6-2017

Field-Grown Transgenic Switchgrass (Panicum virgatum L.) with Altered Lignin Does Not Affect Soil Chemistry, Microbiology, and Carbon Storage Potential

Jennifer M. DeBruyn
University of Tennessee, Knoxville, jdebruyn@utk.edu

David A. Bevard
University of Tennessee, Knoxville

Michael E. Essington
University of Tennessee, Knoxville

Julie Y. McKnight
University of Tennessee - Knoxville, jmcknig8@vols.utk.edu

Sean M. Schaeffer
University of Tennessee, Knoxville, sschaef5@utk.edu

See next page for additional authors

Follow this and additional works at: http://trace.tennessee.edu/utk_biospubs

Recommended Citation

Authors

Jennifer M. DeBruyn, David A. Bevard, Michael E. Essington, Julie Y. McKnight, Sean M. Schaeffer, Holly L. Baxter, Mitra Mazarei, David G.J. Mann, Richard A. Dixon, Fang Chen, Chunliu Zhou, Zeng-Yu Wang, and Charles Neal Stewart Jr

Field-grown transgenic switchgrass (*Panicum virgatum* L.) with altered lignin does not affect soil chemistry, microbiology, and carbon storage potential

JENNIFER M. DEBRUYN[1], DAVID A. BEVARD[1], MICHAEL E. ESSINGTON[1], JULIE Y. MCKNIGHT[1], SEAN M. SCHAEFFER[1], HOLLY L. BAXTER[2,3], MITRA MAZAREI[2,3], DAVID G. J. MANN[2,3,*], RICHARD A. DIXON[3,4], FANG CHEN[3,4], CHUNLIU ZHUO[4], ZENG-YU WANG[3,5] and CHARLES NEAL STEWART JR[2,3]

[1]*Department of Biosystems Engineering & Soil Science, University of Tennessee, Knoxville, TN, USA,* [2]*Department of Plant Sciences, University of Tennessee, Knoxville, TN, USA,* [3]*BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN, USA,* [4]*Department of Biological Sciences, BioDiscovery Institute, University of North Texas, Denton, TX, USA,* [5]*Forage Improvement Division, Samuel Roberts Noble Foundation, Ardmore, OK, USA*

Abstract

Cell wall recalcitrance poses a major challenge on cellulosic biofuel production from feedstocks such as switchgrass (*Panicum virgatum* L.). As lignin is a known contributor of recalcitrance, transgenic switchgrass plants with altered lignin have been produced by downregulation of caffeic acid *O*-methyltransferase (COMT). Field trials of COMT-downregulated plants previously demonstrated improved ethanol conversion with no adverse agronomic effects. However, the rhizosphere impacts of altering lignin in plants are unknown. We hypothesized that changing plant lignin composition may affect residue degradation in soils, ultimately altering soil processes. The objective of this study was to evaluate effects of two independent lines of COMT-downregulated switchgrass plants on soils in terms of chemistry, microbiology, and carbon cycling when grown in the field. Over the first two years of establishment, we observed no significant differences between transgenic and control plants in terms of soil pH or the total concentrations of 19 elements. An analysis of soil bacterial communities via high-throughput 16S rRNA gene amplicon sequencing revealed no effects of transgenic plants on bacterial diversity, richness, or community composition. We also did not observe a change in the capacity for soil carbon storage: There was no significant effect on soil respiration or soil organic matter. After five years of establishment, δ^{13}C of plant roots, leaves, and soils was measured and an isotopic mixing model used to estimate that 11.2 to 14.5% of soil carbon originated from switchgrass. Switchgrass-contributed carbon was not significantly different between transgenic and control plants. Overall, our results indicate that over the short term (two and five years), lignin modification in switchgrass through manipulation of COMT expression does not have an adverse effect on soils in terms of total elemental composition, bacterial community structure and diversity, and capacity for carbon storage.

Keywords: biodiversity, bioenergy, lignin, microbial communities, soil carbon, switchgrass

Introduction

Switchgrass (*Panicum virgatum* L.) is a promising bioenergy crop due to high biomass production with relatively low agronomic inputs. Native to North America, it is a warm-season, perennial C_4 grass. One of the main challenges in cost-efficient conversion of switchgrass and other lignocellulosic biomass into biofuels is recalcitrance of plant cell walls. Lignin, a complex phenolic polymer, is a primary contributor to this recalcitrance as it inhibits access to fermentable polysaccharides. One approach to address the challenges of conversion of lignocellulosic biomass is modification of the bioenergy crops themselves. Genetic manipulation of lignin biosynthetic pathway genes has successfully altered lignin characteristics in switchgrass and other species, ultimately resulting in improved conversion to biofuels in some transgenic lines (Piquemal *et al.*, 2002; Chen & Dixon, 2007; Fu *et al.*, 2011; Xu *et al.*, 2011; Shen *et al.*, 2013; Baxter *et al.*, 2014).

While transgenic bioenergy crops hold a great deal of promise from an energy production standpoint, it is unknown whether there maybe trade-offs when grown

*Present address: Dow AgroSciences, 9330 Zionsville Road, Indianapolis, IN 46268, USA

Correspondence: Jennifer M. DeBruyn, tel. 865 974 7266, fax 865 974 4515, e-mail: jdebruyn@utk.edu

in the field. Plants with altered cell walls might affect soils directly through altered plant nutrient use and/or changes in residue and root exudate composition, altering decomposition rates and nutrient turnover (Motavalli *et al.*, 2004; Kolseth *et al.*, 2015). Transgenic crops can also indirectly affect their environment as a result of increased biomass or altered management practices (e.g., pesticide application) (Kolseth *et al.*, 2015). Studies on *Bacillus thuringiensis (Bt)* and herbicide-tolerant (HT) food crops have generally revealed no major differences in soil microbial community structure, enzyme activities, residue decomposition rates, and other aspects of soil functioning (Griffiths *et al.*, 2007; Lupwayi *et al.*, 2007; Liu *et al.*, 2008; Yanni *et al.*, 2010; Fang *et al.*, 2012; Dohrmann *et al.*, 2013; Kolseth *et al.*, 2015). However, unlike the *Bt* and HT modifications, downregulating lignin pathways is carried out with the specific goal of changing the recalcitrance of plant tissues. Thus, it remains unknown what affect the altered root and residue composition has on rhizosphere processes, particularly with respect to soil carbon storage.

There is a growing body of research showing that soils are influenced by bioenergy crops. For example, researchers have documented altered soil microbial communities (Mao *et al.*, 2011; Jesus *et al.*, 2016; Liang *et al.*, 2016) and increased soil organic carbon stocks (Gauder *et al.*, 2016) under switchgrass. Genetically modifying switchgrass to downregulate lignin biosynthetic pathways has changed lignin quantity and composition in plant tissues (Fu *et al.*, 2011; Baxter *et al.*, 2014). These modifications can also have other effects on cell wall carbohydrates, for example, increased cellulose, hemicellulose, and xylose (Fu *et al.*, 2011; Baxter *et al.*, 2014). Plant litter recalcitrance is a key factor determining decomposition products and soil organic matter formation (Cotrufo *et al.*, 2013). Therefore, we hypothesized that the altered composition of lignin in transgenic switchgrass roots and residues would affect soil microbes and their nutrient and carbon cycling processes. Switchgrass has a large capacity for belowground carbon sequestration (Lemus & Lal, 2005; Agostini *et al.*, 2015). Research has shown that different (nontransgenic) cultivars of switchgrass can have variable soil carbon inputs; for example, cultivars with longer specific root lengths resulted in greater plant-derived carbon in the soils (Adkins *et al.*, 2016). Therefore, it is important to determine whether an alteration of lignin and carbohydrate composition in switchgrass adversely impacts belowground carbon storage and turnover.

In a previous study, two lines of field-grown transgenic switchgrass plants were evaluated for their potential in biofuels production (Fu *et al.*, 2011; Baxter *et al.*, 2014, 2016). These switchgrass plants had downregulated expression for one of the genes coding for the lignin biosynthesis enzyme caffeic acid *O*-methyltransferase (COMT). The study revealed that field-grown transgenic plants had altered cell wall chemistry and improved ethanol yields, with no deficiencies in agronomic performance in terms of biomass yield or disease susceptibility (Baxter *et al.*, 2014, 2016). Given the promising performance of these two transgenic lines for biofuels production, the objective of our study was to examine these same field-grown transgenic plants for possible effects on the soils. Due to faster decomposition rates of lower lignin plant material, we hypothesized that the altered lignin characteristics of COMT-downregulated switchgrass would have a significant effect on soil chemistry, biology, and potential for soil carbon storage. We evaluated differences between two independent COMT-downregulated transgenic lines and their nontransgenic controls over the first five years of establishment, examining root lignin characteristics, soil elemental composition, soil bacterial community diversity and structure, and soil carbon storage and turnover.

Materials and methods

Field experimental design and soil sampling

The field experiment design has been described in Baxter *et al.* (2014). Briefly, two T1-generation events of switchgrass (*Panicum virgatum* L.) plants generated by RNAi-mediated gene silencing of COMT (Fu *et al.*, 2011) were grown at the University of Tennessee East Tennessee Research and Education Center, Knoxville, TN, USA. The soils at this location are in the Shady series (fine-loamy, mixed, subactive, thermic Typic Hapludults). Plants were grown in a completely randomized experimental design consisting of 10 replicate plots of transgenic plants and five replicate plots of control plants (nontransgenic) for two different lines (COMT2 and COMT3), for a total of 30 plots with 160 cm between plots. Each replicate plot had nine vegetatively propagated plants placed 80 cm apart. Border plants were included to reduce shading effects. Plants were grown for five consecutive growing seasons (2011–2015).

Soil samples were collected seasonally throughout the first two years of establishment (2011–2012), then again during the fifth growing season (2015). Soil cores were collected using sterilized 19-mm-diameter soil probes from each of the 30 plots to a depth of 0–15 cm. In addition, deeper horizons (15–30 cm) were sampled from 12 of the plots. Fifteen soil cores were taken from each plot and composited in sterile Whirl-Pak bags. Probes and gloves were cleaned with 70% ethanol and dH$_2$O between each plot to prevent cross-contamination. Soil samples were sieved through 2-mm mesh and then subdivided for analysis. In June of the fifth growing season, samples of plant leaves and roots were collected for lignin and carbon isotope analysis. In each plot, three of the nine plants were sampled: Four-cm sections of leaf tissue were cut approximately 10 cm from the leaf tip. To collect roots, a 19-mm soil auger was used to retrieve four 15 cm deep soil cores from near the base of

each switchgrass plant. Roots were manually separated from the soils, washed with distilled water, and air-dried.

Chemical analyses of root lignin

To quantify and characterize the lignin content and composition of the roots, thioacidolysis was conducted according to the method described previously (Lapierre *et al.*, 1985, 1986). Air-dried root samples were ground to fine powder with a freeze mill under liquid nitrogen and then extracted with chloroform/methanol (2 : 1 v/v), 100% methanol, and water two times each successively. About 12 mg of freeze-dried samples was reacted with 3 mL of 0.2 M BF_3-etherate in an 8.75 : 1 dioxane/ethanethiol mixture at 100 °C for 4 h. After cooling, the lignin monomers were extracted with dichloromethane, derivatized with BSTFA + 1% TMCS (Sigma Aldrich Co., St. Louis, MO, USA), and then subjected to GCMS analysis on a Hewlett-Packard 5890 series II gas chromatograph with a 5971 series mass-selective detector and HP-1 column (60 m × 0.25 mm, 0.25-μm film thickness). Lignin monomers were quantified using docosane as an internal standard. All the analyses were performed on three biological replicates from each plot.

Soil moisture, pH, and elemental composition

Gravimetric soil moisture was determined after drying at 105 °C for at least 48 h. Soil pH was determined after mixing soils 1 : 2 with distilled H_2O and letting the mixture settle for 3 min. To determine concentrations of major elements, soils were digested using a microwave version of the Bernas methods (Ammons *et al.*, 1995) and analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES). In brief, 200 mg of air-dried soils was digested in 2 mL of hydrofluoric acid (HF) at room temperature for 16 h, followed by digestion in a microwave with a 5 mL aliquot of *aqua regia* (3 : 1 : 1 HCl : HNO_3 : H_2O). A 1 mL aliquot of boric acid was then added to each tube to neutralize unreacted HF. Samples were filtered through 0.45-μm syringe filters and then diluted 10× and 100× in deionized water. Samples were then analyzed on a Spectro CIROS ICP-OES. Elemental concentrations were reported as mass per mass dry soil.

Soil bacterial communities

A subset of samples was selected for examination of the bacterial communities via 16S rRNA gene amplicon sequencing. Samples from three of the five control plots and three of the ten transgenic plots were selected from both plant lines (COMT2 and COMT3) at four time points (summer and fall of the first two years), for a total of 48 libraries. Bacterial community structure was analyzed by high-throughput sequencing of 16S rRNA gene amplicons (V4 region) on the MiSeq platform as previously described (Cobaugh *et al.*, 2015). Briefly, extracted DNA was sent to the Hudson Alpha Bioinformatics Institute Genomic Services Laboratory (Huntsville, AL, USA), where the V4 region of the 16S rRNA gene was amplified with bar-coded primers 515F and 806R (Caporaso *et al.*, 2012). Amplicon libraries were pooled, and 250 base pair paired-end sequence reads were

obtained on an Illumina MiSeq. Reads were processed using the open source bioinformatic software package MOTHUR v 1.33.3 following the MiSeq SOP protocol (Schloss *et al.*, 2009). Briefly, sequences with homopolymers longer than eight nucleotides or containing ambiguous bases were removed. Remaining sequences were aligned to a SILVA reference library and trimmed to 445 bases that started and ended at the same alignment position. The reads were subjected to the UCHIME chimera removal algorithm. Reads were classified using the Ribosomal Database Project database using at least 80% similarity to define taxonomy and binned into operational taxonomic units (OTUs) according to their taxonomic classification at the genus level (phylotype clustering). Sequences that classified as something other than bacteria were removed. One library had very low coverage and was deemed a failed run and excluded from further analysis. After screening, 8 661 357 reads remained, with a mean of 184 284 sequences per sample. Sequences were deposited in NCBI short read archive (BioProject: PRJNA327812).

Soil respiration

Respiration was determined using an incubation-headspace CO_2 method. We incubated 50 g of soil for 24 h in sealed, 1 pint (473.2 mL) mason jars fitted with butyl rubber septa (20 mm, Wheaton). After 24 h, 500 μL of headspace air was sampled with a syringe (5-mL gastight, SGE Analytical Science). Headspace air was analyzed for CO_2 concentration (ppm) using an infrared gas analyzer (Gashound LI-820, LiCor Inc., Lincoln, NE, USA).

Soil carbon

Organic matter content of soils was determined using mass loss on ignition: Oven-dried soils (105 °C) were heated to 400 °C for 16 h and then cooled in a desiccator, and weight loss recorded and expressed as a percentage of the oven-dried weight of soil. For isotopic analysis of soil and leaf material, samples were dried at 65 °C and homogenized into a fine powder. Soil carbon content was determined using an elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA). Carbon isotope composition ($\delta^{13}C$) values were measured on a cavity ring-down spectrometer (G2121-I, Picarro Inc., Santa Clara, CA, USA). All isotope values were expressed as the ratio of $^{13}C/^{12}C$ of a sample relative a standard (PeeDee Belemnite) and multiplied by 1000 to produce units of ‰. To determine how much newly added C from switchgrass contributed to total soil organic C, we used an isotopic mixing model (Phillips & Gregg, 2003) with two sources: nonswitchgrass soil $\delta^{13}C$ and switchgrass leaf $\delta^{13}C$. Nonswitchgrass soil was collected from plots adjacent to the study plots that were not planted with switchgrass or any other C_4 crop for at least 30 years.

Data analysis and statistics

Statistical analysis of data was performed in R (R Core Team, 2013). Alpha-diversity and coverage estimates were calculated in Mothur after subsampling the libraries to an even size

(n = 81 934 reads per sample). To examine bacterial community structure, Bray–Curtis distances between samples were calculated and visualized with nonmetric multidimensional scaling using Plymouth Routines In Multivariate Ecological Research (PRIMER) v6 software (Lutton, UK) (Clarke & Gorley, 2006). Analysis of similarity (ANOSIM) was used to determine whether there was significant multivariate clustering of community structure based on treatments.

Results

Plant root lignin

COMT-downregulated switchgrass plants used in this study had been previously evaluated for lignin content and composition in aboveground biomass, finding that the transgenic plants had decreased lignin content and lower syringyl/guaiacyl (S/G) lignin monomer ratios over a two-year field trial (Baxter et $al.$, 2014). After five years, we evaluated the lignin content and composition in the roots of these same plants. Transgenic plants did not differ significantly from controls in terms of total lignin monomer yield released by thioacidolysis (P > 0.05). However, transgenic plants had significant lower concentrations of syringyl (S) monomers, resulting in significant reductions in S/G ratios (P < 0.01) (Fig. 1). S/G ratios were 40.1% and 42.7% less than control plants for COMT2 and COMT3 lines, respectively.

Soil chemistry

Over the first two years of establishment, mean soil pH ranged from 6.48 to 7.15 in the upper (0–15 cm) soil and 6.39 to 7.32 in the deeper (15–30 cm) soil (Fig. S1). There was a significant change in soil pH by sampling date (F = 29.05, P < 0.001), with a small but significant increase in soil pH between the first and second seasons. However, there was no significant difference in soil pH between COMT2 and COMT3 lines, or between the transgenic and control plants (P > 0.05). We also examined the concentrations of 19 soil elements, but did not see a significant difference in elemental composition between lines or between transgenic and control plants (Table 1 and Fig. S2).

Soil bacterial communities

Sequencing of 16S rRNA gene libraries from the plots yielded a mean of 184 284 quality reads per sample. Prior to calculation of diversity metrics, libraries were subsampled to an equal number of reads (n = 81 934 reads per sample). Rarefaction curves showed this was good coverage for these samples (Fig. S2); coverage estimates ranged from 0.9990 to 0.9994. The number of OTUs ranged from 351 to 471, with a mean of 421. Diversity, as estimated by the inverse Simpson's Index, ranged from 2.71 to 7.36, with a mean of 3.88 (Fig. S3). There were statistically significant differences in richness and diversity by sampling time, with a lower richness but higher diversity in the second year. However, there was no statistical difference between the transgenic and control plots in terms of bacterial diversity or richness (Table 2). The bacterial communities were dominated by Proteobacteria (mean relative abundance ranged from 44.3 to 51.6%) and Acidobacteria (26.0 to 36.7%), with lesser abundances of Actinobacteria (2.88 to 7.06%), Verrucomicrobia (3.74 to 4.36%), Bacteroidetes (1.14 to 4.05%), Planctomycetes (1.14 to 4.05%), Firmicutes (1.05 to 4.76%), Armatimonadetes (1.10 to 1.81%), Chloroflexi (0.86 to 2.39%), and Gemmatimonadetes (0.94 to 1.75%) (Fig. 2). Other phyla were
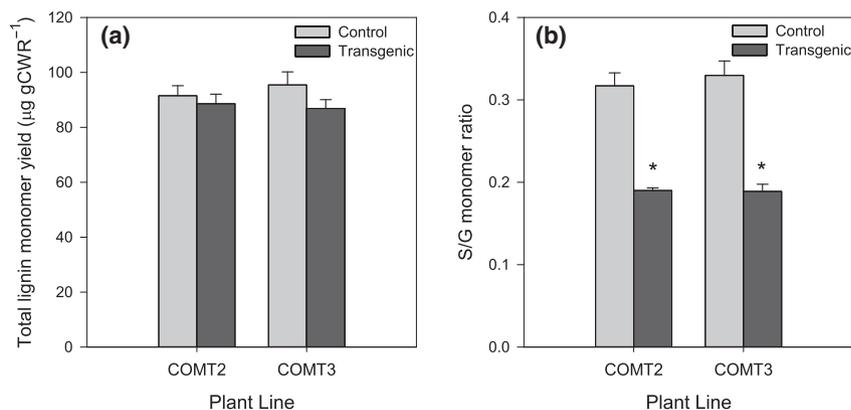
Fig. 1 Quantity and composition of lignin in switchgrass plant roots, in terms of (a) mean total lignin content (p-hydroxyphenyl, guaiacyl, and syringyl monomers) per gram cell wall residue (CWR) and (b) mean syringyl/guaiacyl (S/G) ratio. Error bars denote standard errors of the mean (transgenic n = 10 and control n = 5). *denotes statistically significant differences between transgenic and control (t-test P < 0.05).

Table 1 Elemental concentrations (mg per kg dry mass soil) in soils (0 – 15 cm) of control and transgenic switchgrass plots. Data are means and standard error of seven seasonal sampling times during the first two years of establishment (controls $n = 35$; transgenic $n = 70$). The P values are the results of T-tests comparing transgenic to control plots

	COMT2			COMT3		
	Control	Transgenic	*t*-test *P*	Control	Transgenic	*t*-test *P*
Al	58 800 ± 1200	57 100 ± 7300	0.201	56 900 ± 900	57 500 ± 670	0.631
Ba	1600 ± 50	1570 ± 39	0.646	1590 ± 52	1630 ± 41	0.646
Ca	5410 ± 65	5400 ± 53	0.860	5425 ± 84	5480 ± 87	0.685
Cd	1.22 ± 0.04	1.19 ± 0.03	0.599	1.24 ± 0.05	1.21 ± 0.03	0.487
Co	26.1 ± 2.2	26.4 ± 1.7	0.919	25.8 ± 2.4	25.7 ± 1.7	0.972
Cr	38.5 ± 1.1	37.3 ± 0.8	0.373	36.6 ± 0.8	37.1 ± 0.7	0.673
Cu	15.2 ± 1.1	14.8 ± 0.7	0.704	15.8 ± 1.0	15.2 ± 0.8	0.699
Fe	26 700 ± 400	25 900 ± 200	0.063	25 800 ± 300	26 200 ± 260	0.445
K	23 400 ± 300	22 500 ± 310	0.063	23 100 ± 700	22 600 ± 340	0.548
Mg	4200 ± 66	4100 ± 41	0.064	4140 ± 58	4210 ± 53	0.383
Mn	1320 ± 18	1290 ± 16	0.281	1280 ± 31	1300 ± 16	0.582
Mo	20.1 ± 3.9	20.7 ± 2.8	0.904	19.8 ± 4.0	19.9 ± 2.8	0.971
Na	11 580 ± 250	11 570 ± 230	0.985	11 600 ± 290	12 000 ± 240	0.360
Ni	17.9 ± 0.7	16.7 ± 0.3	0.090	16.2 ± 0.4	17.1 ± 0.4	0.129
P	890 ± 12	900 ± 13	0.630	923 ± 30.4	895 ± 14	0.336
S	240 ± 14	226 ± 9	0.371	226 ± 13	240 ± 9.9	0.409
Se	27.2 ± 0.8	27.1 ± 0.7	0.917	24.9 ± 1.2	27.2 ± 0.7	0.055
Si	240 000 ± 8000	241 000 ± 5000	0.866	255 000 ± 9000	238 000 ± 5000	0.087
Zn	540 ± 19	533 ± 15	0.764	539 ± 17	538 ± 13	0.977

detected at <1% mean relative abundance. The structure and β-diversity of the bacterial communities was examined through NMDS plots of Bray–Curtis distances and ANOSIM (Fig. 3 and Table 2). The structure of the communities varied significantly by season. At one time point (fall of the first year), there was a significant difference in the community structures between transgenic and control plants for COMT2 only. At all other dates, no significant differences were detected for either line.

Soil carbon storage potential

Soil organic matter content ranged from 2.97 to 3.42% for the upper soils (0–15 cm) and 2.74 to 2.95% for the deeper soils (15–30 cm) (Fig. 4). There were no significant differences in organic matter content between transgenic and control plant plots in either the upper ($t = -1.334$, $P = 0.187$) or lower soils ($t = 0.934$, $P = 0.551$). There was also no significant difference in respiration rates between treatments: Respiration rates as estimated from CO_2 accumulation after a 24-h incubation ranged from 0.025 to 0.249 µg C per gram dry weight soil per h, with a mean of 0.108 (data not shown).

We measured the $\delta^{13}C$ of the soil, plant roots, and plant leaves. Using an isotopic mixing model, we estimated that the switchgrass plants contributed 11.2 to 14.5% of the total soil organic carbon (Fig. 5 and

Table 3). There was no significant difference in total soil organic carbon between transgenic and controls ($F = 20.36$, $P = 0.179$ and $F = 0.821$, $P = 0.383$ for COMT2 and COMT3, respectively). We observed a decrease in the proportion of plant-derived C in transgenic COMT2 (11.2 ± 0.1%) relative to control plots (14.2 ± 1.9%). Although this represents a large decrease in absolute terms (nearly 20%), the observed means were not statistically significant ($F = 1.436$, $P = 0.317$). There was also no significant difference between transgenic and control for COMT3 ($F = 0.156$, $P = 0.719$).

Given the lack of observed effect in most of the parameters examined, we performed a post hoc power analysis to determine what treatment effects were detectable. The effect sizes observed for organic matter content and % carbon derived from switchgrass were $d = 0.35$ and 0.47, respectively, resulting in a power of 0.39 and 0.56, respectively. Thus, lack of differences should not be interpreted as suggesting there are no true differences, just that our chance of detecting true differences was low.

Discussion

In this study, we examined the effects of two COMT-downregulated transgenic switchgrass lines on soils. Our hypothesis was that changes in the plant physiology, specifically with respect to lignin content and

Table 2 Analyses of variance (ANOVA) and analysis of similarity (ANOSIM) reveal bacterial community richness, diversity, and structure changed seasonally, but was not significantly different by plant line or between transgenic and control plots

Factor	Levels	Richness (number of OTUs) F	Diversity (Simpsons index) F	Community structure (Bray–Curtis similarities) Global R
Year	2011, 2012	**23.49***[***]	**14.68***[***]	**0.363**[***]
Season	Summer, fall	**7.188**[*]	0.071	**0.22**[***]
Sample date	Summer 2011, fall 2011, summer 2012, fall 2012	**14.24**[***]	**7.685**[***]	**0.516**[***]
Plant line	COMT2, COMT3	0.221	0.031	−0.023
Transgenic	Transgenic, control	0.038	0.161	−0.01

Significant differences between factor levels are in bold *$P < 0.05$; ***$P < 0.001$.
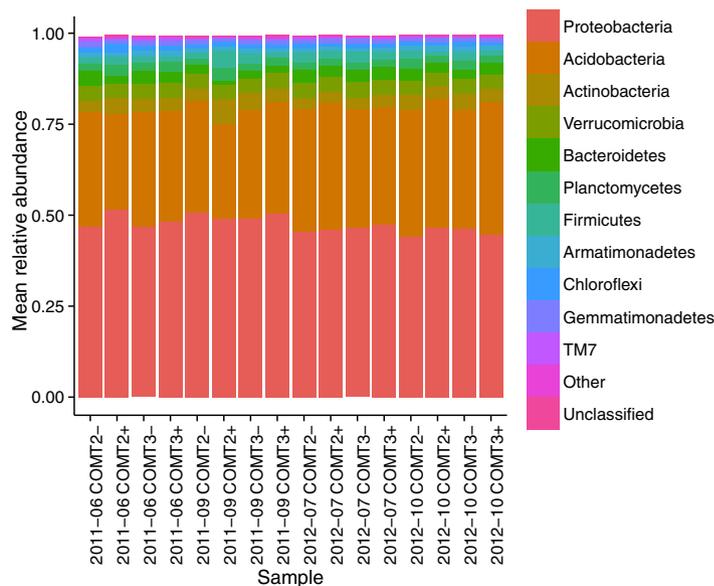
Fig. 2 Mean relative abundance of bacterial phyla by sampling date and transgenic ('+') vs. control ('-') plant lines ($n = 3$).

composition, would result in changes to soil chemistry, biology, and carbon storage potential. Previous work showed that over the first two growing seasons, these transgenic plants were significantly altered in terms of the lignin content and composition, specifically the ratio of S/G monomers, of both green and senesced aboveground tissues (Baxter *et al.*, 2014). Here, we show that the belowground biomass also had physiological alterations. Unlike the aboveground tissues, we did not observe a significant difference in total lignin content of the roots. The COMT RNAi construct was under the control of the maize ubiquitin 1 promoter, which is active in shoots and roots; however, root lignin has seldom been studied. In addition, the main effect endowed by the COMT downregulation is a decreased S/G ratio

(Fu *et al.*, 2011), which was observed in the roots (40–43% reduction in S/G). These are comparable to the 43–46% reductions for aboveground biomass for these same plant lines when grown under greenhouse conditions (Fu *et al.*, 2011). In this field study, slightly lower reductions were observed for aboveground biomass (35–39% in year two) (Baxter *et al.*, 2014); the higher reductions in the roots may be due to a longer period of establishment (we measured the roots in year five). For engineering carbon sequestration capabilities in feedstocks, it will be important to assess root traits, such as altered lignin, as a possible mechanism for increasing soil organic carbon.

Despite the compositional changes of the transgenic plants, there was very little effect on soil chemistry (pH
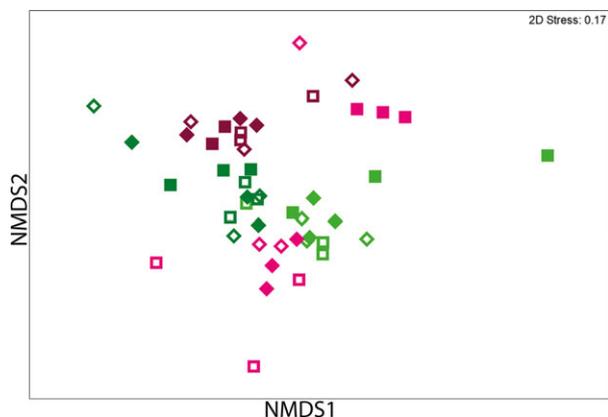
Fig. 3 Nonmetric multidimensional scaling of Bray–Curtis similarities reveals variation in bacterial community structure. For three of the four dates, there is no difference between transgenic (filled) and control (open) plant plots; shapes designate the two lines of switchgrass (squares are COMT2; diamonds are COMT3). There was variation by sampling date: summer 2011 (light green), fall 2011 (pink), summer 2012 (dark green), and fall 2012 (purple).

and elemental content), microbial activity, or bacterial communities, at least during the study period. We saw no significant effects on soil pH or any of the major elements measured over the first two years of establishment. Plants can alter the available or bioaccessible concentrations of soil elements either directly through uptake and crop removal or indirectly during decomposition of plant residues (e.g., Pourhassan *et al.*, 2016). Total concentrations of soil elements are generally not impacted by plants, unless the plants are hyperaccumulators (e.g., Ma *et al.*, 2001), and even then it can require several years to elicit a change in soil metal levels (Lasat, 2002). To alleviate demands on arable soils for food crop production, there has been interest in growing bioenergy crops on marginal lands; thus, the

demonstrated phytoextraction of select heavy metals (e.g., Cd) from contaminated soils is a desirable quality of switchgrass (Zhang *et al.*, 2015). Based on our results, we conclude that the transgenic plants tested here do not alter the elemental content of soil, at least over the short term, and thus, this genetic modification has not altered the plants' capacity for phytoextraction of heavy metals.

Soil microbial communities perform critical processes in soils and dictate the fate of carbon and nutrients. Thus, one major objective of the study was to determine the influence of genetically modified switchgrass plants on soil microbial communities. The soil bacterial communities in the switchgrass plots showed seasonal variation: Diversity increased significantly over time and each of the four dates had significantly different bacterial community structure. Seasonal variation in soil bacterial communities in agroecosystems is not uncommon (e.g., DeBruyn *et al.*, 2011; Lauber *et al.*, 2013). In addition to seasonal changes, these communities were also likely affected by the establishment of a new crop. Other studies have shown that soil microbial communities can undergo shifts during establishment of new crops; for example, an increase in copies of bacterial nitrogen fixing genes was observed in the second year of establishment of switchgrass (Mao *et al.*, 2011). There may be several factors driving the changes in the soil microbiome. First, we noted a slight, but not significant, increase in soil pH over time, and other studies have shown pH to be one of the strongest drivers of soil microbial community composition (Lauber *et al.*, 2009). Second, as these perennial plants continued to grow through the seasons, root biomass increased, which would have resulted in an increase in the amount of root exudates and dead tissue available to soil microbes. Plants can also change exudate composition depending on developmental stage, and this may have additionally
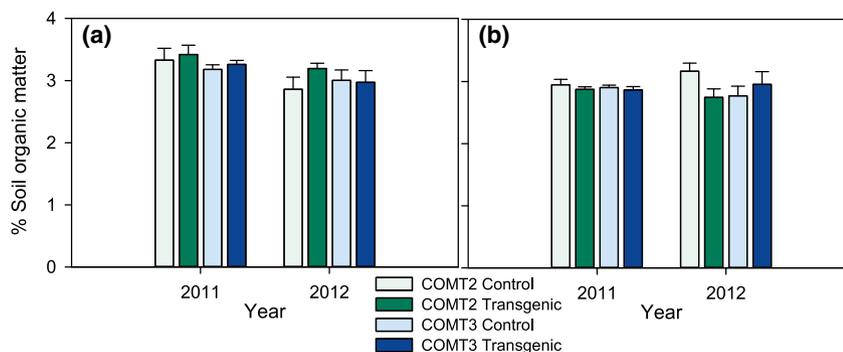
Fig. 4 Percent soil organic matter in plots with transgenic switchgrass plants. Mean soil organic matter in (a) upper 0 to 15 cm and (b) deeper 15 to 30 cm horizons during the first two years of establishment in soils below transgenic (dark colors) and control (light colors) switchgrass plants. Error bars denote standard errors of the mean (transgenic $n = 10$ and control $n = 5$). There were no significant differences between transgenic and control for either line.
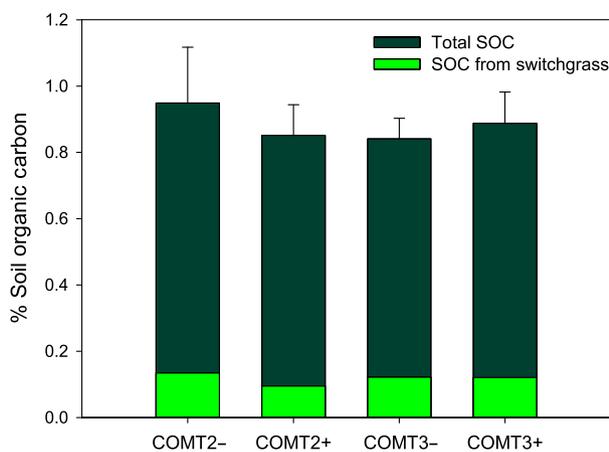
Fig. 5 Total soil organic carbon (SOC) and the proportion of the total SOC contributed by transgenic (COMT2+ and COMT3+) and control (COMT2- and COMT3-) switchgrass plants during the fifth growing season (2015), as estimated based on isotopic mixing models. Data are means and standard deviation ($n = 10$ for transgenic; $n = 5$ for controls). There were no significant differences in total SOC or proportion from switchgrass between transgenic and control for either line.

influenced soil microbiome composition (Lakshmanan, 2015).

Despite the seasonal differences in the soil microbiome, there was no significant effect of transgenic switchgrass plants on soil bacterial community structure, richness, or diversity. We did note that in the fall of the first year of establishment, the communities in the transgenic and control plots were significantly different from each other. Interestingly, a study on transgenic canola also noted that while the rhizosphere microbial communities were altered under transgenic plants, the affect was only temporary and did not persist past the growing season (Dunfield & Germida, 2003). Thus, it may be that this was only a temporary perturbation, as the differences were not apparent in the second year of our study. In general, the type of plant (transgenic vs. control) was not the main driver of

bacterial community structure. This has been observed with other types of genetically modified crops as well: Management (tillage, pesticide application) and season or plant growth stage exerted stronger influences on soil microbial communities than plant cultivars in multiple studies (Sessitsch et al., 2005; Griffiths et al., 2007; Lupwayi et al., 2007; Liu et al., 2008; Hannula et al., 2012).

There is a great deal of interest in switchgrass for its capacity to sequester carbon below ground. In this study, the transgenic switchgrass plants had lower lignin and increased cellulose and hemicellulose in aboveground tissues and an altered lignin composition in both aboveground and belowground tissues. Thus, a main objective of this study was to determine whether these modifications would affect the potential for carbon sequestration in the soils. Over the first two years, we did not see significant changes in soil organic matter, suggesting no effect on carbon sequestered. We did not observe significant changes in soil respiration, although it should be noted that respiration rates were based on laboratory incubations at discrete time points. Longer term *in situ* respiration rates should be measured to determine an effect on carbon turnover. Additional analyses in the fifth year revealed no significant changes in total soil organic carbon after five years of establishment. A mixing model of carbon isotope values predicted that the plants had contributed 11.2–14.5% of the soil organic carbon. This corroborates other studies that have demonstrated increases in soil organic carbon under switchgrass (Chimento et al., 2016; Ferchaud et al., 2016; Gauder et al., 2016). Although the mixing model appeared to show that the transgenic COMT2 plants had contributed slightly less to soil organic carbon pools than the control COMT2 plant lines, these differences were not statistically significant. There was also no significant effect of transgenic COMT3 on predicted carbon contribution.

It is important to note that due to the sample sizes, smaller effects may have not been detected and it is possible that this is a case of a type II error (i.e., false

Table 3 Mean carbon (C) content (%) and $\delta^{13}C$ (‰) for switchgrass leaves and soil from experimental plots. Also shown is the mean % of soil organic carbon (SOC) derived from switchgrass after five years of cultivation. Numbers after the mean correspond to the standard error of the mean. There was no significant differences between control and transgenic for either line ($P > 0.1$)

	COMT2		COMT3	
	Control	Transgenic	Control	Transgenic
Leaf C content (%)	35.9 ± 0.7	35.1 ± 0.7	35.6 ± 0.6	35.6 ± 0.5
Leaf $\delta^{13}C$ (‰)	−14.0 ± 0.1	−14.4 ± 0.1	−14.0 ± 0.1	−14.1 ± 0.2
SOC (%)	0.83 ± 0.05	0.78 ± 0.05	0.84 ± 0.05	0.94 ± 0.1
Soil $\delta^{13}C$ (‰)	−20.2 ± 0.2	−20.5 ± 0.1	−20.2 ± 0.1	−20.3 ± 0.1
Calculated switchgrass-derived SOC (%)	14.2 ± 1.9	11.2 ± 0.1	14.5 ± 1.8	13.6 ± 1.6

negative). In addition, this study was limited to the top 30 cm of soil. Switchgrass can have a large root mass, extending up to 330 cm below the surface, so it is unknown whether the transgenic plants may have had an impact on the deeper soils (>30 cm). We would expect the contribution of the deep soils to be minimal, given that the majority of soil organic carbon is found in the top 40 cm of soils beneath switchgrass (Garten & Wullschleger, 2000).

Overall, we observed negligible impacts of COMT-downregulated transgenic switchgrass plants on soil pH or total elemental content, microbiology, and carbon storage potential over the first five years of establishment. Studies of other genetically modified crops have generally found little effect on soil nutrient cycling, although there are very few studies that directly address carbon storage potential (Kolseth *et al.*, 2015). As our study was one of the first trials of field-grown transgenic switchgrass and was based on a limited number of samples, it is unknown whether smaller effects may have gone undetected and would be revealed with a larger sample size and/or longer term monitoring. A comparison of newly established to >10 year (nontransgenic) switchgrass sites revealed no significant differences in soil microbial communities (Liang *et al.*, 2016), suggesting that the communities measured here may be similar to those in the future. We can conclude that at least over the short term, there are no anticipated adverse large effects of these transgenic switchgrass plants on soil processes and soil organic matter stability.

Acknowledgements

References

Adkins J, Jastrow JD, Morris GP, Six J, de Graaff MA (2016) Effects of switchgrass cultivars and intraspecific differences in root structure on soil carbon inputs and accumulation. *Geoderma*, **262**, 147–154.

Agostini F, Gregory AS, Richter GM (2015) Carbon sequestration by perennial energy crops: is the jury still out? *Bioenergy Research*, **8**, 1057–1080.

Ammons JT, Essington ME, Lewis RJ, Gallagher AO, Lessman GM (1995) An application of a modified microwave total dissolution technique for soils. *Communications in Soil Science and Plant Analysis*, **26**, 831–842.

Baxter HL, Mazarei M, Labbe N *et al.* (2014) Two-year field analysis of reduced recalcitrance transgenic switchgrass. *Plant Biotechnology Journal*, **12**, 914–924.

Baxter HL, Mazarei M, Fu C *et al.* (2016) Time course field analysis of COMT-downregulated switchgrass: lignification, recalcitrance, and rust susceptibility. *Bioenergy Research*, **9**, 1087–1100.

Caporaso JG, Lauber CL, Walters WA *et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME Journal*, **6**, 1621–1624.

Chen F, Dixon RA (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology*, **25**, 759–761.

Chimento C, Almagro M, Amaducci S (2016) Carbon sequestration potential in perennial bioenergy crops: the importance of organic matter inputs and its physical protection. *Global Change Biology Bioenergy*, **8**, 111–121.

Clarke KR, Gorley RN (2006) *PRIMER v6: User Manual/Tutorial*. PRIMER-E Ltd, Plymouth.

Cobaugh KL, Schaeffer SM, DeBruyn JM (2015) Functional and structural succession of soil microbial communities below decomposing human cadavers. *PLoS ONE*, **10**, e0130201.

Cotrufo MF, Wallenstein MD, Boot CM, Denef K, Paul E (2013) The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Global Change Biology*, **19**, 988–995.

DeBruyn JM, Nixon LT, Fawaz MN, Johnson AM, Radosevich M (2011) Global biogeography and quantitative seasonal dynamics of Gemmatimonadetes in soil. *Applied and Environmental Microbiology*, **77**, 6295–6300.

Dohrmann AB, Kuting M, Junemann S, Jaenicke S, Schluter A, Tebbe CC (2013) Importance of rare taxa for bacterial diversity in the rhizosphere of Bt- and conventional maize varieties. *ISME Journal*, **7**, 37–49.

Dunfield KE, Germida JJ (2003) Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). *Applied and Environmental Microbiology*, **69**, 7310–7318.

Fang H, Dong B, Yan H, Tang F, Wang B, Yu Y (2012) Effect of vegetation of transgenic Bt rice lines and their straw amendment on soil enzymes, respiration, functional diversity and community structure of soil microorganisms under field conditions. *Journal of Environmental Sciences*, **24**, 1259–1270.

Ferchaud F, Vitte G, Mary B (2016) Changes in soil carbon stocks under perennial and annual bioenergy crops. *Global Change Biology Bioenergy*, **8**, 290–306.

Fu C, Mielenz JR, Xiao X *et al.* (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proceedings of the National Academy of Sciences*, **108**, 3803–3808.

Garten CT, Wullschleger SD (2000) Soil carbon dynamics beneath switchgrass as indicated by stable isotope analysis. *Journal of Environmental Quality*, **29**, 645–653.

Gauder M, Billen N, Zikeli S, Laub M, Graeff-Honninger S, Claupein W (2016) Soil carbon stocks in different bioenergy cropping systems including subsoil. *Soil and Tillage Research*, **155**, 308–317.

Griffiths BS, Caul S, Thompson J, Birch ANE, Cortet J, Andersen MN, Krogh PH (2007) Microbial and microfaunal community structure in cropping systems with genetically modified plants. *Pedobiologia*, **51**, 195–206.

Hannula SE, de Boer W, van Veen J (2012) A 3-year study reveals that plant growth stage, season and field site affect soil fungal communities while cultivar and GM-trait have minor effects. *PLoS ONE*, **7**, e33819.

Jesus ED, Liang C, Quensen JF, Susilawati E, Jackson RD, Balser TC, Tiedje JM (2016) Influence of corn, switchgrass, and prairie cropping systems on soil microbial communities in the upper Midwest of the United States. *Global Change Biology Bioenergy*, **8**, 481–494.

Kolseth AK, D'Hertefeldt T, Emmerich M *et al.* (2015) Influence of genetically modified organisms on agro-ecosystem processes. *Agriculture Ecosystems and Environment*, **214**, 96–106.

Lakshmanan V (2015) Root microbiome assemblage is modulated by plant host factors. In: *Plant Microbe Interactions* (eds Bais H, Sherrier J), pp. 57–79. Academic Press Ltd-Elsevier Science Ltd, London.

Lapierre C, Monties B, Rolando C (1985) Thioacidolysis of lignin: comparison with acidolysis. *Journal of Wood Chemistry and Technology*, **5**, 277–292.

Lapierre C, Monties B, Rolando C (1986) Thioacidolysis of poplar lignins: identification of monomeric syringyl products and characterization of guaiacyl-syringyl lignin fractions. *Holzforschung*, **40**, 113–118.

Lasat MM (2002) Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality*, **31**, 109–120.

Lauber CL, Hamady M, Knight R, Fierer N (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, **75**, 5111–5120.

Lauber CL, Ramirez KS, Aanderud Z, Lennon J, Fierer N (2013) Temporal variability in soil microbial communities across land-use types. *ISME Journal*, **7**, 1641–1650.

Lemus R, Lal R (2005) Bioenergy crops and carbon sequestration. *Critical Reviews in Plant Sciences*, **24**, 1–21.

Liang C, Jesus ED, Duncan DS, Quensen JF, Jackson RD, Balser TC, Tiedje JM (2016) Switchgrass rhizospheres stimulate microbial biomass but deplete microbial necromass in agricultural soils of the upper Midwest, USA. *Soil Biology and Biochemistry*, **94**, 173–180.

Liu W, Hao L, Wu W, Kun Wei Q, Xu Chen Y, Thies JE (2008) Transgenic Bt rice does not affect enzyme activities and microbial composition in the rhizosphere during crop development. *Soil Biology and Biochemistry*, **40**, 475–486.

Lupwayi NZ, Hanson KG, Harker KN *et al.* (2007) Soil microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat–canola rotations under low-disturbance direct seeding and conventional tillage. *Soil Biology and Biochemistry*, **39**, 1418–1427.

Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y, Kennelley ED (2001) A fern that hyperaccumulates arsenic - A hardy, versatile, fast-growing plant helps to remove arsenic from contaminated soils. *Nature*, **409**, 579.

Mao YJ, Yannarell AC, Mackie RI (2011) Changes in N-transforming Archaea and Bacteria in soil during the establishment of bioenergy crops. *PLoS ONE*, **6**, e24750.

Motavalli PP, Kremer RJ, Fang M, Means NE (2004) Impact of genetically modified crops and their management on soil microbially mediated plant nutrient transformations. *Journal of Environmental Quality*, **33**, 816–824.

Phillips DL, Gregg JW (2003) Source partitioning using stable isotopes: coping with too many sources. *Oecologia*, **136**, 261–269.

Piquemal J, Chamayou S, Nadaud I *et al.* (2002) Down-regulation of caffeic acid O-methyltransferase in maize revisited using a transgenic approach. *Plant Physiology*, **130**, 1675–1685.

Pourhassan N, Bruno S, Jewell MD, Shipley B, Roy S, Bellenger JP (2016) Phosphorus and micronutrient dynamics during gymnosperm and angiosperm litters decomposition in temperate cold forest from Eastern Canada. *Geoderma*, **273**, 25–31.

R Core Team (2013) *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, **75**, 7537–7541.

Sessitsch A, Gyamfi S, Tscherko D, Gerzabek MH, Kandeler E (2005) Activity of microorganisms in the rhizosphere of herbicide treated and untreated transgenic glufosinate-tolerant and wildtype oilseed rape grown in containment. *Plant and Soil*, **266**, 105–116.

Shen H, Poovaiah CR, Ziebell A *et al.* (2013) Enhanced characteristics of genetically modified switchgrass (*Panicum virgatum* L.) for high biofuel production. *Biotechnology for Biofuels*, **6**, 1–15.

Xu B, Escamilla-Treviño LL, Sathitsuksanoh N *et al.* (2011) Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New Phytologist*, **192**, 611–625.

Yanni SF, Whalen JK, Ma BL (2010) Crop residue chemistry, decomposition rates, and CO$_2$ evolution in *Bt* and non-*Bt* corn agroecosystems in North America: a review. *Nutrient Cycling in Agroecosystems*, **87**, 277–293.

Zhang CL, Guo J, Lee DK, Anderson E, Huang H (2015) Growth responses and accumulation of cadmium in switchgrass (*Panicumvirgatum* L.) and prairie cordgrass (*Spartinapectinata*). *RSC Advances*, **5**, 83700–83706.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Mean soil pH (0–15 cm) over the first two growing seasons in plots with transgenic (open) and control (black) switchgrass plants.

Figure S2. Rarefaction curves for 16S rRNA gene sequence libraries.

Figure S3. Diversity (inverse Simpson's index) of the 16S rRNA gene sequence libraries.

Figure S4. PCA of soil elemental composition (19 elements) shows no difference between transgenic (filled) and control (open) plants.