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Two-year field analysis of reduced recalcitrance transgenic switchgrass

Holly L. Baxter[1,2], Mitra Mazarei[1,2], Nicole Labbe[3], Lindsey M. Kline[3], Qunkang Cheng[4], Mark T. Windham[4], David G. J. Mann[1,2], Chunxiang Fu[2,5], Angela Ziebell[2,6], Robert W. Sykes[2,6], Miguel Rodriguez Jr[2,7], Mark F. Davis[2,6], Jonathan R. Mielenz[2,7], Richard A. Dixon[2,8], Zeng-Yu Wang[2,5] and C. Neal Stewart Jr[1,2,*]

[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]*Center for Renewable Carbon, University of Tennessee, Knoxville, TN 37996, USA*
[4]*Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996, USA*
[5]*Samuel Roberts Noble Foundation, Ardmore, OK 73401, USA*
[6]*National Renewable Energy Laboratory, Golden, CO 80401, USA*
[7]*Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA*
[8]*Department of Biological Sciences, University of North Texas, Denton, TX 76203, USA*

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**Correspondence* (Tel +1 865 974 7324;
fax +1 865 946 1989;
email nealstewart@utk.edu)

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Summary

Switchgrass (*Panicum virgatum* L.) is a leading candidate for a dedicated lignocellulosic biofuel feedstock owing to its high biomass production, wide adaptation and low agronomic input requirements. Lignin in cell walls of switchgrass, and other lignocellulosic feedstocks, severely limits the accessibility of cell wall carbohydrates to enzymatic breakdown into fermentable sugars and subsequently biofuels. Low-lignin transgenic switchgrass plants produced by the down-regulation of caffeic acid *O*-methyltransferase (COMT), a lignin biosynthetic enzyme, were analysed in the field for two growing seasons. COMT transcript abundance, lignin content and the syringyl/guaiacyl lignin monomer ratio were consistently lower in the COMT-down-regulated plants throughout the duration of the field trial. In general, analyses with fully established plants harvested during the second growing season produced results that were similar to those observed in previous greenhouse studies with these plants. Sugar release was improved by up to 34% and ethanol yield by up to 28% in the transgenic lines relative to controls. Additionally, these results were obtained using senesced plant material harvested at the end of the growing season, compared with the young, green tissue that was used in the greenhouse experiments. Another important finding was that transgenic plants were not more susceptible to rust (*Puccinia emaculata*). 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.

Introduction

Switchgrass (*Panicum virgatum* L.) is a warm-season C4 perennial grass native to the prairies of North America. High biomass production, minimal water and nutritional requirements, widespread adaptability and high net energy gain have made switchgrass a leading dedicated bioenergy crop. In addition, switchgrass is relatively tolerant to a range of biotic and abiotic stresses and provides numerous environmental benefits including reduced soil erosion, improved surface water quality and the potential to reduce greenhouse gas emissions due to a high rate of carbon sequestration (McLaughlin and Kszos, 2005; McLaughlin and Walsh, 1998; Parrish and Fike, 2005; Schmer *et al.*, 2008). Despite these advantages, high costs associated with the conversion of lignocellulosic biomass into biofuels currently prevent feedstocks such as switchgrass from being economically competitive fuel sources (Himmel *et al.*, 2007).

A major barrier to cost-efficient biofuel production from lignocellulosic plants is the resistance of the cell wall to chemical, microbial or enzymatic deconstruction into fermentable sugars, also known as cell wall recalcitrance (Himmel *et al.*, 2007). The primary structural components of switchgrass cell walls are cellulose (30%–40%), hemicellulose (25%–35%) and lignin (15%–20%) (David and Ragauskas, 2010). The cellulose microfibrils are integrated into a highly cross-linked matrix of lignin and hemicellulose (Iiyama *et al.*, 1994). Lignin is a complex phenolic polymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units, which are derived from the monolignols *p*-coumaryl, coniferyl and sinapyl alcohol, respectively (Boerjan *et al.*, 2003). Lignin contributes structural stability to the plant, aids in water transportation in vascular tissues and protects the cell wall from pathogen penetration. Because it inhibits enzymatic and microbial access to fermentable cell wall polysaccharides, lignin has been identified as a primary contributor to biomass recalcitrance. Recalcitrance can be influenced by the amount of

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lignin in the cell walls, as well as the S/G lignin monomer ratio (Chen and Dixon, 2007; Corredor *et al.*, 2009; Davison *et al.*, 2006). Expensive and energy-inefficient thermochemical pretreatments are generally required to disrupt the lignin–polysaccharide matrix and increase the accessibility of carbohydrates for hydrolysis prior to fermentation (Mosier *et al.*, 2005). In addition to being costly, many pretreatment processes also generate degradation products that can be inhibitory to downstream microbial fermentation (Klinke *et al.*, 2004).

One approach to reduce recalcitrance is by genetically modifying cell wall characteristics to develop transgenic feedstocks that are more amenable to enzymatic hydrolysis. Manipulating genes in the lignin biosynthetic pathway can decrease lignin content and/or modify its composition, which could reduce or eliminate the need for pretreatment prior to fermentation (Chen and Dixon, 2007; Hisano *et al.*, 2009). The down-regulation of lignin biosynthetic enzymes has been successful in improving saccharification in lignocellulosic grass species including Brachypodium (*Brachypodium distachyon*), sugarcane (*Saccharum* spp. hybrids) and switchgrass (Bouvier d'Yvoire *et al.*, 2013; Fu *et al.*, 2011a,b; Jung *et al.*, 2012; Saathoff *et al.*, 2011).

Caffeic acid *O*-methyltransferase (COMT) is an enzyme functioning late in the lignin biosynthetic pathway, catalysing the *O*-methylation of the 5-hydroxyl groups of monolignol precursors. The preferred substrates of COMT are 5-hydroxyconiferyl aldehyde and 5-hydroxyconiferyl alcohol, two metabolites involved in the synthesis of the S-monomer subunit sinapyl alcohol (Humphreys *et al.*, 1999; Louie *et al.*, 2010; Osakabe *et al.*, 1999). Reduced COMT activity results in a decreased flux of lignin precursors towards the synthesis of sinapyl alcohol, thus reducing the S/G lignin monomer ratio and the total lignin content. These characteristics have been observed in COMT-deficient brown midrib mutants of maize (bm3) and sorghum (bmr12), as well as in transgenic plants with antisense or RNAi-mediated down-regulation of COMT (Guo *et al.*, 2001; Jung *et al.*, 2012; Palmer *et al.*, 2008; Vignols *et al.*, 1995). Consistent with these studies, greenhouse-grown T_1 generation transgenic COMT-RNAi switchgrass exhibits a reduction in total lignin content (11.4%–13.4%) and the S/G lignin monomer ratio (44%–46%). These plants, without pretreatment, also exhibit an increase in the enzymatic release of sugars (29%–38%) and up to 42% more ethanol yield per unit of biomass than controls. With pretreatment, a 17%–22% increase in enzymatic sugar release and 30%–38% increase in ethanol yield per unit of biomass compared with controls are observed (Fu *et al.*, 2011a). These improvements could be a result of the reduction in lignin content, altered S/G ratio and/or reduced cross-linking of lignin to polysaccharides as a result of the presence of 5-hydroxyguaiacyl residues in the lignin (Simmons *et al.*, 2010).

Although COMT-down-regulated switchgrass performs well in the greenhouse, there has been considerable speculation about how lignin-altered biomass feedstocks would perform in the field (Pedersen *et al.*, 2005). Given the important roles of lignin in plant development and stress-related processes, evaluating the performance of low-lignin plants in the field is essential. The synthesis and deposition of lignin is influenced by a range of biotic and abiotic stresses, including herbivory, pathogen infection, temperature stress, light and oxidative stress, water deficiency and mechanical injury (Moura *et al.*, 2010). As lignin-modified plants respond and adapt to stresses in the field, the consequent physiological changes could cause results to vary from those observed in plants grown under greenhouse conditions where stresses are minimized. Considering this potential for variation, field trials are necessary to ensure that the altered lignin profiles and associated improvements in saccharification and ethanol yield can be achieved under field conditions without negatively impacting biomass yield or disease susceptibility. Furthermore, these improvements should be demonstrated in senesced biomass harvested at the end of the growing season, as this is the material that is most preferable for commercial biofuel production (Heaton *et al.*, 2009).

We conducted a two-year field study using two independent transgenic lines (COMT2 and COMT3) from the previous greenhouse study (Fu *et al.*, 2011a) with the following objectives: (i) to verify that RNAi-mediated silencing of COMT is stable in transgenic tissues and that the expected modifications to lignin are retained in field-grown plants, (ii) to characterize the chemical composition of the cell wall and to assess whether the improved sugar release efficiency and ethanol yield can be demonstrated in field-grown plants and (iii) to investigate potential impacts of COMT down-regulation on agronomic performance and disease susceptibility. To our knowledge, this is the first reported field study evaluating the biofuel potential of transgenic switchgrass with reduced cell wall recalcitrance.

Results

Field plants and design

Plants were generated by RNAi-mediated gene silencing of COMT and were previously characterized under greenhouse conditions (Fu *et al.*, 2011a). Two T_1-generation events, COMT2 and COMT3 (Table S1), were analysed in the field. The experimental design was a completely randomized design with ten replicates for each transgenic event and five replicates for each control. Each replicate contained nine vegetatively propagated plants (Figure S1). The field study was performed for two consecutive growing seasons (2011–2012) (Figure S2).

Analysis of COMT transcript levels

COMT transcript abundance for transgenic T1-generation field-grown lines, COMT2 and COMT3, was analysed by qRT-PCR. COMT transcript levels were reduced by up to 97% in both transgenic lines for 2011 and 2012 (Figure S3).

Lignin content and composition

Pyrolysis molecular beam mass spectrometry (py-MBMS) of cell wall residues was used to estimate the total lignin content and the S/G lignin monomer ratio for whole tillers harvested at mid-growing season (green) and whole aboveground biomass harvested at the end of the growing season (senesced) each year. COMT down-regulation resulted in 10.1%–14.5% less lignin in biomass harvested at the green tissue stage for both years (Figure 1a,c; Table S2). The green tissue S/G ratio was 22%–27% less than that of controls in year one and 33%–36% less in year two (Figure 1b,d; Table S2). In senesced tissue transgenic samples, an 8.4%–10.6% decrease in lignin content was observed in year one, accompanied by a 19%–20% reduction in the S/G ratio (Figure 2a,b; Table S2). In year two, senesced tissue from transgenic lines had an 11.6%–12.0% decrease in lignin content and a 35%–39% reduction in the S/G ratio compared with the controls (Figure 2c,d; Table S2).

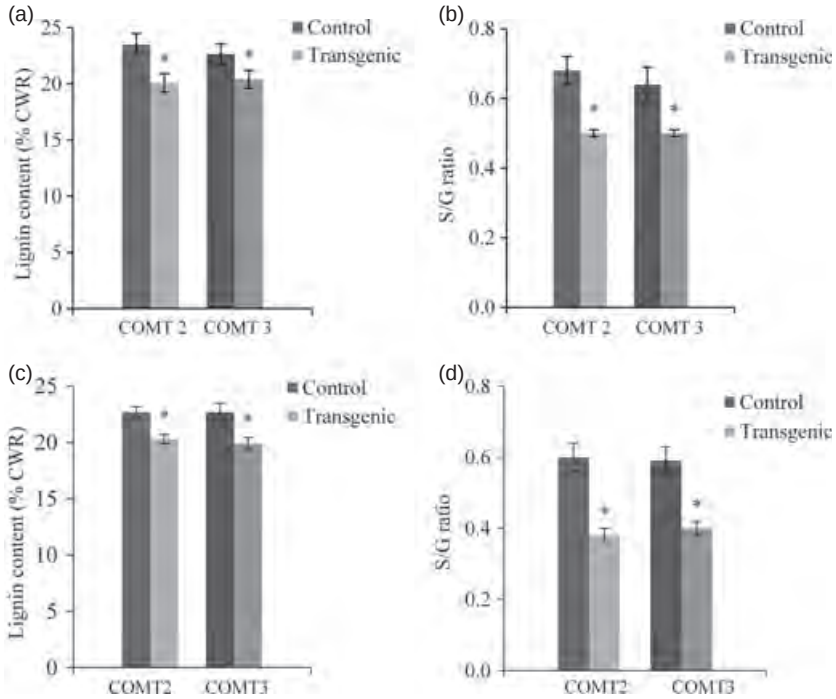

Figure 1 Lignin content and S/G ratio of green tissue samples, as estimated by py-MBMS, for 2011 (a, b) and 2012 (c, d). All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues.

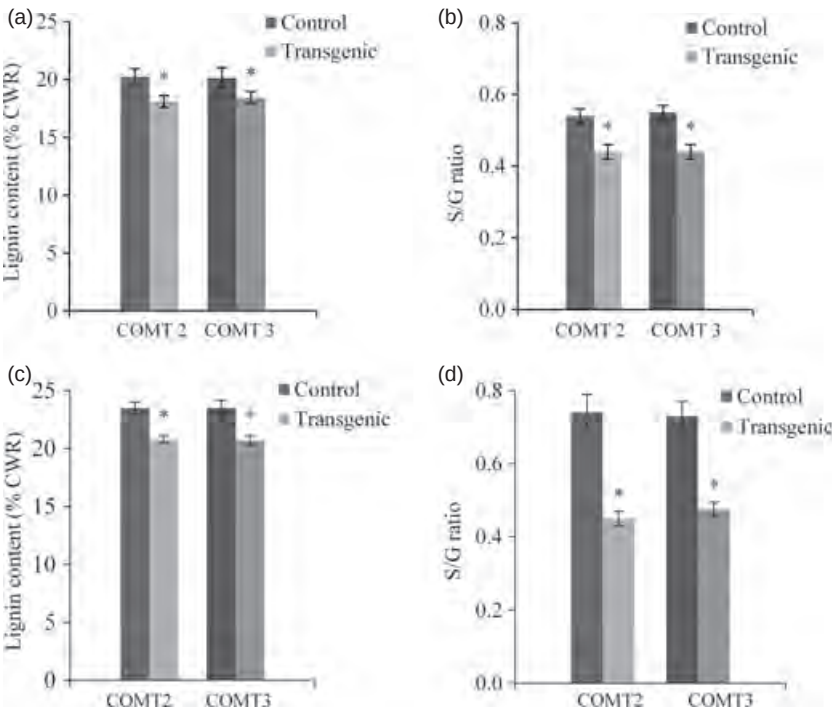

Figure 2 Lignin content and S/G ratio of senesced tissue samples, as estimated by py-MBMS, for 2011 (a, b) and 2012 (c, d). All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues

Cell wall composition and index of cellulose crystallinity

The chemical composition of the cell wall and the index of cellulose crystallinity were determined for biomass harvested after senescence. Sugar profiles were similar between transgenic and control plants harvested in the first year. Cellulose and hemicellulose comprised approximately 39% and 28%–30% of the cell wall, respectively. Other compositional differences between transgenic and control plants were minor, and there were no significant changes in the index of cellulose crystallinity (Table 1; Figure 3). In second-year harvested samples, cellulose and hemicellulose accounted for 38%–39% and 30%–32% of the cell wall, respectively. Similar to the first year, no differences in cellulose content were detected; however, transgenic events had slightly higher hemicellulose levels (6.4%–7.4%), primarily due to an increase in xylose. Additionally, an 11.6% increase in acetyl

Table 1 Chemical composition of senesced biomass on an extractives-free basis in 2011 and 2012

	Control	COMT2	Control	COMT3
Year 1 (2011)				
Structural ash	5.1 ± 0.7	5.3 ± 0.6	**4.6 ± 0.7**	**5.6 ± 0.6***
Lignin	**22.2 ± 0.9**	**20.9 ± 1.3***	**22.4 ± 0.7***	**21.3 ± 0.6***
Acetyl	2.6 ± 0.2	2.4 ± 0.3	2.7 ± 0.2	2.6 ± 0.3
Total carbohydrates	67.0 ± 3.1	67.5 ± 2.2	70.1 ± 1.1	69.2 ± 3.2
Cellulose	39.0 ± 1.6	39.4 ± 1.3	39.7 ± 0.9	39.3 ± 1.6
Hemicellulose	28.0 ± 1.8	28.1 ± 1.6	30.4 ± 0.2	29.9 ± 1.9
Xylan	22.9 ± 1.6	22.7 ± 1.6	25.3 ± 0.2	24.4 ± 1.6
Galactan	1.9 ± 0.2	2.0 ± 0.3	**1.7 ± 0.2**	**2.1 ± 0.3***
Arabinan	2.9 ± 0.7	3.0 ± 0.3	2.8 ± 0.7	3.0 ± 0.6
Mannan	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
Year 2 (2012)				
Structural ash	2.2 ± 0.4	2.4 ± 0.3	2.6 ± 0.2	2.3 ± 0.6
Lignin	**22.1 ± 0.2**	**20.5 ± 0.3***	**22.4 ± 0.7**	**20.5 ± 0.3***
Acetyl	4.6 ± 0.2	4.6 ± 0.3	**4.3 ± 0.2**	**4.8 ± 0.3***
Total carbohydrates	68.9 ± 1.1	70.9 ± 2.5	**67.5 ± 2.0**	**69.9 ± 1.6***
Cellulose/glucan	39.0 ± 0.9	39.1 ± 1.3	37.8 ± 1.3	38.0 ± 1.3
Hemicellulose	**29.9 ± 0.4**	**31.8 ± 1.3***	**29.7 ± 0.9**	**31.9 ± 0.6***
Xylan	**26.1 ± 0.7**	**27.8 ± 0.9***	**26.0 ± 0.7**	**28.0 ± 0.6***
Galactan	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.4	0.8 ± 0.3
Arabinan	2.7 ± 0.2	2.9 ± 0.3	**2.6 ± 0.2**	**2.9 ± 0.3***
Mannan	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.2	0.3 ± 0.0

Chemical composition data are presented on a percentage dry weight basis. All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). Bold values denote statistical significance.

content was observed in event COMT3 (Table 1; Table S2). The index of cellulose crystallinity was reduced by 18.6% in event COMT2, and 16.0% in event COMT3, compared with controls (Figure 3; Table S2).

Sugar release efficiency

Sugar release by enzymatic hydrolysis was determined for whole tillers harvested at mid-growing season (green) and whole aboveground biomass harvested at the end of the growing season (senesced) each year. Sugar release assays were performed by subjecting cell wall residues to a hot water pretreatment, followed by a 72-h incubation with hydrolysing enzymes. The amounts of glucose, xylose and total sugars (glucose and xylose combined) released per gram of cell wall residues were determined for each sample. In year one, transgenic plants harvested at the green tissue stage exhibited a 9.0%–11.7% increase in total sugar release. Similarly, in year two, the total sugar release for green tissue transgenic plants was improved by 14.7%–18.7% relative to the controls. Sugar release improvements in transgenic plants harvested after senescence were only observed in line COMT2 in year one. In year two, the total sugar release from senesced tissues was improved by 32.0% and 34.2% in lines COMT2 and COMT3, respectively (Table 2; Table S2).

Ethanol yield

Ethanol yield of field-grown senesced biomass was determined using the simultaneous saccharification and fermentation (SSF) approach described in the greenhouse study (Fu *et al.*, 2011a). Briefly, samples were pretreated under moderate dilute acid conditions followed by SSF with *Saccharomyces cerevisiae*. Weight loss from the bottles due to CO_2 release was used to monitor the progression of the fermentation over time. In year one, line COMT3 exhibited a faster fermentation rate and produced 21.2% more ethanol per gram of biomass than its corresponding control, while line COMT did not differ significantly from its control (Figure 4; Table S2). In year two, both transgenic lines fermented more quickly than their controls and exhibited ethanol yield improvements of 28.2% (COMT2) and 21.4% (COMT3) (Figure 5; Table S2).

Agronomic performance

To assess the growth and productivity of COMT-down-regulated switchgrass under field conditions, tiller height and plant diameter were measured at mid-growing season of each year, and tiller number and aboveground dry weight biomass yield were determined at the end of the season. In the first year, line

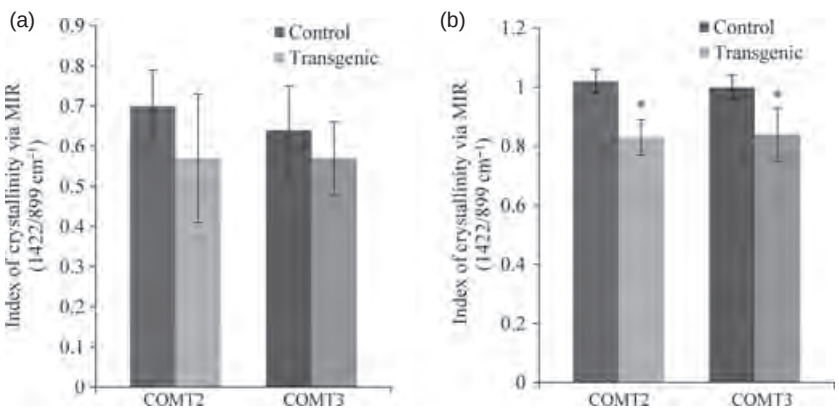

Figure 3 Index of cellulose crystallinity as determined by Fourier transform infrared spectroscopy analysis of senesced samples in 2011 (a) and 2012 (b). MIR, mid-infrared. All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Bars represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

Table 2 Sugars released by enzymatic hydrolysis from green and senesced samples in 2011 and 2012

		Green tissue			Senesced tissue		
Year		Glucose release (g/g CWR)	Xylose release (g/g CWR)	Total release (g/g CWR)	Glucose release (g/g CWR)	Xylose release (g/g CWR)	Total release (g/g CWR)
2011	Control	**0.241 ± 0.019**	0.181 ± 0.019	**0.421 ± 0.039**	**0.234 ± 0.015**	0.162 ± 0.010	**0.396 ± 0.023**
	COMT2	**0.277 ± 0.008***	0.194 ± 0.008	**0.471 ± 0.014***	**0.255 ± 0.013***	0.163 ± 0.008	**0.418 ± 0.012***
	Control	0.249 ± 0.013	0.183 ± 0.012	**0.432 ± 0.025**	0.230 ± 0.027	0.163 ± 0.011	0.393 ± 0.035
	COMT3	0.269 ± 0.020	0.201 ± 0.017	**0.471 ± 0.033***	0.243 ± 0.014	0.170 ± 0.008	0.413 ± 0.020
2012	Control	**0.151 ± 0.016**	**0.158 ± 0.006**	**0.309 ± 0.022**	**0.126 ± 0.010**	**0.163 ± 0.004**	**0.289 ± 0.011**
	COMT2	**0.178 ± 0.014***	**0.176 ± 0.009***	**0.354 ± 0.022***	**0.182 ± 0.013***	**0.200 ± 0.008***	**0.382 ± 0.020***
	Control	**0.147 ± 0.011**	**0.157 ± 0.007**	**0.304 ± 0.014**	**0.132 ± 0.004**	**0.180 ± 0.019**	**0.300 ± 0.012**
	COMT3	**0.183 ± 0.016***	**0.177 ± 0.014***	**0.360 ± 0.029***	**0.195 ± 0.011***	**0.208 ± 0.006***	**0.403 ± 0.014***

All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). CWR, cell wall residues. Bold values denote statistical significance.

COMT2 was not significantly different than its corresponding control in tiller height, plant diameter, tiller number or dry weight biomass yield. However, event COMT3 exhibited a decrease in tiller height (15.3%), plant diameter (8.1%) and dry weight yield (50.5%) relative to the control. In the second year, line COMT2 had an increase in tiller height and plant diameter and a corresponding 18.2% increase in dry weight biomass yield relative to its control, whereas line COMT3 was not different from its control in any of the morphological traits or dry weight biomass yield (Table 3; Table S2).

Disease susceptibility

All plants were monitored throughout both growing seasons for any deleterious effects caused by pathogens or pests. Grasshoppers, ladybugs and spiders were observed on the plants. Insect damage to the leaves of transgenic and nontransgenic plants was minimal. The most prevalent diseases observed were switchgrass rust caused by *Puccinia emaculata* and leaf spot caused by a *Bipolaris* species. For both growing seasons, rust was observed from mid- to late June until the plants began to senesce towards the end of November. Disease severity of rust infection was rated weekly during the second growing season and expressed as the percentage of the leaf covered in rust uredia; it ranged from 3% to 5% in mid- to late June to 25%–30% in late September. COMT down-regulation did not affect rust susceptibility in either transgenic line relative to its control at any of the time points assessed (Figure S4).

Discussion

Overcoming the chemical and structural features of the biomass that contribute to cell wall recalcitrance could significantly lower biomass conversion costs and enable the sustainable production of biofuels from lignocellulosic materials (Himmel *et al.*, 2007). Greenhouse studies have shown that manipulating lignin biosynthesis in lignocellulosic plants can be an effective route for decreasing recalcitrance. However, given the important biological roles of lignin in developmental and defence-related processes, experiments to evaluate the sustainability of such feedstocks in an agronomic setting are indispensable. The current study evaluated the field performance of switchgrass with reduced recalcitrance over the course of two years: the first year (2011), in which the plants were newly transplanted, and the second year (2012), in which the plants were well established and had reached a more steady-state growth pattern. To our knowledge, this is the first reported field study evaluating the potential of transgenic switchgrass with reduced lignin content for use as a bioenergy crop.

Brown midrib (bmr) mutant and transgenic grasses with reduced COMT enzyme activity are characterized by significant reductions in S-lignin, resulting in a lower S/G monomer ratio, as well as a decrease in the total lignin content (Chen *et al.*, 2004; Fu *et al.*, 2011a; Jung *et al.*, 2012; Palmer *et al.*, 2008; Piquemal *et al.*, 2002; Tu *et al.*, 2010; Vignols *et al.*, 1995). In agreement with these studies, COMT down-regulation in field-grown switchgrass was associated with a reduction in the S/G ratio and lower lignin levels. RNAi-mediated gene silencing of COMT gene expression was maintained in the transgenic lines for the duration of the field experiment, showing a reduction of 82%–97% compared with the control plants. This resulted in an 8%–15% decrease in lignin content in green- and senesced-harvested biomass from transgenic lines in years one and two. While differences in lignin levels between transgenic and control plants were relatively consistent across both years, the reduction in the S/G ratio was significantly more pronounced in the second growing season; a 35%–39% reduction was observed in both green and senesced tissues from transgenic lines compared with their controls. This is similar to the 43%–46% reduction that was reported for the COMT transgenic lines when grown under greenhouse conditions (Fu *et al.*, 2011a). An additional observation resulting from COMT down-regulation was a brownish coloration at the basal internode of the stems of the transgenic plants, which was also noted in the greenhouse study (Fu *et al.*, 2011a).

Understanding the broader effects of lignin down-regulation on the synthesis of other cell wall components is also important, as any unintended changes in composition could ultimately affect the quality of the feedstock for bioconversion. Although the mechanisms are not yet well understood, studies have shown that down-regulating lignin genes can affect other metabolic processes, including primary metabolism and the synthesis of cell wall carbohydrates (Coleman *et al.*, 2008; Dauwe *et al.*, 2007; Hu *et al.*, 1999). In the present study, cellulose content remained unchanged between field-grown transgenic and control plants

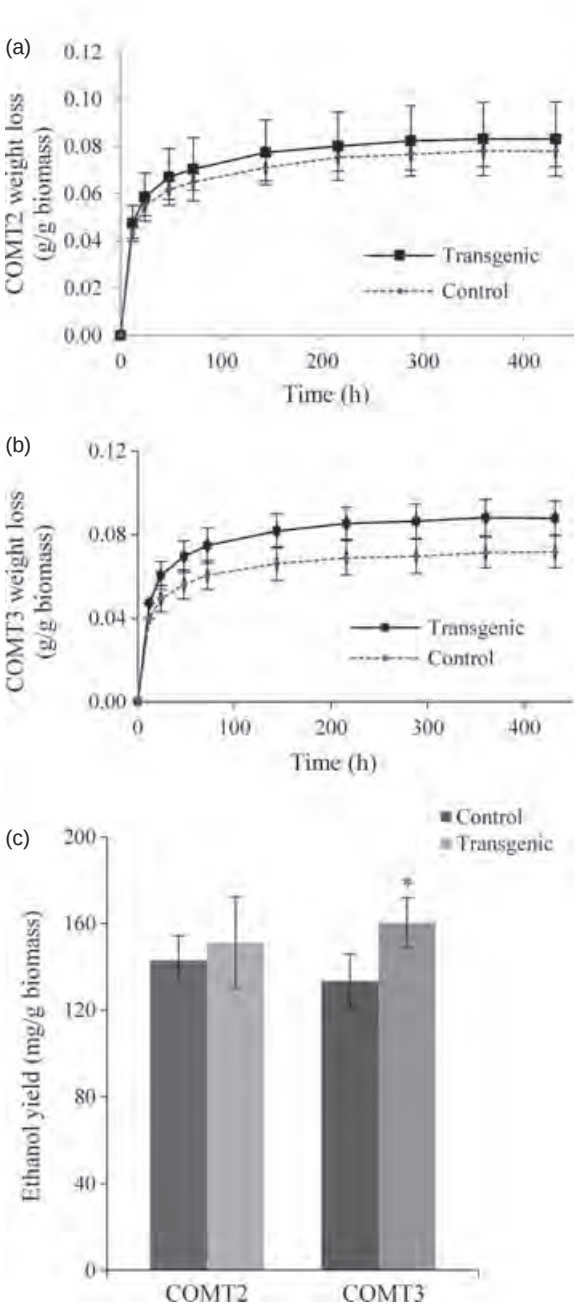

Figure 4 Ethanol yield as determined by simultaneous saccharification and fermentation of senesced samples in 2011. (a, b) Weight loss of fermentation bottles due to CO_2 release over time for lines caffeic acid *O*-methyltransferase COMT2 (a) and COMT3 (b); data points represent the average of the biological replicates within each group ± s.d. (c) Final ethanol yield; bars represent the average of the biological replicates within each group ± s.d. All five biological replicates from each control group, and five randomly selected samples from each transgenic group, were used in the analyses. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

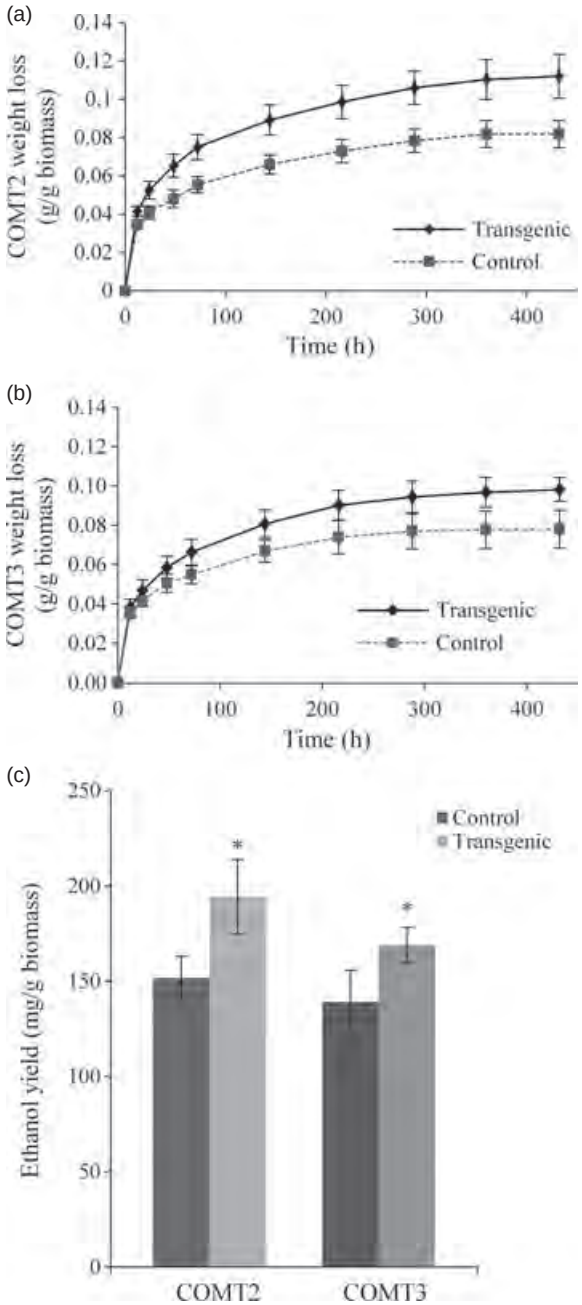

Figure 5 Ethanol yield as determined by simultaneous saccharification and fermentation of senesced samples in 2012. (a, b) Weight loss of fermentation bottles due to CO_2 release over time for lines caffeic acid *O*-methyltransferase COMT2 (a) and COMT3 (b); data points represent the average of the biological replicates within each group ± s.d. (c) Final ethanol yield; bars represent the average of the biological replicates within each group ± s.d. All five biological replicates from each control group, and five randomly selected samples from each transgenic group, were used in the analyses. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$).

for both years. This is consistent with the greenhouse study, which reported minor or negligible impact of lignin reduction on cellulose content in these two lines (Fu *et al.*, 2011a). However, hemicellulose content was increased by 6.4%–7.4% in the field-grown transgenic lines in the second year, primarily due to an increase in xylose, the major monosaccharide component of hemicellulose in grasses. A slight increase in xylose content for these two transgenic lines was also reported in the greenhouse study with these plants (Fu *et al.*, 2011a). Similarly, a field trial

Table 3 Morphology and dry weight biomass yield of field-grown transgenic lines during the 2011 and 2012 growing seasons

Year		Tiller height (cm)	Plant diameter (cm)	Tiller number	DW biomass yield (g/m^2)
2011	Control	69.5 ± 9.4	73.5 ± 8.1	71.5 ± 15.0	38.5 ± 31.2
	COMT2	68.4 ± 6.1	75.5 ± 5.6	85.4 ± 10.1	44.4 ± 10.9
	Control	**77.3 ± 6.3**	**77.9 ± 3.4**	81.3 ± 5.5	**75.8 ± 21.3**
	COMT3	**65.5 ± 5.8***	**71.6 ± 3.4***	88.8 ± 10.3	**37.7 ± 12.2***
2012	Control	**184.9 ± 6.7**	**200.5 ± 22.2**	180.8 ± 24.0	**2150.7 ± 378.8**
	COMT2	**197.2 ± 5.4***	**232.9 ± 11.1***	196.1 ± 28.7	**2628.5 ± 292.8***
	Control	188.2 ± 11.4	225.4 ± 15.9	186.2 ± 9.6	2720.2 ± 711.8
	COMT3	192.1 ± 8.8	217.9 ± 17.0	192.5 ± 31.5	2160.4 ± 390.8

All five biological replicates from each control group, and all 10 biological replicates from each transgenic group, were used in the analyses. Values represent the average of the biological replicates within each group ± s.d. Each transgenic line was compared with its respective control. An asterisk indicates a significant difference as determined by a *t*-test ($P < 0.05$). DW, dry weight. Bold values denote statistical significance.

with COMT-down-regulated sugarcane reported an increase in xylose content for the transgenic line that displayed the strongest reduction in lignin content (Jung *et al.*, 2013). In addition to altered hemicellulose levels, the transgenic lines in this experiment exhibited a 16.0%–18.6% decrease in the index of cellulose crystallinity relative to controls, suggesting that COMT down-regulation in field-grown switchgrass might also have an indirect influence on structural characteristics of cellulose. A lower ratio of crystalline to amorphous cellulose is considered to be a desirable trait in bioenergy feedstocks as it can enhance enzymatic digestion of the cell wall (Himmel *et al.*, 2007).

Greenhouse experiments with switchgrass have demonstrated the advantages of modifying lignin biosynthesis for improving sugar release and ethanol yield in live, green plants (Fu *et al.*, 2011a,b; Saathoff *et al.*, 2011; Shen *et al.*, 2012, 2013). However, the improved traits observed in young green plants grown in low-stress environments may or may not be retained in mature field-grown plants due to environmental and age-related effects. For these reasons, greenhouse studies need to be validated in a realistic growth environment using the more commercially relevant senesced biomass to truly assess the economic potential of lignin-modified switchgrass as a bioenergy feedstock. In the current study, improvements in sugar release were different between the transgenic plant lines by both year and green vs senesced tissue. Most importantly, at the end of year two, senesced tissues of COMT2 and COMT3 events had 32% and 34% higher sugar release, respectively, compared with their controls. The altered lignin and improved sugar release in the transgenic events harvested in year two translated to a faster rate of fermentation and ethanol yield improvements of 28.0% (COMT2) and 21.4% (COMT3) in senesced plants relative to their controls.

While modifications to lignin biosynthesis can significantly improve the accessibility of fermentable sugars, some can be associated with developmental abnormalities and yield reductions which could offset the positive gains in saccharification and ethanol yield (Bonawitz and Chapple, 2012). COMT down-regulation in grass species has been shown to have minimal effects on growth and development, including greenhouse-grown switchgrass and maize and field-grown perennial ryegrass (Fu *et al.*, 2011a; Piquemal *et al.*, 2002; Tu *et al.*, 2010). In the current study, line COMT2 was not different from its control in morphology and dry weight yield in the first growing season, but exhibited an increase in tiller height, plant width and dry weight biomass yield in year two. In contrast, line COMT3 yielded less biomass than its control during the first growing season, but was not significantly different than its corresponding control in the second growing season. Because this line showed normal growth and development under greenhouse conditions (Fu *et al.*, 2011a) and under field conditions once adequately established, it is likely that the yield reduction observed in the first growing season was an effect of establishment year. Switchgrass can be slow to establish and can require multiple years to reach its full yield potential (Perrin *et al.*, 2008; Samson, 2007); consequently, switchgrass stands that exhibit poor growth during the establishment year often produce significantly higher yields in the following growing seasons (Samson, 2007). From this, it can be inferred that measures of biomass yield obtained from plants harvested in the subsequent years following establishment may be more realistic and reliable than those obtained in the initial year of planting.

In addition to biomass yield effects, another concern when manipulating lignin is that plant defence could be compromised. Lignin and its precursors play a central role in defence against pathogens, both as a physical barrier against pathogen entry and as an induced response to infection (Nicholson and Hammerschmidt, 1992; Vance *et al.*, 1980). Given the potential threat posed by pathogens and insect pests to the successful establishment and sustainability of bioenergy feedstocks (Stewart and Cromey, 2011), the possible consequences of reduced lignin on plant stress susceptibility must be thoroughly evaluated in a field setting. Of particular relevance, the fungal pathogen *Puccinia emaculata* has been reported to cause rust disease in switchgrass and has recently been observed in agronomic fields in Tennessee (Zale *et al.*, 2008) and Arkansas (Hirsch *et al.*, 2010). The down-regulation of COMT has been shown to have varying effects on the susceptibility of field-grown transgenic crops to different rust species; COMT down-regulation in perennial ryegrass increased the susceptibility of field-grown transgenic plants to rust (Tu *et al.*, 2010), but had no effect on rust susceptibility in sugarcane or poplar grown in the field (Jung *et al.*, 2013; Pilate *et al.*, 2002). In this study, the most common pathogens observed in the field were rust (*Puccinia emaculata*) and a *Bipolaris* species. The severity of *Bipolaris* was minimal, and rust susceptibility did not differ in COMT-down-regulated switchgrass plants relative to their nontransgenic controls. Additionally, no major insect damage to the leaves of control or transgenic plants was observed.

In order for modified lignocellulosic biofuel feedstocks to be economically viable, they must perform satisfactorily in appropriate agronomic field environments. The results of this experiment demonstrate that once established, COMT-down-regulated transgenic switchgrass had similar gains in sugar release (up to 34% higher) and biofuel production (up to 28% higher) as observed when comparing transgenic and control plants grown in the greenhouse. Importantly, these results were achieved using the more commercially relevant senesced biomass harvested at the end of the growing season, compared with the young green tissue used in the prior greenhouse experiments. Also important was that COMT down-regulation did not impact disease susceptibility. This first two-year field trial of a transgenic herbaceous cellulosic feedstock shows that genetic engineering of an appropriate lignin target, in this case decreasing the expression of caffeic acid *O*-methyltransferase, can confer significant real-world improvements in biofuel yield without negatively impacting biomass yield or disease susceptibility. When we combine the biofuel and biomass gains observed in the second year of the study, the COMT2 transgenic line yielded over 50% more litres of ethanol per hectare compared with its control, which demonstrates that such transgenic feedstocks could make important improvements in lignocellulosic biofuels.

Experimental procedures

Green and senesced sample collection and preparation

Green tissue samples

Samples were collected in mid-growing season from green plants at the same developmental stage. The tallest tiller from each plant was selected, and the whole tiller (including stem and leaves) was cut from 10 to 15 cm above the soil surface. For first-year analyses, green tissue samples were collected in October 2011 from plants at the E6 growth stage [growth stages determined using the criteria described by Moore *et al*. (1991)]. For second-year analyses, green tissue samples were collected in August 2012 from plants at the R0 growth stage.

Senesced tissue samples

Whole aboveground senesced biomass was harvested after the killing frost in December of each year. After determination of dry weight yield, subsamples were taken from each replicate to be used for the senesced tissue analyses.

All green and senesced tissue samples were oven-dried at 43 °C for 96 h. The dried samples were allowed to equilibrate at ambient conditions prior to size reduction. The switchgrass samples were milled with a Wiley mill (Thomas Scientific, Model 4, Swedesboro, NJ) through either a 20-mesh (1.0 mm) or 40-mesh (0.425 mm) screen. For the senesced tissue samples, the milled material was divided among smaller 100-g bags with a PT100 sample divider (Retsch, Hann, Germany) to ensure the composition of each fraction analysed in this study was representative of that of the original material and without loss of material.

Lignin content and composition by py-MBMS

Green and senesced biomass samples were dried and ground to a 20-mesh (1.0 mm) particle size for determination of lignin content and S/G lignin monomer ratio by pyrolysis molecular beam mass spectrometry, following the protocol described by Sykes *et al.* (2009). All ten replicates for each transgenic line and all five replicates for each corresponding control were assessed. Briefly, cell wall residues of the biomass were prepared by removing soluble extractives and starch. Approximately 4 mg of biomass per sample was loaded in 80-μL stainless steel cups and pyrolysed at 500 °C in a quartz reactor using a Frontier py2020 autosampler. The resulting pyrolysis vapours were analysed using a custom Extrel single quadrupole molecular beam mass spectrometer. The relative intensities of the peaks identified as lignin precursors were summed to estimate total lignin content. S/G lignin monomer ratio was determined by summing the intensity of the syringyl peaks and dividing by the sum of the intensity of the guaiacyl peaks.

Cell wall composition by wet chemistry

For cell wall compositional analyses, all ten replicates for each transgenic line and all five replicates for each corresponding control were assessed. Senesced samples were dried and ground to a 40-mesh (0.425 mm) particle size. Sample fractions to be analysed for chemical composition were first extracted in a Dionex (Sunnyvale, CA) accelerated solvent extractor (ASE) 350, following the method described in NREL Laboratory Analytical Procedure (LAP) 'Determination of Extractives in Biomass' to remove nonstructural extractives. In this process, 5 g of raw milled biomass containing <10% moisture was added to a 33-mL extraction cell and sequentially extracted by pressurized water then ethanol under 1500 psi, 100 °C, 5 min heating time, 7 min static time, at three static cycles. The material was then allowed to air dry to less than 10% moisture content by weight and a change in weight <1% in 24 h, using a subsample that was dried in a 105 °C convection oven for a minimum of 4 h to determine the percentage of total solid. The extractives-free material was stored in polyethylene bags at ambient temperatures until further analyses were performed.

The quantification of cellulose, hemicellulose, lignin and structural ash in the switchgrass was performed using the protocols developed by NREL, 'Determination of Structural Carbohydrates and Lignin', 'Determination of Total Solids in Biomass' and 'Determination of Sugars, Byproducts and Degradation Products in Liquid Fraction Process Samples', using three replicates. A two-stage acid-catalysed hydrolysis was performed to fractionate the sample into soluble and insoluble matter, and the two fractions were separated through vacuum filtration and ceramic fine-porosity filtering crucibles. The insoluble solids fraction consisted of acid-insoluble lignin and ash. The acid-insoluble lignin was quantified gravimetrically after combustion of the residue at 575 °C for 24 h. The monomeric units of polysaccharides within the soluble liquid fraction were quantified via a Flexar high-pressure liquid chromatography (HPLC) (Perkin Elmer, Shelton, CT) with a refractive index (RI) detector. The system was equipped with an Aminex HPX-87P carbohydrate column (300 × 7.8 mm ID, 9 μm particle size) and de-ashing guard column (125-0118) from Bio-Rad (Hercules, CA), using deionized water at 0.25 mL/min at 85 °C. Acetyl quantification was performed using a Bio-Rad Aminex HPX-87H organic acid column (300 × 7.8 mm ID, 9 μm particle size) at 55 °C, using 0.1 N sulphuric acid at 0.6 mL/min. The acid-soluble lignin content of the soluble liquid fraction was measured using a two-beam Lambda 650 series spectrometer (Perkin Elmer), and this value combined with the gravimetric value for acid-insoluble lignin provided the total lignin content. The structural ash content of the extracted sample was determined gravimetrically after combustion of the nonash materials at 575 °C for 24 h in a

muffle furnace. A total of eight primary components were measured as follows: glucan, xylan, galactan, arabinan, mannan, acetyl, structural ash and lignin.

Fourier transform infrared spectroscopy (FTIR)

FTIR analyses were performed on all 10 replicates for each transgenic line and all 5 replicates for each corresponding control. Senesced samples were dried and ground to a 40-mesh (0.425 mm) particle size. FTIR was utilized to collect spectral data on the raw biomass via a Spectrum One FTIR spectrometer (Perkin Elmer). Spectra from 4000 to 600 per cm were collected in absorbance mode with 8 scans per spectrum at 1 per cm resolution using an ATR attachment. Ten spectra were collected for each sample. An ATR correction was employed to account for less than optimal sample/crystal contact, and normalization was performed in the Spectrum software. The index of crystallinity of each sample was calculated by the intensity ratio between the bands of 1422 and 899 per cm, assigned to CH_2 bending mode and deformation of anomeric CH, respectively (Kataoka and Kondo, 1998).

Sugar release

Green and senesced biomass samples were dried and ground to a 20-mesh (1.0 mm) particle size for determination of sugar release, following the high-throughput pretreatment and enzymatic hydrolysis protocol described by Studer *et al.* (2009). All ten replicates for each transgenic line and all five replicates for each corresponding control were assessed. Briefly, cell wall residues were prepared by removing soluble extractives and starch. Samples were loaded in triplicate into a custom-made 96-well metal plate. A hot water pretreatment at 0.5%–1% w/w solids loading was conducted at 180 °C for 17.5 min, using condensing steam for heating. After pretreatment, enzymatic hydrolysis was performed in the well plate on the pretreated slurry by incubation with Ctec2 enzyme cocktail (70 mg protein/g biomass) at 40 °C for 72 h. Following a 72-h hydrolysis, the amount of glucose and xylose released to liquid was measured using a colorimetric assay. Sugar release data were reported in grams of sugars released per gram of cell wall residues.

Quantitative saccharification, pretreatment and fermentation

Ethanol analyses were performed on senesced samples. Five randomly selected replicates for each transgenic line and all five replicates for each corresponding control were dried and milled to a 20-mesh (1.0 mm) particle size. Prior to fermentation, all samples were pretreated at 180 °C for 7.5 min in 0.5% H_2SO_4 following the procedure described by Fu *et al.* (2011a). Briefly, dry milled biomass was soaked in a ninefold excess of 0.5% H_2SO_4 for 18 h and centrifuged at 8000 rpm, 30 min and 4 °C in a Sorvall RC-5B (DuPont instruments) centrifuge. After removing the supernatant, the biomass was loaded at approximately 2.5 g dry biomass per cylinder into 10 cm × 1 cm hastelloy steel tubular pretreatment reactors (Industrial Alloys Plus, Inc.). Cylinders were placed in boiling water for 2 min before heating to 180 °C in a sand bath (Omega FSB1: Techne Co.) for 7.5 min. Directly after removing from the sand bath, biomass was cooled by placing cylinders in an ice bath for 2 min. Pretreated biomass was washed with 100 mL Milli-Q water per gram dry biomass and stored in −20 °C until fermentation. Following the protocol described in ASTM E 1758–01 (ASTM 2003) and HPLC method (NREL/TP 51–42623), quantitative saccharification was performed on the pretreated biomass in triplicate to obtain the cellulose contents. Simultaneous saccharification and fermentation was conducted in triplicate for each replicate following the procedure described by Fu *et al.* (2011a), using *Saccharomyces cerevisiae* D5A (ATCC200062) and enzymes Spezyme CP and Accellerase BG provided by Genencor International. The procedures used to monitor weight loss, and a description of the bottles used for the fermentation, have been previously described by Mielenz *et al.* (2009).

Agronomic performance

As plants were at varying growth stages after being transplanted to the field in May 2011, all aboveground biomass was cut back in August to synchronize growth and allow for uniform comparisons. After a sufficient period of regrowth, tiller height and plant width were measured in October 2011. Tiller height was measured by selecting the tallest tiller (approximately E6 growth stage) from each individual plant and measuring from the base of the soil to the tip of the top leaf. Plant diameter was determined by measuring around the mid-section of each whole plant. In the second year, new shoots began to emerge in March 2012. Tiller height and plant width were measured in August 2012, following the same criteria described above for first-year measurements, on plants at the R0 growth stage.

For both growing season, plants remained green until mid- to late November, after which they began to senesce. The first frost occurred on November 1 for both 2011 and 2012. Tiller number and dry weight biomass yield were determined after senescence in December. The biomass was oven-dried at 43 °C for 96 h. After equilibration at ambient temperature, aboveground dry biomass was weighed to determine total dry weight biomass yield.

Statistical analyses

For each independent line, the transgenic group was compared with its corresponding control group using the PROC TTEST procedure in SAS version 9.3 (SAS Institute Inc., Cary, NC). The data for each analysis fit the assumptions of a normal distribution (Shapiro–Wilk test >0.05), and variances were not different (folded *F*-test >0.05). Differences were considered significant where *P*-values (based on a two-sided *t*-test) were <0.05.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 COMT switchgrass field design.
Figure S2 Pictures of the field taken at different time points across the two growing seasons.
Figure S3 Analysis of COMT transcript levels for 2011 (a) and 2012 (b).
Figure S4 Rust susceptibility of field-grown transgenic plants.
Table S1 Segregation analysis of T_1 progeny derived from reciprocal crosses between transgenic and wild-type switchgrass plants.
Table S2 Summary of the differences between each transgenic line and its corresponding control for years one (2011) and two (2012) in the field experiment.

