgenic and control lines were not significantly different during the time course of the field experiments. These results suggest that an optimized level of transgene expression is needed to produce transgenic plants with the desired growth under field conditions. It further demonstrates increase biofuel potential with no growth penalty or increased susceptibility to rust is feasible in this biofuel feedstock.
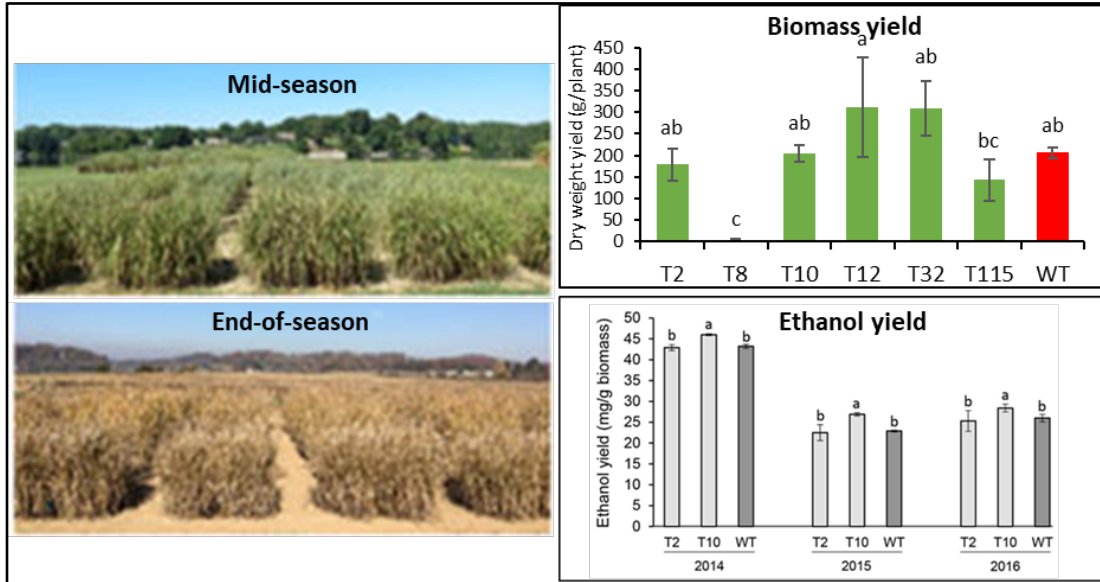
Figure 5. The 2016 field trials of reduced recalcitrance FPGS1-KD switchgrass planting at the UTK (Knoxville, TN) field site. Bars represented by different letters are significantly different at *p*≤0.05 as tested by LSD method with SAS software. WT: wild-type control.

Field experiments of transgenic hybrid switchgrass (COMT x Rambo) (MYB4 x Rambo) lines

Overcoming biomass recalcitrance and increasing biomass yield are two major goals for improving switchgrass as a biofuel feedstock. We sought to transfer the low-recalcitrance traits associated with the COMT-KD or MYB4-OE lines into high-yielding hybrid field varieties in an attempt to improve growth-related traits. Low-recalcitrant COMT or MYB4 switchgrass lines were crossed with high-yielding switchgrass field wild-type accessions (so-called 'Rambo') to evaluate parents and COMT and MYB4 hybrid families under field conditions for agronomic performance and biomass quality. Analyses with fully established field-grown of these lines (Alexander et al., 2020) showed that for COMT hybrids, biomass yield of the transgenic hybrids was similar to or greater than the wild-type parents selected for high biomass. Lignin content of COMT transgenic hybrids was reduced by 10%, S/G ratio decreased by 27%, and sugar release increased between 20% and 44% compared to their wild-type parents. These results indicate that hybridization of transgenic COMT lines with a high-yielding locally selected genotype resulted in both improved agronomic performance and enhanced biomass quality in the offspring. On the other hand, the MYB4 transgenic hybrid showed a 10% reduction in biomass yield compared with its wild-type parent in Year 1 (2014), but not in Year 2 (2015). The lignin S/G ratio was not reduced in MYB4 transgenic hybrids, nor was sugar release increased. These data indicate that the MYB4 transgene may not be suitable for an agronomic setting and that generalizations concerning the use of transgenics materials in hybrid cross will be dependent of the type of genes used to create the transgenic lines (Figure 6).
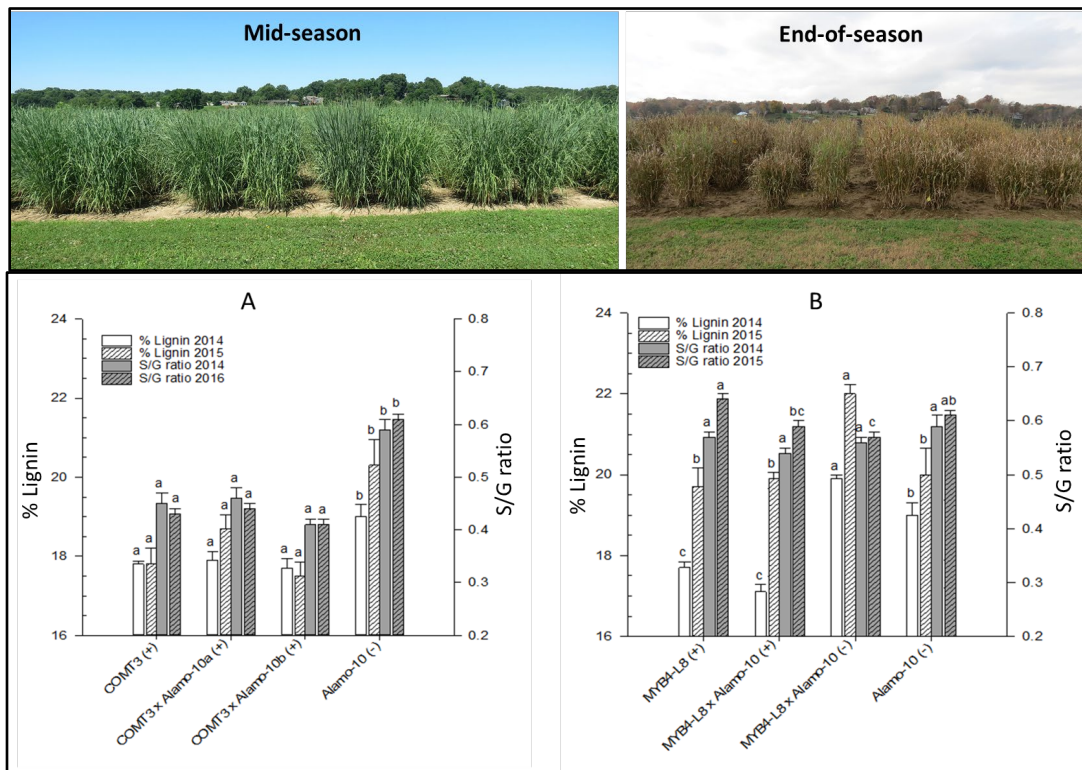
Figure 6. Field trials of COMT and MYB4 switchgrass hybrid families planting at the UTK (Knoxville, TN) field site. Lignin content and S/G ratio of parents and hybrid families over two growing seasons (2014 and 2015); A) COMT x Rambo (Alamo-10), B) MYB4 x Rambo (Alamo-10). Bars represented by different letters are significantly different at $p \leq 0.05$ as tested by LSD method with SAS software.

Effect of field-grown transgenic switchgrass on soil properties

To understand potential environmental impacts of cultivating transgenic bioenergy crops in the field, we evaluated the effects of transgenic switchgrass COMT-KD plants with altered lignin content and composition on soils in terms of chemistry, microbiology, and carbon cycling when grown in the field (Chauhan et al., 2014; DeBruyn et al., 2017). The transgenic lines grown in the field across five growing seasons, during which root lignin characteristics, soil elemental composition, bacterial community diversity and structure, and soil carbon storage and turnover were examined. No differences were observed between transgenic and control plants in terms of soil pH or the total concentrations of 19 soil-based elements. In addition, there were no detectable effects of the transgenic plants on bacterial diversity, richness, or community composition were observed. Moreover, no changes in the capacity for soil carbon storage were observed. Finally, switchgrass-contributed carbon was not significantly different between transgenic and control plants (Figures 7 & 8). Our results suggest that at least over the short-term there is no anticipated

adverse large effects of these transgenic switchgrass plants on soil processes and soil organic matter stability.
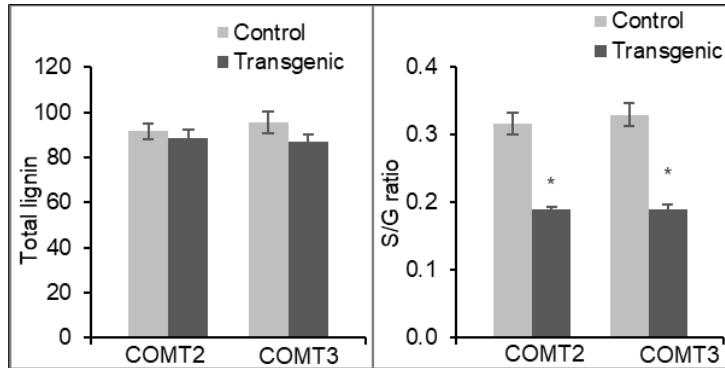

Figure 7. Total lignin content and S/G ratio in the roots of transgenic switchgrass COMT-KD lines (COMT2 and COMT3) and corresponding non-transgenic controls. Bars with asterisk are significantly different from controls at $p \leq 0.05$ as calculated using *t*-tests for pairwise comparison with SAS software.
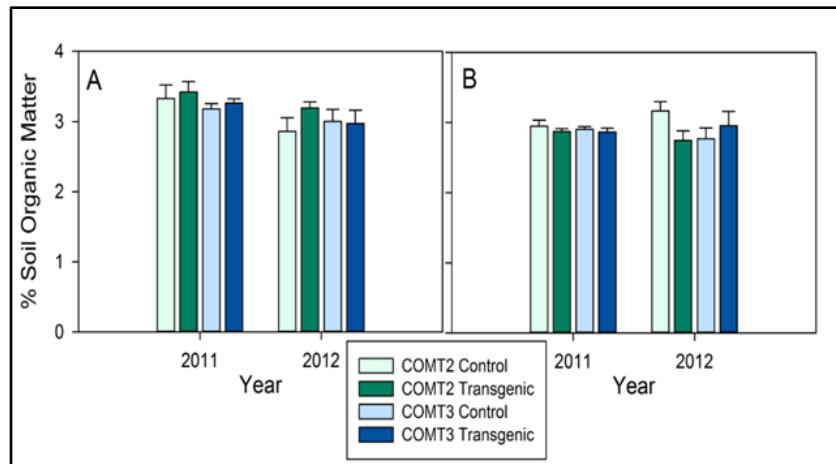

Figure 8. Mean soil organic matter in the upper 0-15 cm (A) and deeper 15-30 cm (B) during the first two growing seasons (2011 and 2012) in soils below transgenic switchgrass COMT-KD lines (COMT2 and COMT3) (dark colors) and corresponding non-transgenic controls (light colors).

Still, it has been reported that soil organic carbon is not changed in the short-term after switchgrass establishment and it may take several years of switchgrass culture before any changes are detectable (Ma et al., 2000). In order to explore a longer time after switchgrass establishment, we evaluated the effects of field-grown transgenic switchgrass COMT-KD and MYB4-OE lines on soil organic carbon during the eighth growing season (2018) (Xu et al, 2019). Again, no differences in total soil organic carbon, dissolved organic carbon, and permanganate oxidizable carbon were detected between transgenic and non-transgenic treatments for both COMT and MYB4 lines. Soil

CO₂-C production from the COMT2 transgenic line was not significantly different from its non-transgenic control. In contrast, the COMT3 transgenic line had greater soil CO_2-C production than its non-transgenic control. On the other hand, the MYB4 transgenic line L8 released more CO_2 than the control (Figure 9). Our results suggest that long-term *in situ* monitoring of transgenic switchgrass systems on a suite of soil and environmental variables is needed to determine the sustainability of growing genetically modified bioenergy crops and that these effects can vary by transgenic lines used to in the test.
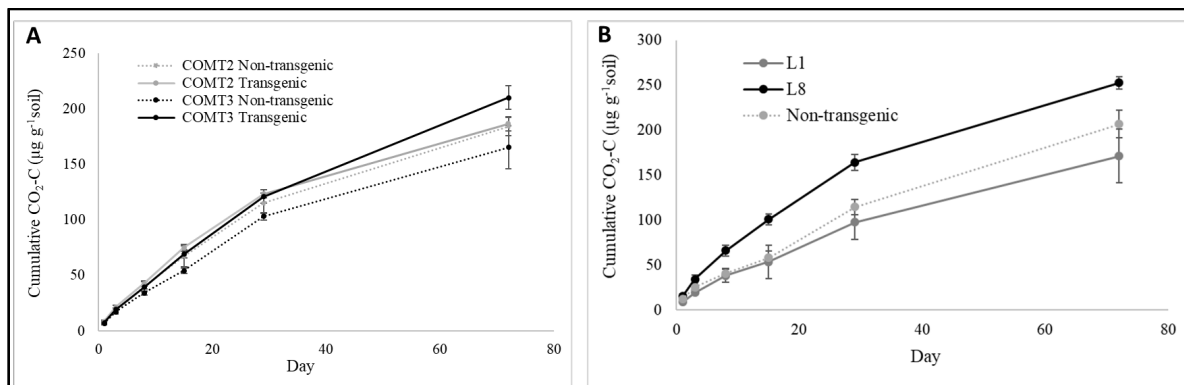

Figure 9. Cumulative soil CO_2-C production from transgenic switchgrass COMT-KD lines (COMT2 and COMT3) and corresponding non-transgenic controls (**A**), and from transgenic switchgrass MYB4-OE lines (L1 and L8) and the non-transgenic control (**B**).

Field experiments of switchgrass natural variants

Several switchgrass natural variant lines have been evaluated under field conditions. Analyses with fully established field-grown plant materials from these lines showed that one natural variant line (NV3) had significantly more biomass (31% more), while another line (NV2) had significantly lower biofuel production(14% more), both relative to the controls (Figure 10).
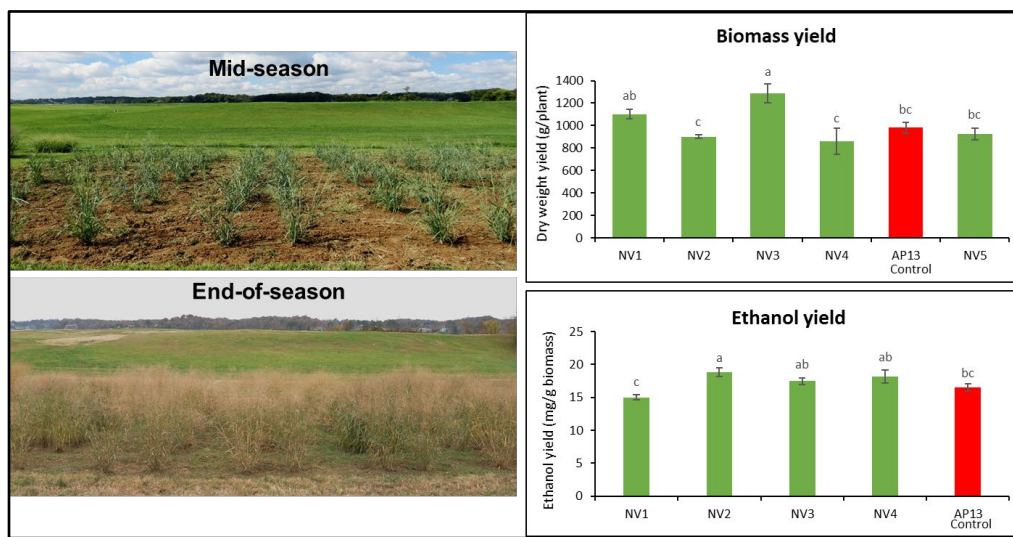
Figure 10. The 2015 field trials of natural variants switchgrass planting at the UTK (Knoxville, TN) field site. Bars represented by different letters are significantly different at $p \leq 0.05$ as tested by LSD method with SAS software.

In summary, we demonstrated that switchgrass varying in multiple cell wall-recalcitrance genes grown under field conditions can lead to perennial bio-feedstocks with enhanced deconstruction and conversion potentials for biofuels production. Results of our field studies also proved that target modification of plant cell walls via biotechnological approaches hold the most promise to reduce biomass recalcitrance. These results also provide a roadmap for improving feedstocks directly or by genetically assisted breeding. Furthermore, we showed that combining low-lignin transgenic switchgrass with a breeding and selection program for biomass yield will allow for the deployment of effective transgenes in high-yielding genetic backgrounds. Together, our results provide insights into the possible improvement of switchgrass productivity via biotechnology paired with plant breeding.

3. Switchgrass variants for QTL and GWAS studies

Establishing natural and breeding populations for a QTL mapping population

Switchgrass mapping projects were initiated to identify genetic loci associated with important traits and to initiate a marker-assisted breeding program. A mapping population was developed by crossing two distinct switchgrass genotypes, 'AP13' and 'VS16'. Genetic linkage maps were constructed for both the parents. The female parent (AP13) map consisted of 18 linkage groups and covers 1,901 cM of the switchgrass genome. Comparative analysis of mapped loci with other grasses revealed high synteny of switchgrass with the sorghum and foxtail millet genomes. The

mapping population was evaluated at three locations in Oklahoma and Georgia for three years, and phenotypic data on biomass yield, yield components, lignin content, and sugar release were measured. Wide variability was observed in the population for many traits. This inter-institutional partnership constructed genetic linkage maps of a lowland AP13 × upland VS16 switchgrass population using simple sequence repeats (SSR), sequence tagged sites, and Diversity Arrays Technology markers. A subset of the population was sequenced at JGI. Eleven quantitative trait loci (QTLs) associated with biomass yield were mapped on seven linkage groups. The additive effect of the QTLs mapped for biomass yield, with the percent variance explained ranging from 6-24%. Additional QTLs were also found associated with lignin content and glucose/xylose release. Identification of additional genetic loci associated with important traits is in progress.

Identifying switchgrass reduced-recalcitrance feedstock via analysis of natural variation and gene mapping

A total of 352 genotypes of the switchgrass association mapping population were evaluated in two locations for three years. From this set, 20,233 single nucleotide polymorphisms (SNPs) were obtained following a genotyping by sequencing protocol of the population. More than 500 SNPs were found associated with important biomass traits. Six genotypes were identified from switchgrass natural variants with superior sugar release, reduced lignin content and high biomass yield across multi-environments. A scatter plot of the sugar release against biomass yield of the candidate genotypes is presented in Figure 11. Two such genotypes were selected as TOP performing lines and QTLs for biomass yield, plant height, regrowth, days to heading, vegetative growth length, lignin content, and sugar release (glucose and xylose) were detected in the biparental (AP13 x VS16) population. Eleven genomic regions were identified that control biomass yield and/or plant height in the population. Markers flanking the QTL for biomass-related and composition traits have been identified. Identification of genes and causal alleles contributing to biomass yield and uniformity across different environments using high-resolution GWAS is currently underway, along with additional new field studies to evaluate switchgrass cultivars and genotypes for biomass and sustainability traits (see below).
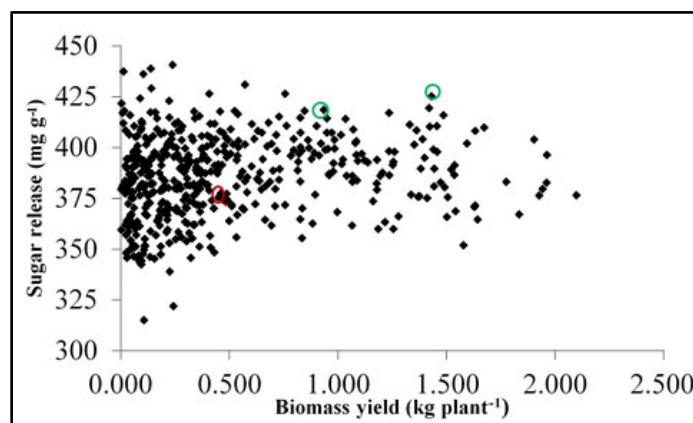
Figure 11. Scatter plot of mean sugar release (mg g^{-1}) and biomass yield (kg plant^{-1}) in the association mapping population. Dots with green circle are top two selected genotypes, SGAMP19_05 and SGAMP15_14. Dot with red circle is the control genotype AP13.

Establishment of switchgrass GWAS fields at UGA and UTK sites

Currently, switchgrass field experiments of a GWAS panel consisted of up to 400 diverse accessions are being performed to enable rapid domestication and increased sustainability in switchgrass. Three switchgrass GWAS fields have been established at: UGA (Tifton, GA), UTK (Knoxville, TN), and UGA (Watkinsville, GA) field sites. For UGA-Tifton field site, a total of six replicated plots (three replicates per drought and control treatments) were established for use in water-use efficiency (WUE) study. Genotypes were planted in a complete randomized block design with 1 m spacing between plants. For UTK-Knoxville field site, a total of four replicated plots (two replicates per low and moderate nitrogen fertility treatment, respectively) were established for use in nitrogen-use efficiency (NUE) study. Genotypes were planted in a honeycomb design with 2.5 m spacing between plants (Figure 12). For UGA-Watkinsville field site, one replicate plot was established. Genotypes were planted in a honeycomb design with 2 m spacing between plants.
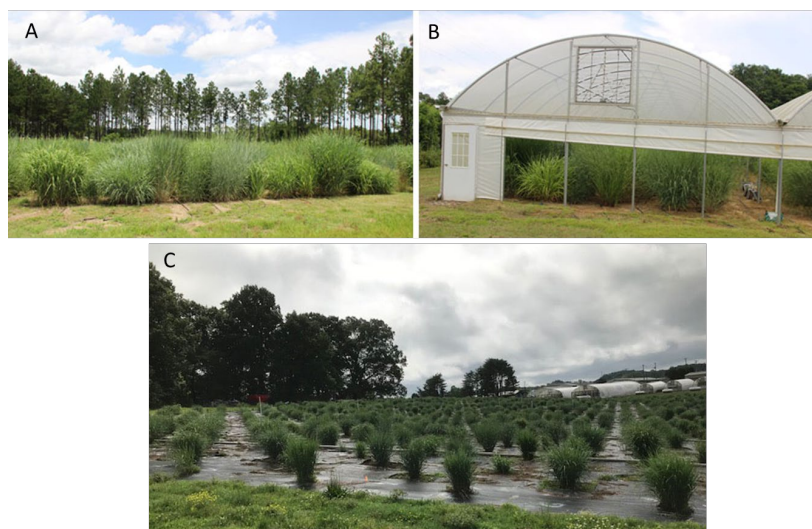

Figure 12. The 2019 field trials of switchgrass GWAS planting at UGA (Tifton, GA) (**A**: uncovered; **B**: under rain-out shelter) and UTK (Knoxville, TN) (**C**) field sites.

These established switchgrass GWAS fields are being evaluated for various phenotypic and sustainability traits such as plant height, plant perimeter, tiller number, dry biomass production, rust disease severity, WUE, and NUE, among others. Our current results indicate wide phenotypic variation across the GWAS panel. Furthermore, beyond the anticipated and known rust disease, two other new diseases were detected at the GWAS field at UTK (Knoxville, TN) field site and with

collaboration with Gary Bergstrom at Cornell University were identified as false smut disease caused by fungal *Cerebella andropogonis* and ergot disease caused by *Claviceps sp.* (Figure 13).
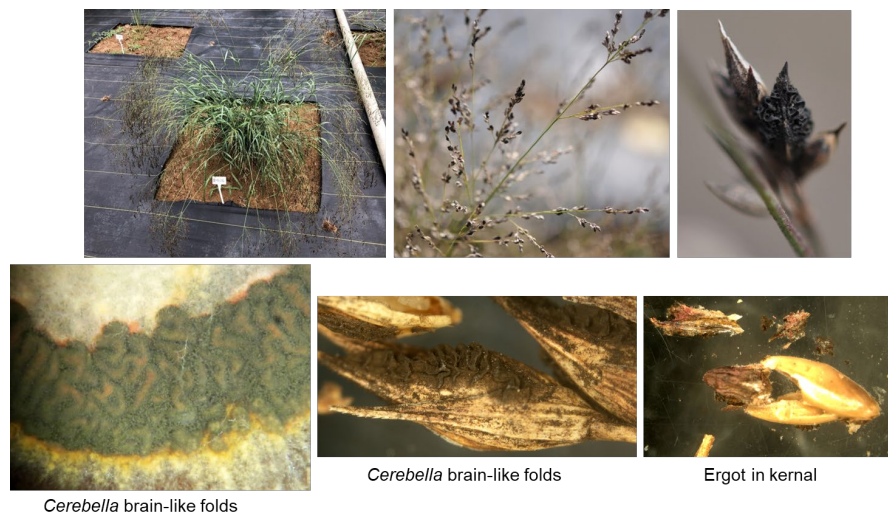

Cerebella brain-like folds

Cerebella brain-like folds

Ergot in kernal

Cerebella brain-like folds

Figure 13. Detection of false smut disease caused by *Cerebella andropogonis* and ergot disease caused by *Claviceps sp.* in 2019 switchgrass GWAS field at UTK (Knoxville, TN) field site.

In summary, the use of the natural variation and identification tools in the GWAS switchgrass studies allow rapid identification of gene candidates. Our established GWAS fields in different environmental conditions enable rapid discovery of environmentally stable genetic controls. Using a GWAS approach, many measurements (including growth, composition and biomass convertibility) of the broad variation across the switchgrass population can be correlated with specific SNPs in specific genes. This approach will lead to the identification of a number of important genetic regulators of biomass recalcitrance and other plant traits bringing the increased yield and sustainability in long-lived perennial switchgrass.

4. Establishment and use of breeding populations

Mapping of phenotypic and metabolic QTL in an F$_2$ validation population and identification of candidate genes underlying the QTL

An F$_2$ field population of 350 genotypes was established in 2016, generated by crossing two F$_1$ sibs from the cross AP13 (lowland) × VS16 (upland) (as noted above). These genotypes are being used as a tool to validate associations that will be identified during Years 4–5 in the GWAS panel. Analysis of the F$_2$ population is being carried out now to maximize data acquisition and validation within the time frame of CBI. QTLs have been identified for a number of phenotypic traits, including rate of spring emergence, heading date, anthesis, plant height, stem diameter, tiller number, crown area, chlorophyll content and rust resistance. The majority of the QTL explain <15%

of the variation, indicating that these traits are controlled predominantly by genes of small effect. In collaboration with the CBI CompBio Team, we mapped a QTL for a set of 205 metabolites found in leaf tissue harvested from greenhouse-grown plants. A preliminary analysis identified 134 QTL for 101 metabolites that explained between 4% and 87% of the genetic variation for the mapped traits. We aim to conduct similar analyses for other biochemical traits, including milled samples of the F_2 biomass harvested in 2017 and 2018 (two replicates of ~300 plants for each year) which have recently been provided to NREL (CBI Deconstruction Team) for cell wall composition analyses. Candidate metabolic genes underlying the mapped QTL, noted above, are being investigated. One example is shikimate *O*-hydroxycinnamoyl transferase, which underlies a co-mapped QTL for the metabolites 4-*O*-coumaroyl quinic acid and 4-*O*-caffeoyl quinic acid (Figure 14). These metabolites are hydroxycinnamate-quinate esters similar to chlorogenic acid (3-*O*-caffeoyl quinic acid), which are thought to be an intermediate of the lignin biosynthetic pathway. Examination of aligned resequencing reads from the lowland accessions 'Alamo' and 'Kanlow' and the upland accessions 'Summer' and 'Dacotah' indicates the presence of deletions at both the 5' end and 3' end of the gene in the upland genotypes. The large number of SNPs and indels in the coding region in the upland compared with the lowland accessions suggests that this gene is inactive in the upland ecotype. The level of the two metabolites is 20-fold higher in the lowland parent (AP13, a derivative of Alamo) than in the upland parent (VS16, a derivative of Summer) of the F_2 population. RNA-seq analysis also showed that the candidate gene was more highly expressed in lowland than in upland accessions. CRISPR-KOs are being created by the CBI Rapid Genetics Team to validate this hypothesis.
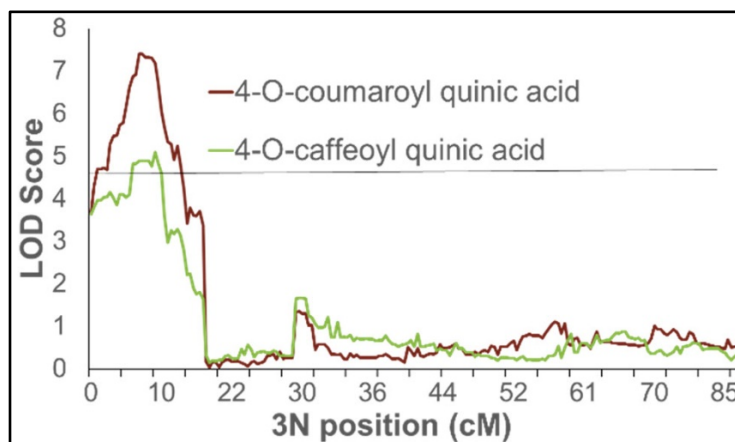

Figure 14. QTL on chromosome 3N explaining 19% and 16% of the genetic variation for the metabolites 4-*O*-coumaryl quinic acid and 4-*O*-caffeoyl quinic acid, respectively.

In summary, QTL mapping enables researchers to discover genetic regulators for metabolic pathways in plants. Manipulation of the regulators leads to reduced recalcitrance, increased digestibility and biofuels production. Our QTL mapping of phenotypic traits and metabolites further show the rapid gene identification, opening up opportunities for discovery of novel genetic determinants of economically

important but complex traits in plants.

5. New phenotyping approaches for switchgrass field trials

High-throughput switchgrass phenotyping and biomass modeling by UAV

Unmanned aerial vehicle (UAV) technology is an emerging and powerful approach for high-throughput plant phenotyping for field-grown crops. Conventional phenotyping studies have been implemented to identify plant traits. However, these trials are performed manually, which is resource-intensive and time consuming. With remote sensing costs decreasing, this quickly advancing technology provides a powerful, efficient tool set to aid in data collection for many morphological and phenological traits. To develop UAV-based phenotyping and sustainability for switchgrass field trials, we first conducted a pilot experiment in a small scale. In this pilot study, a total of 120 switchgrass GWAS accessions was transplanted in 4 replicated field plots. Each plot contains 30 switchgrass plants planted in 15 pairs with variable plant spacing (i.e., 1.5 m, 1.75 m, 2.0 m, and 2.25 m) and plot spacing (i.e., 6.1 m, 9.1 m, and 12.2 m). Multiple UAV flights on the field were performed. UAV-based analyses were performed using an RGB camera to determine plant height and perimeter and a multispectral camera to detect chlorophyll (an indicator for nitrogen status in plant) through photogrammetric image analysis (Figure 15). UAV-based plant height measurements correlated strongly with manual measurements (r=0.92), with similar correlation strength for manual vs. automated plant perimeter measurements (r=0.94) (Figure 16). Thus, we conclude that UAV-based multispectral camera can be used to detect the chlorophyll in leaves of small two-month-old GWAS plants (Figure 17).

Figure 15. Pilot field experiment for UAV-based sensor optimization at UTK (Knoxville, TN) field site. **A**) The 2018 GWAS field trial, **B**) UAV-based image of the GWAS field.
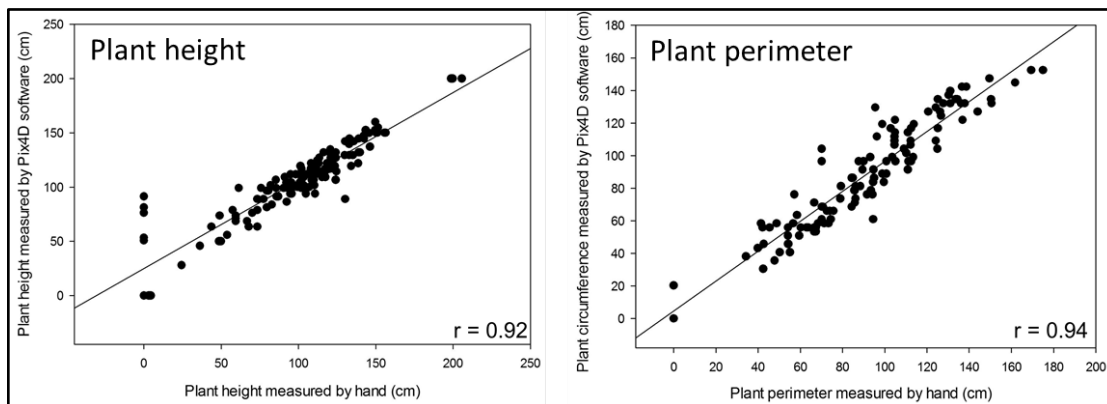
Figure 16. Simple correlation between manual and UAV-based plant height and plant perimeter measurements.
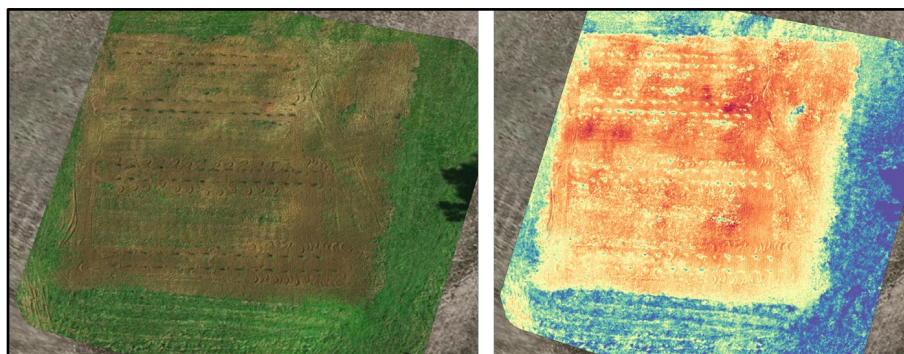

Figure 17. UAV-based chlorophyll map of pilot switchgrass field experiment at UTK (Knoxville, TN) field site using a multispectral camera. Atlas software was used to generate the chlorophyll map using the Normalized Difference RedEdge (NDRE, NIR – RedEdge/NIR + RedEdge) vegetation index that is sensitive to chlorophyll.

After the successful pilot study, we integrated the methods developed in this study in a larger switchgrass GWAS field trial consisting of 1320 plants (a total of four replicated plots per 330 switchgrass GWAS accessions, two replicates per low and moderate nitrogen fertility treatment). We performed numerous UAV flights equipped with LiDAR, multispectral, and a high-definition camera for automated phenotyping and sustainability traits of the switchgrass GWAS population (Figure 18). We employed UAV-based imagery to measure plant canopy height, plant canopy perimeter, and biomass yield. We validated automated UAV results with manual ground truth measurements. UAV-based measurements correlated strongly with manual measurements for plant height (r=0.93) and plant perimeter (r=0.95). We developed a robust and standardized biomass yield model by combining plant canopy height and canopy perimeter parameters with spectral index. The biomass yield obtained from this model strongly correlated with manual measurements (r=0.90) (Figure 19).
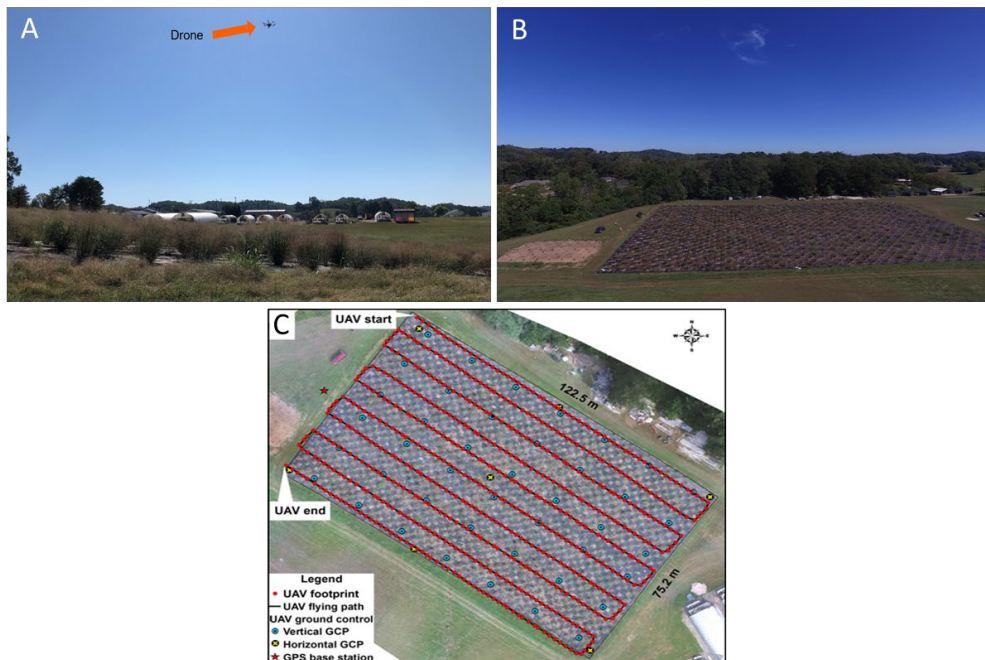
Figure 18. UAV-based phenotyping of the switchgrass GWAS field at UTK (Knoxville, TN) field site. **A**) The 2019 GWAS field trial, **B**) UAV-based image of the GWAS field, **C**) UAV route over the GWAS field.
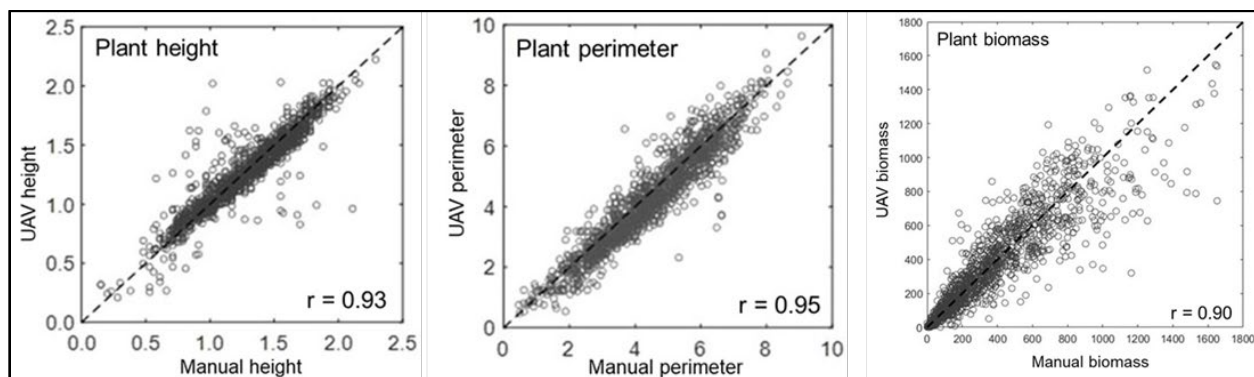

Figure 19. Simple correlation between manual and UAV-based plant height (m), plant perimeter (m), and plant biomass (g) measurements.

In summary, our study provides insights into the capacity of UAV-based remote sensing for switchgrass high-throughput phenotyping in the field. Large-scale GWAS panels require accurate phenotyping of thousands of plants. Automated phenotyping, especially to predict end-of-season biomass yield would be a significant advance in the bioenergy field. The UAV-based approaches proposed in our study facilitate identification of superior genotypes and beneficial gene

targets. To our knowledge, this is the first report on UAV technology for high-throughput phenotyping of switchgrass grown under field conditions.

6. Modeling and simulation of field performance

Process-based simulation of marginal land crop performance

We are currently estimating the relative benefit of increased feedstock crop yield versus yield resilience across different marginal lands and climates for switchgrass using a process-based ecosystem model. We modified our DayCent spatial assessment model to specify switchgrass green-up and senescence dates as a function of latitude and extend our simulations across regional scales. We used abandoned agricultural land area as an estimate of marginal land available for potential switchgrass production (Figure 20). Simulating biomass yields and the associated greenhouse gas (GHG) footprint of cultivation on this marginal land, we compared the performance of higher-yielding (i.e., higher photosynthetic efficiency) versus more-resilient (i.e., more drought-tolerant) switchgrass varieties. The resulting spatial distribution is currently being used to guide switchgrass breeding efforts.
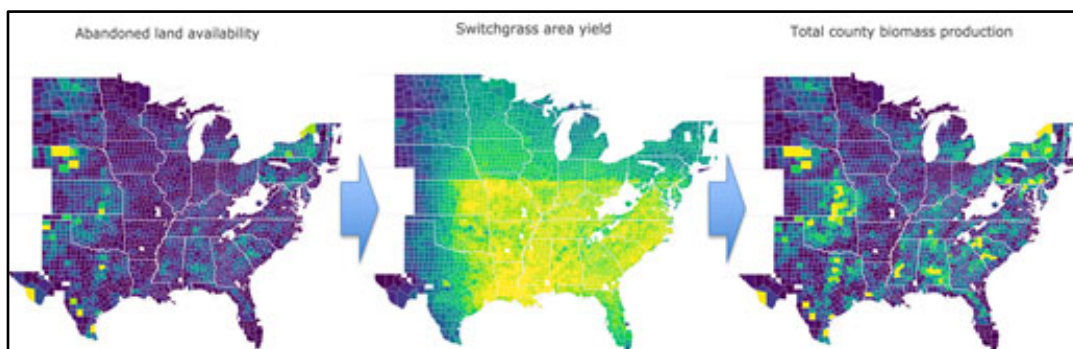

Figure 20. Ecosystem modeling workflow showing marginal land identification, process-based biomass yield potential prediction and the resulting feedstock production maps.

System-level assessment of mitigation potential

Many bioenergy critiques focus on land availability and potential losses of ecosystem carbon storage resulting from energy crop cultivation. To understand the GHG mitigation potential of a biofuel system relative to that of alternate land management options (e.g., reforestation), we coupled DayCent simulations of crop growth and carbon cycling with conversion technologies. Interestingly, our assessment of per-acre GHG mitigation potential shows current-day cellulosic ethanol production from switchgrass is equal to reforestation and greater than grassland restoration.

In summary, parameterization using collected switchgrass data has been able to provide insights and estimates of lifecycle and land-use impacts of this potential biomass feedstock.

7. Concluding Remarks

BESC and CBI have conducted greenhouse studies on a collection of switchgrass lines with reduced recalcitrance arising from both directed transgenics and natural variants (detailed in a former report). A limited number of these lines were selected as TOP Lines for field experiments to evaluate agronomic performance and sustainability across multiple growing seasons. Many of these transformed switchgrass plantings had demonstrated low recalcitrance and normal growth in a greenhouse setting. However, we showed that if the plant's MYB4 or microRNA156 gene expression is *too high* or FPGS1 gene expression is *too low* in a field setting, switchgrass productivity is negatively affected. But, when expression is optimal, "*just right*", the altered switchgrass produces both higher biomass growth and biofuel yields. The results were shown over multiple growing seasons, demonstrating that one transgenic line from field grown MYB4, microRNA156, or FPGS1 with optimal transgene expression had gains in biofuel and/or biomass, while some lines had substantial biomass loss or did not survive the winter. In sum total, a key observation is that we can achieve both lower recalcitrance and higher biomass simultaneously in some lines (e.g., GAUT4) or achieve increase biofuel potential with no cost to biomass (e.g., COMT). We also showed that further improvement in growth-related traits can be achieved by transferring the low-recalcitrance traits associated with transgenic switchgrass line (e.g., COMT and MYB4) into high-yielding field varieties (wild-type 'Rambo') via biotechnology coupled with plant breeding.

Biomass yield is the single most important factor impacting economic sustainability of biofuels and bioproducts. Our switchgrass GWAS fields established in Georgia and Tennessee field sites provide information on genetic variation in switchgrass and identify genotypes that have high and uniform biomass yield across diverse environments. GWAS analyses also enable CBI researchers to identify genetic loci associated with high biomass yield and sustainability, with subsequent integration into genomic selection models. Also, QTL and association mapping, as powerful tools, further enable CBI researchers for rapid gene discovery. Consistently, we identified QTL for phenotypic traits and metabolites in an F_2 switchgrass validation population.

UAV-based remote sensing holds great promise as a high-throughput phenotyping tool. We reported the first study on UAV technology for switchgrass grown under field conditions. Our developed UAV-based system enables phenotyping of thousands of switchgrass plants with precise predication of end-of-season biomass yield, an important trait in the bioenergy field.

In summary, our results emphasize the importance of performing field experiments for successful establishment and sustainability of switchgrass feedstock for biofuels production. Our in-field testing demonstrated that plants have great plasticity in their cell wall properties than reported

in the literature. Data compared from the greenhouse and field experiments successfully validated the degree of congruency for cell wall traits, sugar release efficiency, ethanol yield as well as agronomic performance and rust susceptibility.

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