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Time Course Field Analysis of COMT-Downregulated Switchgrass: Lignification, Recalcitrance, and Rust Susceptibility

Holly L. Baxter[1,2] · **Mitra Mazarei**[1,2] · **Chunxiang Fu**[2,3] · **Qunkang Cheng**[4] · **Geoffrey B. Turner**[2,5] · **Robert W. Sykes**[2,5] · **Mark T. Windham**[4] · **Mark F. Davis**[2,5] · **Richard A. Dixon**[2,6] · **Zeng-Yu Wang**[2,3] · **C. Neal Stewart Jr.**[1,2]

Abstract Modifying plant cell walls by manipulating lignin biosynthesis can improve biofuel yields from lignocellulosic crops. For example, transgenic switchgrass lines with down-regulated expression of caffeic acid O-methyltransferase, a lignin biosynthetic enzyme, produce up to 38 % more ethanol than controls. The aim of the present study was to understand cell wall lignification over the second and third growing seasons of COMT-downregulated field-grown switchgrass. COMT gene expression, lignification, and cell wall recalcitrance were assayed for two independent transgenic lines at monthly intervals. Switchgrass rust (*Puccinia emaculata*) incidence was also tracked across the seasons. Trends in lignification over time differed between the 2 years. In 2012, sampling was initiated in mid-growing season on reproductive-stage plants and there was little variation in the lignin content of all lines (COMT-downregulated and control) over time. COMT-downregulated lines maintained 11–16 % less lignin, 33–40 % lower S/G (syringyl-to-guaiacyl) ratios, and 15–42 % higher sugar release relative to controls for all time points. In 2013, sampling was initiated earlier in the season on elongation-stage plants and the lignin content of all lines steadily increased over time, while sugar release expectedly decreased. S/G ratios increased in non-transgenic control plants as biomass accumulated over the season, while remaining relatively stable across the season in the COMT-downregulated lines. Differences in cell wall chemistry between transgenic and non-transgenic lines were not apparent until plants transitioned to reproductive growth in mid-season, after which the cell walls of COMT-downregulated plants exhibited phenotypes consistent with what was observed in 2012. There were no differences in rust damage between transgenics and controls at any time point. These results provide relevant fundamental insights into the process of lignification in a maturing field-grown biofuel feedstock with down-regulated lignin biosynthesis.

Keywords Biomass · Caffeic acid O-methyltransferase (COMT) · Lignin · Lignocellulosic biofuel · Switchgrass

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✉ C. Neal Stewart, Jr.
nealstewart@utk.edu

[1] Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996, USA

[2] BioEnergy Science Center (BESC), Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

[3] Samuel Roberts Noble Foundation, Ardmore, OK 73401, USA

[4] Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996, USA

[5] National Renewable Energy Laboratory, Golden, CO 80401, USA

[6] Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA

Introduction

Plant biomass represents an abundant and renewable source of material for biofuel production, which could help alleviate dependence on petroleum-based fuels and reduce greenhouse gas emissions [1]. Recent research in this area has focused on developing non-food crops, such as perennial grasses and trees, as the raw material for bioconversion [2]. Switchgrass (*Panicum virgatum* L.) is a C4 perennial bunchgrass that produces abundant biomass and is broadly adapted throughout the USA, making it an attractive candidate for a dedicated lignocellulosic biofuel feedstock [3]. A major goal of

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switchgrass breeding and biotechnology efforts is to improve the feedstock quality of lignocellulosic biomass, which primarily involves optimizing the cell wall structure for more efficient conversion into fuel [4–9].

The cell walls of switchgrass and other lignocellulosic feedstock crops, and indeed all plants, are composed predominantly of cellulose, hemicelluloses, and lignin, with minor amounts of pectin and structural proteins [10]. The cellulose polymer is assembled into a network of microfibrils surrounded by hemicelluloses which are cross-linked with lignin. Lignin, a complex phenolic polymer composed of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monomer units, provides mechanical strength and structural reinforcement to the plant, aids in water transport, and is involved in biotic and abiotic stress responses [11, 12]. The carbohydrates (cellulose and hemicelluloses) in the cell wall can be broken down into simple sugars, which can be processed into biofuels and other bioproducts. However, the intrinsic resistance of lignocellulosic cell walls to deconstruction, defined as "recalcitrance," impedes such processes [13]. Lignin in particular is detrimental to bioconversion as it creates a hydrophobic and impenetrable barrier that limits the utilization of cell wall carbohydrates during hydrolysis and downstream processing. While various types of biomass pretreatments can be implemented to improve bioconversion efficiency, they add significant costs to the process and can also generate environmental pollution in the form of chemical waste [14]. Furthermore, pretreatments can liberate compounds that inhibit downstream microbial fermentation [15]. Consequently, genetic engineering approaches have focused on modifying the lignin biosynthetic pathway with the goal of developing transgenic plant lines that are more easily converted into biofuel with minimal or no pretreatment. Manipulating lignin biosynthesis to reduce lignin content, modify its structure, or reduce its degree of cross-linking with structural carbohydrates has proven to be a successful route for improving enzymatic hydrolysis efficiency and biofuel yields in switchgrass and other important biofuel feedstocks [5–9, 16, 17].

Given the substantial impact of lignin on feedstock quality for biofuel production, understanding cell wall development, especially lignification and how it relates to recalcitrance, has been an important endeavor of biofuel-related research. The primary cell walls of plants are formed during cell elongation and are relatively thin and hydrated. After elongation ceases, thickened secondary cell walls are deposited in layers. Secondary cell walls are less hydrated and richer in lignin, thus strengthening and reinforcing the cell wall structure [10]. The amount of lignin deposited in the cell wall, as well as the relative proportions of the subunits (H, G, and S) that comprise the lignin polymer, are controlled in a spatial and temporal manner. In grasses, the overall concentration of lignin within the plant increases with progressive developmental stages, and along the tiller from top to bottom internodes [18–20]. G and minor amounts of H units are incorporated into the lignin polymer during the initial stages of lignification, while later stages of lignification and secondary cell wall formation primarily involve the incorporation of G units with increasing amounts of S units [21]. Grass cell walls also contain especially high amounts of ester- and ether-linked hydroxycinnamates, predominantly p-coumaric and ferulic acid, which accumulate during secondary cell wall development [22, 23]. Field and greenhouse studies examining lignification and cell wall digestibility or recalcitrance in grasses have shown that cell walls become increasingly more difficult to break down as they progress in development [19, 24–26]. This has mainly been attributed to the accumulation of lignin as plants age, but might also be influenced by lignin structure, composition, and degree of crosslinking with other cell wall components [21].

To date, most cell wall characterization experiments with lignin-modified transgenic switchgrass have been limited to a single developmental stage [5–9]. Despite what has been learned through these studies, we currently lack a general understanding of how overexpressing or downregulating genes involved in lignin biosynthesis might affect lignification and cell wall chemistry over growing seasons in field conditions. Since the senesced biomass harvested at the end of the season is generally considered to be the most agronomically relevant tissue in terms of biofuel production, it is especially important to understand the changes that occur in cell wall chemistry and recalcitrance as biomass accumulates over the season. Studying cell wall chemistry dynamics in the field, where environmental conditions are constantly fluctuating, is of particular importance since this accounts for potential environmentally induced variation in cell wall composition across the season. Such information could also help determine if there are other optimal time points at which to harvest switchgrass biomass for biofuel production. For example, it might be preferable to harvest at some time point between mid- and late-season, where there is adequate biomass available and saccharification is highest. Tracking lignin levels over time and understanding how recalcitrance changes across the season could aid in this effort.

In the present study, a time-course analysis was carried out using two independent switchgrass lines with attenuated caffeic acid O-methyltransferase (COMT) levels. COMT is an enzyme in the lignin biosynthetic pathway that is involved in the synthesis of S monolignols. Prior research has shown that COMT-downregulation in switchgrass decreases lignin content, reduces the S/G ratio, and improves sugar release and ethanol yield with no negative effects on disease susceptibility or biomass yield in the greenhouse [5] and the first two growing seasons in the field [27]. These studies set the stage for the present research, for which the goal is to understand lignification and cell wall recalcitrance in field grown transgenic switchgrass over the growing season.

Materials and Methods

Description of Field Experiments

Generation, selection, and greenhouse characterization of the T1-generation COMT-downregulated and corresponding null segregant control plants used in this study have been described previously [5]. The experimental design of the field and transplantation of plants are described in Baxter et al. [27]. Briefly, two independent transgenic lines, COMT2 and COMT3, and a corresponding null-segregant control were used for each line. Ten replicates for each transgenic line and five replicates for each control were distributed in a completely randomized design. Each replicate contained nine vegetatively propagated clones. The experimental plants were surrounded by a border of wild-type switchgrass plants of the Alamo2 genotype derived from the 'Alamo' cultivar to reduce any shading effects. Data for the current study were collected across the second (2012) and third (2013) growing seasons at the University of Tennessee ETREC field site in Knoxville, TN, USA (Fig. 1). No pesticides or herbicides were applied during this period. Weeds were removed by tilling or hand weeding. In both growing seasons (2012 and 2013), aboveground biomass emerged between late March and early April. The first panicle was observed on June 15 in the 2012 season, and June 25 in the 2013 season. There were no differences in days to flower between transgenic and non-transgenic control lines. Emerging panicles were removed from all plants during the early reproductive stage by cutting the top portion of the plant from below the second node, following the guidelines of the USDA APHIS BRS release into the environment permit for the regulated articles. For both years, plants began to senesce between October and November. The first freeze occurred on November 1 in 2012 and October 25 in 2013. Once reaching full senescence in early-to-mid December each year, all aboveground biomass was harvested from the field.

Analysis of COMT Gene Expression

Samples for COMT gene expression analyses were collected once per month from August through November in 2012, and from May through October in 2013. High-quality RNA for gene expression analysis could not be extracted from samples collected after November of 2012, and after October of 2013, due to age-related leaf degradation. For each sampling date, fresh tissue samples were collected from 10 transgenic replicates and 5 control replicates at the same date and time. Single tillers of the same growth stage were selected at random from each replicate. The top portion of the tiller (top two leaves intact) was removed by cutting below the second internode and subsequently placed in liquid nitrogen in the field. Samples were transported to the laboratory on dry ice and stored at −80 °C until analyses were performed. The methods for total RNA isolation, quantitative real-time (qRT) PCR analysis, and the gene-specific primer sequences used are described in Baxter et al. [27]. Briefly, reverse transcription was performed on isolated RNA from leaves using an Omniscript® Reverse Transcription Kit (Qiagen, Valencia, CA) and the resulting cDNA was used for qRT-PCR analysis. The ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) was used to determine cycle thresholds and the data were normalized using ELF1A transcript levels.

Growth Traits and Biomass Yield

Growth traits and biomass yield were determined following the criteria described in Baxter et al. [27]. Briefly, tiller height and width were measured in mid-growing season (August) of each year. Tiller number and aboveground biomass yield were determined at the end of the growing season after plants reached full senescence in December. Aboveground biomass of all nine plants within each replicate was pooled to represent a single replicate. Harvested biomass was oven dried at 43 °C for 96–120 h and weighed to determine total dry weight yield.

Sample Collection and Preparation for Cell Wall Analyses

Samples were collected once per month from August through December in 2012, and from May through December in 2013. The developmental stage of the harvested tiller depended on the date of sampling (Table 1). Using previously described criteria, the elongation stages were determined by counting the number of visible and/or palpable nodes along the stem, where E1 = 1 node present, E2 = 2 nodes present, etc. [20, 28]. Tillers were considered to be of the first reproductive stage (R1) when the inflorescence began to emerge from the boot stage [20]. In our study, developmental stages after R1 (R2 and R3) were referred to as "post R1" since panicles were removed from each tiller at the R1 stage because of USDA regulatory requirements.

For sampling dates prior to December, tillers of the same developmental stage were collected from each of nine vegetatively propagated clones within each replicate. Each whole tiller (leaves and stem intact) was removed by cutting from 10 to 15 cm above the soil surface. All nine tillers collected from each replicate were pooled to represent a single biological replicate. In total, there were 10 biological replicates generated in this way for the transgenic lines and 5 biological replicates for the corresponding control lines. Tiller samples were oven-dried at 43 °C for 72–96 h. For the December sampling, the entire aboveground senesced biomass for each biological replicate was harvested, pooled, and oven-dried at 43 °C for 168 h. All samples (single tiller samples and aboveground senesced harvest) were milled with a Wiley mill (Thomas Scientific, Model 4, Swedesboro, NJ, USA) through a 20-

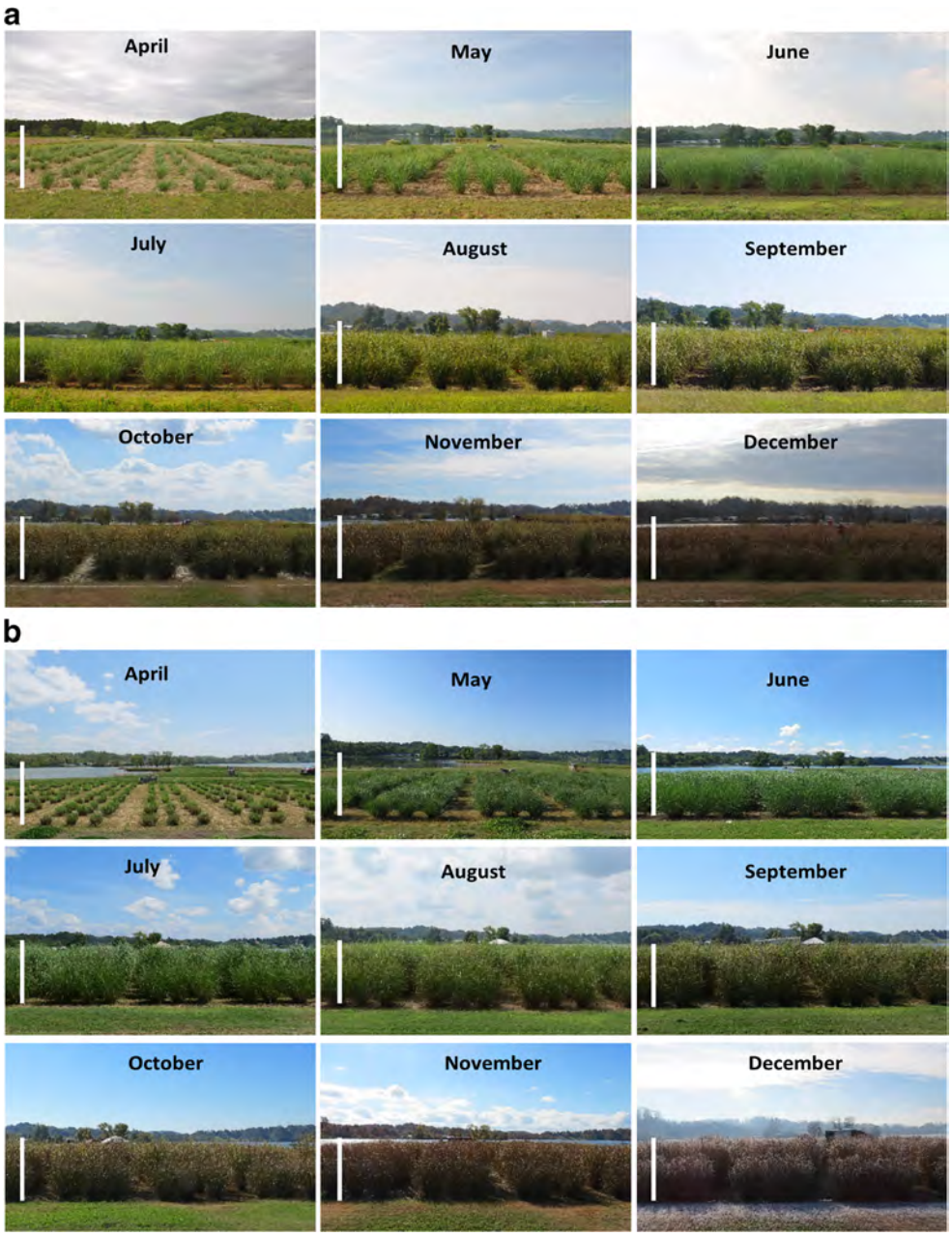

Fig. 1 Pictures of the field taken at various time points across the 2012 (**a**) and 2013 (**b**) growing seasons. *Vertical bar* = 2 m

mesh (1.0 mm) screen for cell wall analyses (lignin content, S/G ratio, and sugar release).

Py-MBMS for Lignin Content and S/G Ratio

Pyrolysis molecular beam mass spectrometry (py-MBMS) was used to determine lignin content and the S/G lignin monomer ratio following previously described methods [29]. After removing soluble extractives and starch from samples [30], approximately 4 mg of cell wall residues (CWR) were pyrolyzed at 500 °C and the vapors were analyzed with a custom Extrel single quadrapole molecular beam mass spectrometer. Total lignin content was estimated as the sum of the relative intensities of all peaks identified as lignin precursors. The S/G lignin monomer ratio was determined as the sum of the intensity of the syringyl peaks divided by the sum of the intensity of the guaiacyl peaks.

Enzymatic Sugar Release

Sugar release was measured using the previously described NREL high-throughput pretreatment and enzymatic hydrolysis assay [31] with some modifications. In brief, starch- and soluble extractives- free CWR were prepared as previously described [30]. CWR were pretreated with condensing steam at 180 °C for 17.5 min, then incubated at 50 °C for 70 h with

Table 1 Sampling dates and corresponding developmental stages for each time point in 2012 and 2013

Year	Date	Developmental stage of harvested material
2012	August 2	R1
	September 6	R1
	October 10	post R1
	November 8	post R1
	December 4	post R1
2013	May 1	E2
	June 3	E5
	July 1	R1
	August 1	R1
	September 3	R1
	October 1	post R1
	November 4	post R1
	December 11	post R1

Single tillers of the same developmental stage were harvested from each biological replicate for each time point prior to December. Whole aboveground senesced biomass samples were harvested from each biological replicate in December. Developmental stages were determined using the criteria described by Hardin et al. (2013). E2, second elongation stage; E5, fifth elongation stage; R1, first reproductive stage

70 mg protein/g biomass Ctec2 enzyme cocktail (Novozymes, Franklinton, NC, USA). The amounts of glucose and xylose released into the liquid were determined using the D-Glucose Assay Kit (glucose oxidase/peroxidase; GOPOD) and D-Xylose Assay Kit (xylose dehydrogenase; XDH), respectively (Megazyme Intl., Bray, Ireland).

Rust Assessment

Single tillers from three individual plants within each replicate were randomly selected and tagged for weekly assessment of rust infection. Rust disease severity was rated using the method described in Baxter et al. [27]. Briefly, rust disease severity was visually rated as the percentage of rust uredia covering the leaf tops using the following scale: 0 = 0 %, 1 ≤ 5 %, 2 ≤ 10 %, 3 ≤ 25 %, 4 ≤ 50 %, 5 = 50 % leaf area coverage with uredia.

Statistical Methods

All statistics were performed in SAS version 9.4 (SAS Institute Inc., Cary, NC). For comparisons among sampling dates, means within each transgenic or non-transgenic control line were compared with a one-way repeated measures ANOVA. Differences were considered significant where p values were less than 0.05, and Fisher's least significant difference method was used to obtain letter groupings. The PROC TTEST procedure was used for cell wall (lignin content, S/G ratio, and sugar release), rust disease susceptibility, and growth (morphology and biomass yield) comparisons between each independent transgenic event and its corresponding control. Differences were considered significant where p values (based on a two-sided t test) were less than 0.05.

Results

COMT Transcript Levels

COMT transcript levels of transgenic and null-segregant control lines were estimated by qRT-PCR. COMT expression levels varied significantly within each line over the course of the growing seasons (Fig. 2a, b). Within each year, the transgenic lines showed general trends of decreased expression compared to the control lines, although not statistically significant at all time points. In 2012, COMT expression levels in line COMT2 were decreased by 77–91 % relative to its corresponding control at all time points except October. Line COMT3 exhibited reduced COMT expression levels by 65–85 % relative to its control for all time points except September (Fig. 2a). In the subsequent season, COMT expression levels in line COMT2 were generally reduced (85–94 %) prior to September, after which COMT2 showed expression levels similar to that of its control. Line COMT3 maintained reduced expression levels of COMT relative to the control across the season, with the exception of the last (October) time point (Fig. 2b).

Growth Traits and Biomass Yield

In 2012 and 2013, plant height and width were determined at approximately mid-growing season (August), and tiller number and total aboveground biomass yield were measured at the end of the season (December). As reported in Baxter et al. [27], line COMT2 exhibited an increased plant height and width relative to its control and produced 18.2 % more biomass in the 2012 growing season, whereas line COMT3 exhibited similar morphological characteristics and biomass yield relative to its control. Similar growth trends were observed in the 2013 season. Line COMT2 showed increased plant width and tiller number relative to its control; however, there was no difference in total biomass yield between the two lines (Table S1). As in 2012, there were no differences in growth traits or total biomass yield between COMT3 and its control (Table S1).

Lignin Content and Composition

In 2012, monthly sampling took place from August through December and all samples were of reproductive stages (Table 1). There was slight month-to-month variation, but no clear patterns or trends, in lignin content across the growing

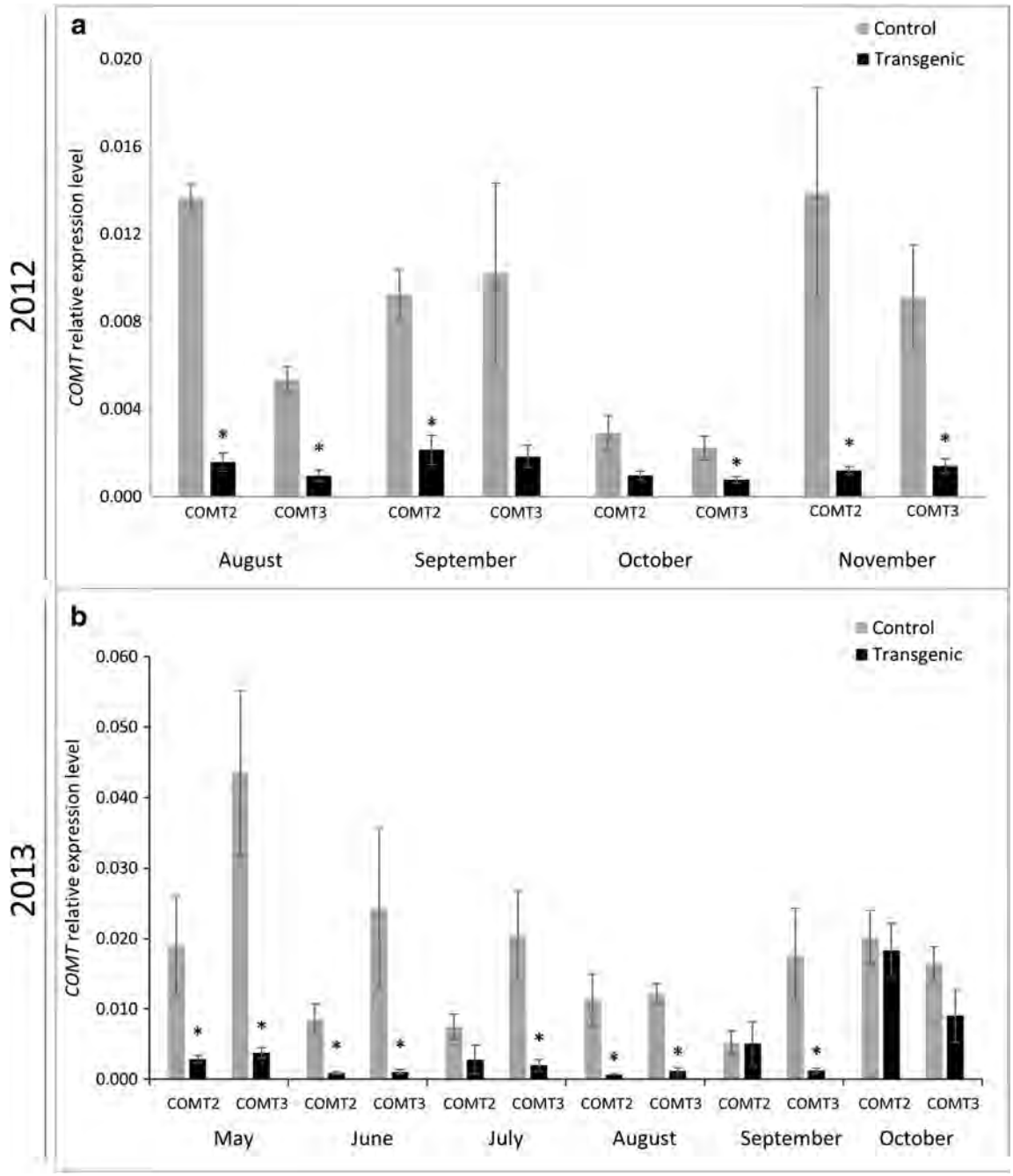

Fig. 2 Relative COMT transcript abundance in control and transgenic lines at different time points in 2012 (**a**) and 2013 (**b**). COMT transcript levels were determined by qRT-PCR. Relative transcript levels were normalized to ubiquitin. *Bars* represent the average of the biological replicates within each control (n = 5) and transgenic (n = 10) line ± standard error. *Asterisks* indicate a significant difference (t test, $p < 0.05$) between the control and transgenic line

season within each transgenic and non-transgenic line (Fig. 3a, b). Lignin content over time ranged from 22.7 to 24.2 % of the cell wall in the controls and 19.8 to 20.8 % of the cell wall in COMT-downregulated plants. For all time points analyzed, lignin content was significantly reduced (11–16 % reduction) in the COMT-downregulated lines compared to their respective controls (Fig. 3a, b). COMT-downregulated and control lines exhibited similar changes in the S/G lignin monomer ratio over time in 2012, which increased between August and September, then stabilized from October through December (Fig. 4a, b). S/G ratios ranged from 0.59 to 0.75 in the control lines, and from 0.38 to 0.47 in the transgenic lines. COMT-downregulated lines maintained a 33–40 % reduction in the S/G lignin monomer ratio relative to their corresponding control lines across all time points (Fig. 4a, b).

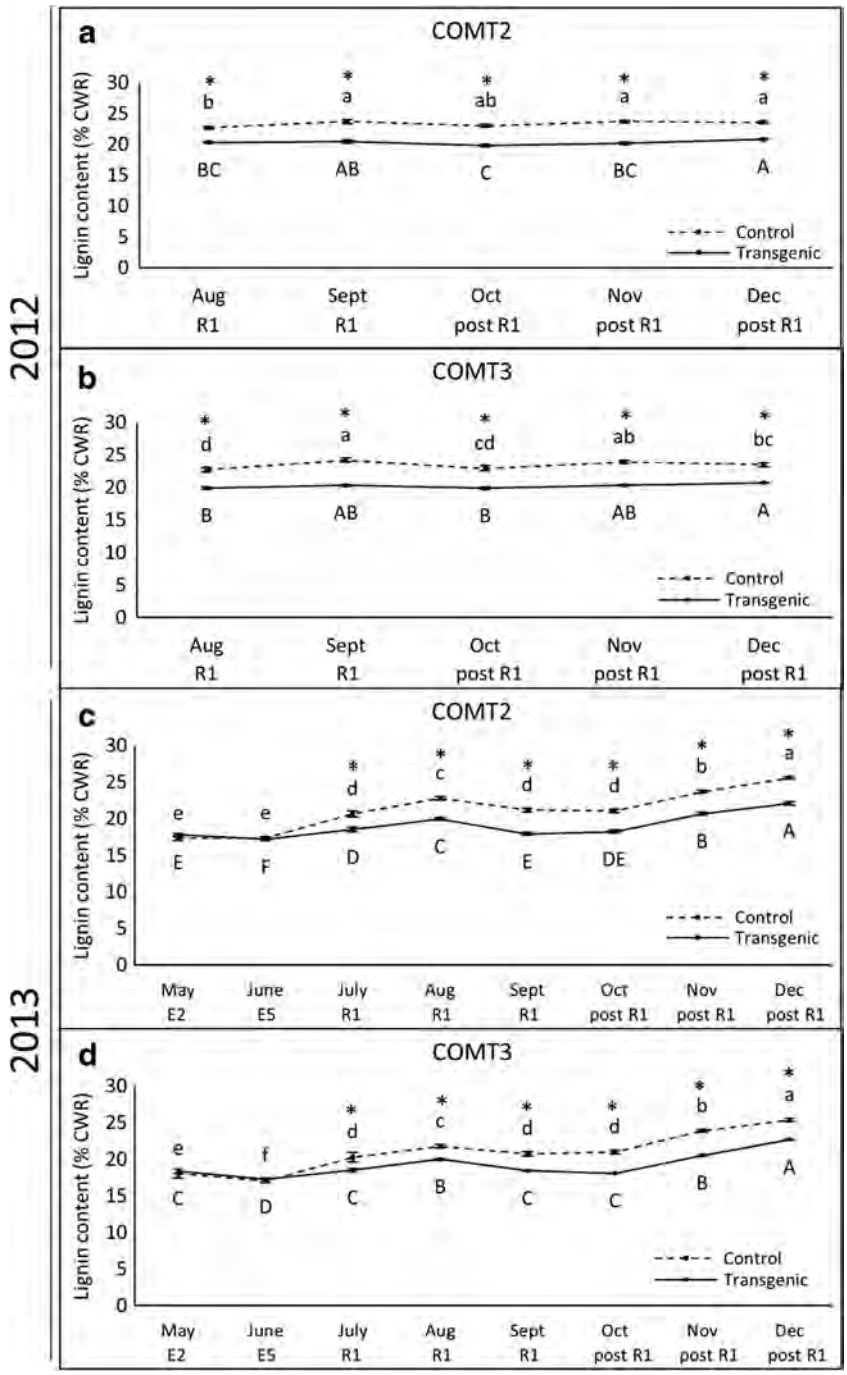

Fig. 3 Lignin content of control and transgenic lines at different time points across the 2012 (**a**, **b**) and 2013 (**c**, **d**) growing seasons. Data points represent the average of the biological replicates within each control ($n = 5$) and transgenic ($n = 10$) line ± standard error. Data points with different letters differ significantly (repeated measures ANOVA, Fisher's LSD, $p < 0.05$) within each control (*lowercase letters*) and transgenic (*capital letters*) line. *Asterisks* indicate a significant difference (*t* test, $p < 0.05$) between the control and transgenic line. *CWR* cell wall residues

In 2013, sampling took place from May through December and included plants from early elongation to late reproductive growth stages (Table 1). In contrast to 2012, the lignin content of transgenic and non-transgenic lines varied significantly across time points (Fig. 3c, d). Lignin generally increased across the season in all plants (transgenic and non-transgenic), with the lowest lignin levels occurring in elongation-stage plants harvested in early season (17–18 % of cell wall) and the highest lignification occurring in the senesced plants harvested at the end of the season (22–26 % of cell wall), as expected (Fig. 3c, d). Lignin differences between COMT-downregulated and control lines did not become apparent until plants reached the reproductive stage in July, after which the transgenic lines maintained an 8–15 % reduction in lignin content relative to their respective controls across the remaining growing season (Fig. 3c, d). Unlike the previous season, control and transgenic lines exhibited different patterns of change in the S/G ratio across the 2013 season. In the control lines, S/G ratios gradually increased from May through September, stabilized from September through November,

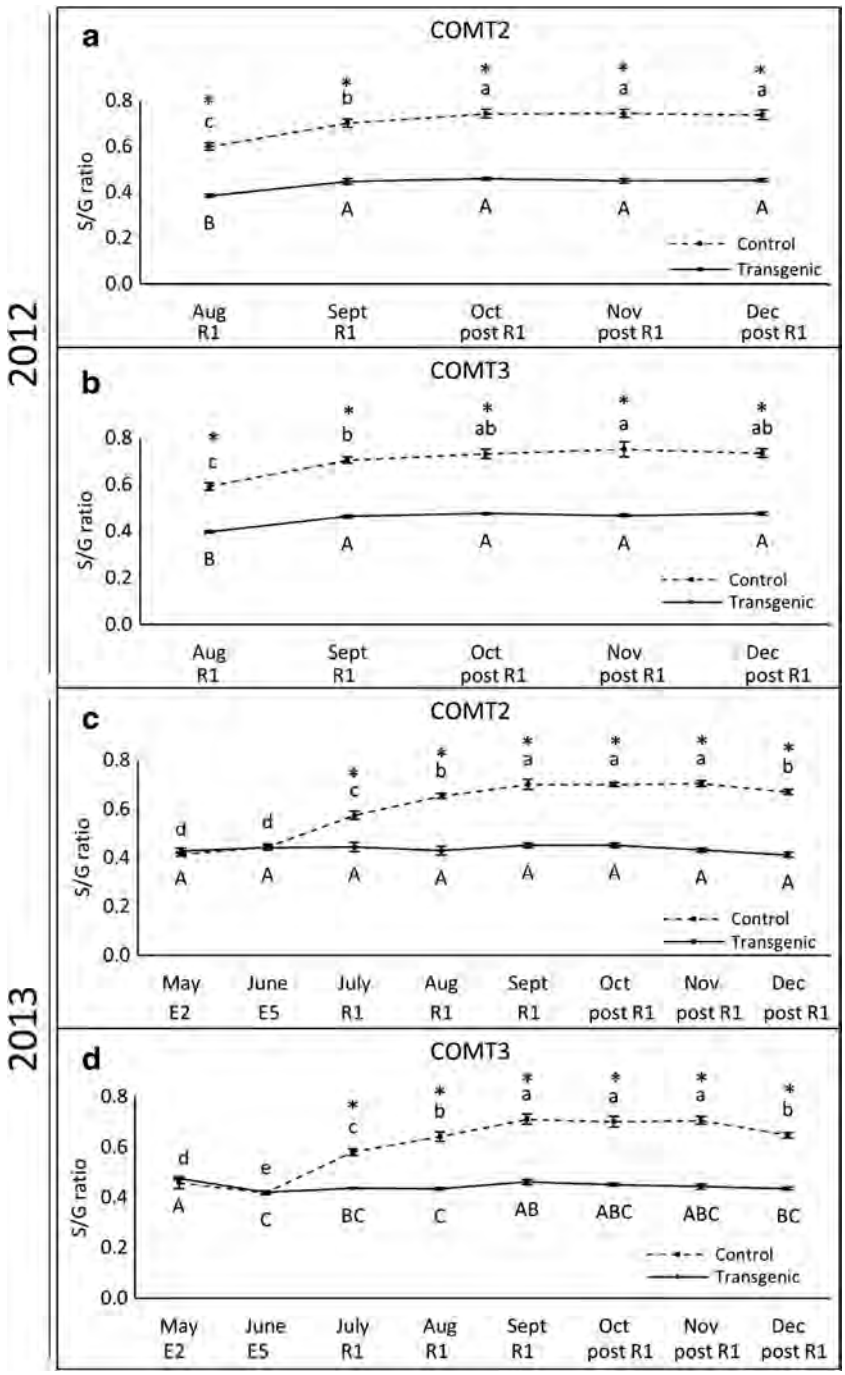

Fig. 4 Syringyl-to-guaiacyl (S/G) lignin monomer ratio of control and transgenic lines at different time points across the 2012 (**a**, **b**) and 2013 (**c**, **d**) growing seasons. Data points represent the average of the biological replicates within each control ($n = 5$) and transgenic ($n = 10$) line ± standard error. Data points with *different letters* differ significantly (repeated measures ANOVA, Fisher's LSD, $p < 0.05$) within each control (*lowercase letters*) and transgenic (*capital letters*) line. *Asterisks* indicate a significant difference (*t* test, $p < 0.05$) between the control and transgenic line

then slightly declined between November and December (Fig. 4c, d). Values ranged from 0.41 (early season) to 0.71 (late season). In contrast to the controls, the S/G ratios of the transgenic lines were relatively stable over time (Fig. 4c, d); values ranged from 0.41–0.47. As with the lignin content, the S/G ratio did not differ between transgenic and non-transgenic plants in the early season during the elongation growth stages. After plants reached the reproductive stage in July, transgenic lines maintained a 23–41 % lower S/G ratio than non-transgenic controls (Fig. 4c, d).

Sugar Release

Enzymatic hydrolysis was performed on hot water-pretreated CWR to measure the total release of sugars (glucose and xylose combined) from the cell wall. In 2012, changes in sugar release across the growing season were similar between transgenic and non-transgenic control lines. All lines exhibited an increase from August through October, no change from October to November, and a significant drop at the end of the season between

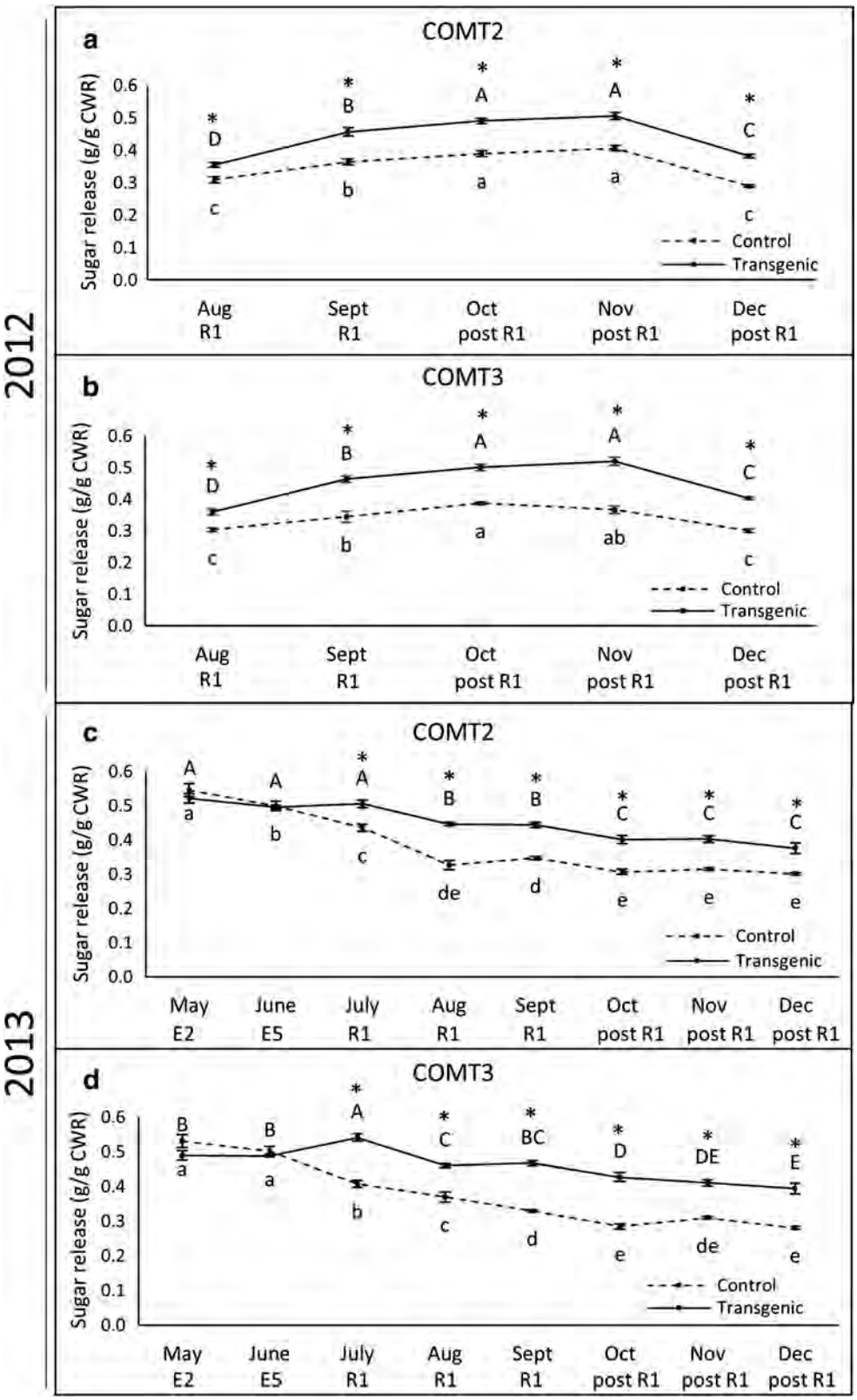

Fig. 5 Total sugar release (glucose + xylose) by enzymatic hydrolysis of hot water-pretreated control and transgenic lines at different time points across the 2012 (**a**, **b**) and 2013 (**c**, **d**) growing seasons. Data points represent the average of the biological replicates within each control ($n = 5$) and transgenic ($n = 10$) line ± standard error. Data points with *different letters* differ significantly (repeated measures ANOVA, Fisher's LSD, $p < 0.05$) within each control (*lowercase letters*) and transgenic (*capital letters*) line. *Asterisks* indicate a significant difference (*t* test, $p < 0.05$) between the control and transgenic line. *CWR* cell wall residues

November and December (Fig. 5a, b). Sugar release ranged from 0.290 to 0.406 g/g CWR in the control lines and 0.354 to 0.520 g/g CWR in COMT-downregulated lines. The transgenic lines had a significantly higher sugar release (15–42 %) compared with their respective controls at all time points (Fig. 5a, b).

In contrast to what was observed in 2012, the total sugar release for all lines (transgenic and non-transgenic) generally decreased across the 2013 season, with the lowest sugar release occurring between October and December (Fig. 5c, d). Sugar release ranged from 0.281 to 0.545 g/g CWR in control plants and 0.375 to 0.541 g/g CWR in transgenic plants. There were no differences in sugar release between transgenic and control lines during the elongation stages of growth (May and June). After reaching the reproductive stage in July, the transgenic lines maintained consistently higher sugar release (16–50 %) relative to their corresponding controls through December (Fig. 5c, d).

Disease Susceptibility

First symptoms of switchgrass rust were observed in late June during the 2012 and 2013 growing seasons. Rust susceptibility of the transgenic and non-transgenic lines was evaluated from June 22 through September 20 in 2012, and from July 12 through October 11 in 2013. In 2012, there were similar levels of rust susceptibility among COMT-downregulated and control lines (Fig. S1; [27]). Likewise, rust data collected across the 2013 growing season did not indicate significant differences in susceptibility (Fig. S2). Leaf spot caused by a *Bipolaris* species was also present, which appeared to affected transgenic and non-transgenic plants to a similar degree based on visual observation.

Discussion

The lignin biosynthetic pathway has been manipulated in switchgrass and other lignocellulosic plant species and the resulting effects on lignin and cell wall recalcitrance have been characterized [5–9, 16, 17]. From these studies, some gene targets for reducing recalcitrance have been identified and transgene expression has been partially optimized for some key genes. However, we currently lack an understanding of how the cell walls of lignin-modified plants develop over time relative to their non-transgenic counterparts; we know very little in this regards in perennial grasses, such as switchgrass.

The COMT-downregulated transgenic lines used in the present study have been characterized under greenhouse conditions [5] and in the field up through the second year of growth [27]. It is well-known that the COMT enzyme primarily catalyzes the methylation steps leading to the synthesis of sinapyl alcohol, the precursor of S lignin subunits [32–34]. Hence, a deficiency in COMT activity results in decreased synthesis of S lignin, thereby reducing the S/G ratio and decreasing the overall lignin content [35–37]. In line with these reports, greenhouse- and field-grown switchgrass plants with COMT-downregulation have decreased lignin amounts and lower S/G ratios compared with controls [5, 27]. In the field, these traits were apparent in green plant tissue harvested at mid-season and senesced tissue harvested at end-of-season [27]. Our goal in the present study was to focus more closely on within-season cell wall, especially lignin, variation and examine the second and third year of growth of these plants in the field. This is the first study to examine lignification and associated recalcitrance over time in a field-grown biofuel feedstock with altered lignin biosynthesis.

COMT Gene Expression

Under greenhouse conditions, RNAi-mediated downregulation of COMT in switchgrass significantly reduced COMT transcript abundance in most transgenic lines relative to the controls [5]. One objective of the current study was to examine the stability of COMT downregulation in the field-grown transgenic lines over time, especially since the expression of COMT and other lignin genes are known to be strongly influenced by external factors such as temperature, light, and biotic stresses [11]. For example, exposure of wheat to a fungal pathogen was associated with increased COMT gene expression [38]. COMT, in addition to other lignin biosynthetic genes, has also been shown to be upregulated in response to cold temperatures [39]. Since field-grown transgenic plants are exposed to fluctuating levels of stress across a growing season, it is important to understand how this fluctuation might influence expression levels of the target gene at various time points and what effects this could have on the desired cell wall phenotype. The field-grown COMT-downregulated lines in our study showed general trends of decreased COMT expression over time, though differences were not statistically significant at all time points, especially in the later part of 2013 (Fig. 2a, b). This variability in gene expression is not surprising in plants grown under natural conditions, especially late in the field season when conditions are becoming less favorable for growth. Despite higher than normal COMT expression in the transgenic lines during this time, the low recalcitrance phenotypes were maintained through December. Late-season increases in COMT expression in the transgenic lines might have had less of an influence on lignin content and composition since most of the lignin deposition was occurring in the previous months, during which COMT activity was still reduced.

Growth and Biomass Yield

Lignin plays an integral role in reinforcing cell walls and providing structural integrity to the plant. Cell wall modifications, particularly those targeting lignin biosynthesis, can result in developmental abnormalities, although this is not always the case [40]. For example, transgenic switchgrass lines with dramatic (40 %) reductions in lignin content due to high levels of MYB4-overexpression did not survive the first

winter in the field, whereas those transgenic lines with comparatively less severe reductions in lignin grew normally across two growing seasons [41]. Likewise, a 1-year study with field-grown COMT-downregulated sugarcane reported reduced yields in the transgenic lines that displayed the strongest reductions in lignin content, while the transgenic line with a relatively moderate lignin reduction produced biomass amounts similar to the control [42]. COMT-downregulated switchgrass lines, which had 8–12 % less lignin than controls, grew normally in the field for two growing seasons and produced at least as much biomass as controls by the end of the second season [27]. Longer-term field studies with transgenic low-lignin grasses to assess the stability of biomass yields across multiple years (> 2 years) have not yet been reported. To gain a better understanding of year-to-year variations in growth, we analyzed growth of the COMT-downregulated plants through the third growing season (2013). In general, our data showed comparable morphology and biomass yield trends between the second and third seasons (Table S1). Most importantly, both transgenic lines continued to exhibit reduced recalcitrance phenotypes relative to controls with no yield penalties compared to the controls.

General Trends over Growing Seasons

When determining the optimal time to harvest switchgrass, it is important to understand how certain aspects of the cell wall—in our case, those related to recalcitrance—change across the growing season. Our study showed different patterns of change in lignification over time between the 2012 and 2013 seasons. Generally, lignin data from the 2012 season showed only slight variation over time (Fig. 3a, b) relative to the subsequent season, which showed increasing trends in all lines as plants progressed from early elongation to late reproductive growth stages (Fig. 3c, d). Temperature, rainfall, humidity, and biotic stress pressures fluctuate from year to year in agricultural systems. Plant responses to combinations of these factors influence gene expression profiles and cell wall chemistry [39]. Therefore, some degree of year-to-year variability in cell wall chemistry and developmental patterns can be expected as a result of different environmental conditions. In our study, plants emerged almost a month earlier in 2012 than in 2013 and reached the reproductive stage slightly sooner, likely due to the warmer-than-average temperatures in early spring (Table S2). It is possible that many of the major cell wall changes, such as those occurring as plants transition from elongation to reproductive growth, took place earlier in the season in 2012 than in the subsequent season. Sampling in 2012 was initiated after plants had already reached the reproductive phase of growth, and cell wall lignin levels were undergoing less change across that developmental timeframe (August–December) than in 2013 (Fig. 3). Another considerable difference between the two growing seasons was the much higher-than-average rainfall that occurred during early and mid-season of 2013. Compared to the preceding growing season, almost twice as much rainfall occurred between April and July in 2013 (Table S2).

In contrast to the 2012 season, data from 2013 was collected across a broader developmental time span, which enabled a more comprehensive view of lignification over the season. Lignin generally accumulated as plants progressed in maturity (Fig. 3c, d), as expected based on prior studies with grasses [19, 20, 24]. There was also a large increase (19 %) in the lignin content of the non-transgenic control plants following the transition from the elongation (E5) growth stage in June to the reproductive (R1) stage in July. This is in line with previous research, which shows that a dramatic increase in lignin content following the transition from elongation to reproductive growth occurs because of the increased deposition of highly lignified secondary cell walls that is initiated after internode elongation ceases [24, 25]. It is notable that the COMT-downregulated lines in our study only exhibited a slight increase (7–8 %) in lignin content following this reproductive transition.

As lignification increases in maturing grass cell walls, there are also associated changes to the relative amounts of H, G, and S units being incorporated into the lignin polymer. While the onset of lignification primarily involves the incorporation of H and G units, later stages of lignification and secondary wall development involve increasing proportions of S units [43]. Consequently, the S/G ratio of grasses steadily increases with progressive developmental stages [20, 24, 25]. During the 2012 field season, control and transgenic lines in our study showed similar patterns of change in the S/G ratio over time, with values increasing between the August and September time points before stabilizing across the remaining season (Fig. 4a, b). In the subsequent growing season, transgenic lines exhibited different trends relative to their respective non-transgenic controls. The S/G ratios of control lines expectedly increased from early-to-mid season (Fig. 4c, d), with a particularly large increase (30–38 %) occurring between the June and July time points as plants transitioned from the elongation to reproductive growth stage. Interestingly, the COMT-downregulated lines exhibited no significant change during this transition, and little to no variation for the remainder of the growing season (Fig. 4c, d). COMT is considered to be preferentially involved in the synthesis of S lignin [35] and its downregulation significantly reduces the S/G ratio in switchgrass [5]. Our results clearly demonstrate the ability of COMT downregulation to consistently suppress S lignin production even into late stages of growth.

Over the course of plant development, cell walls become more recalcitrant as the accessibility of polysaccharides for enzymatic hydrolysis into monosaccharides decreases [19, 26]. The release of sugars from lignocellulosic biomass is strongly influenced by the amount of lignin in the cell walls.

Therefore, a decline in sugar release as plants mature is expected as a result of the increased cell wall lignification. Recalcitrance can also be influenced by other aspects of the lignin polymer, notably the way in which it is integrated into the cell wall and its degree of interaction with other components within the cell wall matrix [13]. In grasses, recalcitrance is also influenced by the presence of hydroxycinnamates, which accumulate within the cell wall matrix as plants age. These phenolics play a role in reinforcing the cell wall structure through the formation of lignin-polysaccharide cross-links, further reducing cell wall digestibility [19, 44]. Hemicelluloses, particularly the xylan component, have also been shown to play important role in the recalcitrance of switchgrass [13]. In the current study, the sugar release of all lines unexpectedly increased prior to leveling off and declining toward the end of the season in 2012 (Fig. 5a, b). Unlike in 2013, the cell wall lignin content in 2012 was not steadily increasing during this time period (Fig. 3a, b), which means that it probably had less of an effect on the trend of sugar release over time. It is possible that the apparent increase in sugar release across this time period (August–October) was being caused by some other factor, either within the plant cell wall itself or a perhaps some component of the environment, that influenced its digestibility. As plants reached full senescence in December, however, this phenomenon was no longer exhibited as demonstrated by the dramatic drop in sugar release between the last two time points for all lines (Fig. 5a, b). In the subsequent growing season, transgenic and non-transgenic lines exhibited a general decrease in sugar release across the season as plants reached progressive developmental stages (Fig. 5c, d). This increase in cell wall recalcitrance over time, as reflected by the decrease in sugar release, is consistent with the steady accumulation of lignin in the cell walls (Fig. 3c, d).

Transgenic and Non-Transgenic Cell Wall Differences

The ideal harvesting timeframe for plant biomass depends on the desired end-product. For bioenergy purposes, a late fall or early winter harvest of switchgrass is preferable due to its high cellulose content and low moisture and extractives content after it reaches senescence [45]. Harvesting late in the season also minimizes fertilizer inputs in the subsequent season by allowing for remobilization of nutrients to root systems [46]. Because each conversion system may have slightly different biomass quality requirements [47], low-recalcitrant transgenic feedstocks should be able maintain their desirable phenotypes across a wide range of dates within the growing season to allow for some flexibility in determining the ideal harvesting time. In the current study, the low-lignin cell wall phenotypes of the COMT-downregulated lines were consistent across the 2012 season and differences in lignin content and composition between COMT-downregulated and control lines were generally in line with previous reports ([5, 27]; Figs. 3a, b, and 4a, b). As with the lignin data, the transgenic lines maintained a consistent phenotype across the growing season in regard to sugar release, which was higher in the COMT-downregulated lines relative to the controls at all time points (Fig. 5a, b). The degree of difference in sugar release between the COMT-downregulated and controls lines fluctuated across the season, but both transgenic lines had a significantly larger increase over their corresponding controls after reaching full senescence (December; 32–24 % increase) compared to the green tissue harvested in mid-season (August; 15–19 % increase).

In contrast to 2012, sampling during the 2013 season was initiated in spring (May) and therefore included data from young elongation-stage plants. Our data showed that the lignin content of COMT-downregulated lines and their controls were similar during elongation stages of growth in early season, suggesting that transgenic and non-transgenic lines initially accumulate lignin at a similar rate (Fig. 3c, d). As described in the above section, the accumulation of lignin during the elongation-to-reproductive growth transition in July was greater in the control lines than the transgenic lines. Consequently, a divergence in lignification between the transgenic and control lines became clear after all plants transitioned to reproductive growth in mid-season, and the COMT-downregulated lines showed cell wall phenotypes consistent with those observed in 2012 (Figs. 3, 4). Sugar release differences between COMT-downregulated and control lines during the 2013 season were also not apparent until after the plants transitioned to reproductive growth in mid-season (Fig. 5c, d), emphasizing the close relationship between lignin content and cell wall recalcitrance. While both transgenic and non-transgenic lines exhibited a decrease in sugar release as plants matured across the season, the reduction was not as severe in the COMT-downregulated lines compared to the controls. Between the first (May) and last (December) time points, the sugar release of the control lines dropped by 45–47 %, while that of the transgenic lines only dropped by 19–28 %. The ability of the COMT-downregulated lines to maintain a relatively higher sugar release after senescence is a valuable trait for those conversion platforms that prefer to process the end-of-season mature biomass.

Disease Susceptibility

Because cell wall modification can affect disease susceptibility [48, 49], switchgrass rust susceptibility across the growing season was analyzed in parallel with cell wall traits. Rust is a major disease of switchgrass in general [50] and has been prevalent within the COMT field site since it was planted in 2011, as well as in other nearby switchgrass plots [27, 41, 51]. In our study, the reduced lignin content and lower S/G ratios in

the COMT-downregulated lines had no apparent effect on rust susceptibility at any time points in 2012 or 2013 (Figs. S1, S2). Leaf spot caused by a *Bipolaris* species was also present for the duration of the field experiment, but transgenic and non-transgenic plants appeared to be equally affected.

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