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REGULAR ARTICLE

Soil microbial community responses to altered lignin biosynthesis in *Populus tremuloides* vary among three distinct soils

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Abstract The development and use of transgenic plants has steadily increased, but there are still little data about the responses of soil microorganisms to these genetic modifications. We utilized a greenhouse trial approach to evaluate the effects of altered stem lignin in trembling aspen (*Populus tremuloides*) on soil microbial communities in three soils which differed in their chemical and physical properties; they included a sandy loam (CO-Colorado), a silt loam (KS-Kansas), and a clay loam (TX-Texas). Three transgenic aspen lines were developed from a natural clone common to the Great Lakes region of North America. The concentrations of stem lignin concentrations were reduced by 35% (Line 23), 40% (Line 141) and 50% (Line 72). Line 72 and Line 141 also had a 40 and 20% increase in syringyl-type stem lignin, respectively. Indirectly, these modifications resulted in increased (5–13%) and decreased (−5 to −57%) levels of root production across the lines and soil types. Responses of the soil microbial communities were investigated using: phospholipid fatty acids (PLFA), neutral lipid fatty acids (NLFA), and 3) extracellular enzyme assays. PLFA analyses indicated that there were large differences in microbial community composition between the three soils. Similarly, there were large differences in total NLFA between soils, with the KS soils having the highest amount and CO the lowest. Enzyme activities did not differ between soils, except for cellubiohydrolase, which was highest in CO soil. Across all three soils, responses to the four genetic lines were not consistent. Interactions between soil type and genetic line make it difficult to assess the potential ecological impacts of transgenic aspen on soil microbial communities and their associated functions. Given these interactions, field trials with transgenic aspen should encompass the wide range of soils targeted for commercial planting in order to determine their effect(s) on the resident soil microbial community.

Keywords Extracellular enzymes · Fungi · Microbial community · NLFA · PLFA · Transgenic aspen

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Introduction

There is a wealth of data showing that soil type can influence plant growth and physiology (Bouma and Bryla 2000; Cox et al. 2006), including altering the composition and functioning of microbial communities (Bardgett et al. 1999a; Frostegård et al. 1991; Hassett and Zak 2005). Individual plant species have been shown to affect soil microbial characteristics (Bardgett et al. 1999b; Belnap and Phillips 2001; Blair et al. 1990; Fang et al. 2001; Knops et al. 2002). Microorganisms control important biogeochemical processes, including carbon (C) cycling (Zak et al. 2006), and differences in microbial communities associated with individual plant species are hypothesized to result from variations in the quantity and quality of plant carbon (C) inputs (Grayston et al. 2001; Marschner et al. 2001). Recent advances in forest biotechnology have the potential to alter both the quantity and quality of plant C entering the soil, but the effects of these changes on soil microbial community structure and function have not been extensively explored.

Trembling aspen (*Populus tremuloides*) is an economically and ecologically important tree (Brunner et al. 2004) across large portions of North America (Barnes and Wagner 2004). Substantial funding is being devoted to developing transgenic lines of poplar species, including trembling aspen, which have better pulping performance (Li et al. 2000; Pilate et al. 2002). Others have speculated that transgenic aspen could be used to increase C storage in soils (Herrera 2005; Lorenz and Lal 2005). Research has focused on modifying genes encoding enzymes involved in the synthesis of lignin monomers, including cinnamate 4-hydroxylase (C4H), 4-coumarte:COA ligase (4CL), and confieryl alcohol dehydrogenase (CAD) (Hu et al. 1999; Li et al. 2003). The introduction of these transgenes can reduce lignin concentrations by as much as 45%, with associated increases in cellulose (Hu et al. 1999). Second, the production of angiosperm lignin monomers syringyl (S) and guaiacyl (G) was modified by over expressing the gene encoding coniferaldehyde 5-hydroxylase (CAld5H), which controls lignin S/G (Li et al. 2003). Increasing the relative proportion of the S monomer (S/G ratio) in stem tissues allows lignin to be more easily removed during the pulping process (Li et al. 2000). These changes could have ecological effects as well.

Lignin, the second most abundant polymer on the planet, is an extremely decay resistant C compound (Halpin et al. 2007), and only a small subset of soil organisms, including some saprophytic fungi, can fully degrade it (Collins and Dobson 1997). Lignin concentrations in plant tissues control decomposition rates and the recycling of C compounds in terrestrial systems (Hobbie et al. 2006). However, the conformation and composition of lignin affects its degradation as well (Webster et al. 2005), so changing S/G ratios is likely to affect accessibility of plant C to soil microorganisms. Experimental work with naturally occurring genetic variation in *Populus* has shown that genetically associated traits, particularly salicylate phenolic glycosides and condensed tannins, can strongly influence nutrient release and decomposition (Madritch et al. 2006; Schweitzer et al. 2004). Given that genetic modifications (Webster et al. 2005) and natural variation (Madritch et al. 2006; Schweitzer et al. 2004) can affect decomposition of plant residues, it is likely that variations in aspen litter lignin quality and quantity resulting from genetic modifications will alter rates of aspen litter decomposition as well as the activity and composition of the decomposer community (Halpin et al. 2007). More research is needed to explore relative impacts of modified plants on soils and soil organisms across a variety of ecological conditions (Halpin et al. 2007; Webster et al. 2005).

Prior results showed that changes in stem lignin concentration did not alter lignin concentrations in fine roots or leaves, but did reduce plant growth, soil C formation (determined using a two source isotope mixing model) and microbial biomass C (Hancock et al. 2007). In particular, aspen with increased S/G ratios had 40% less total biomass, which was likely associated with the higher C costs of syringyl production (Amthor 2003). These results demonstrated that single gene changes have the potential to affect whole plant and ecosystem level processes, particularly primary production and soil C storage.

Recent field trials have shown that *Populus* sp. with altered lignin production have higher rates of root decomposition than the wild type (Pilate et al. 2002), but a follow-up experiment with coarse wood sampled from the earlier field trials showed no consistent effects of the genetic manipulations on

wood decomposition rate (Tilston et al. 2004). While soil microbial biomass and respiration rates were unaffected by lignin modifications to *Populus* sp. in the first study (Pilate et al. 2002), there were significant differences in soil microbial biomass between the transgenic lines in a field trial, although the response depended on soil type (Tilston et al. 2004). Overall, the effects of individual genetic alterations in plants on soil microbial community composition and functioning have not been well characterized (Halpin et al. 2007; Pilate et al. 2002). Further, while plant and microbial responses to genetic alterations appear to vary with soil type (Tilston et al. 2004), data are extremely limited (Halpin et al. 2007).

We used greenhouse trials to assess the effects of altered stem lignin concentrations in four different lines of transgenic aspen on soil microbial community composition and function in three different soils. The four lines of *Populus* included one control line, one derived line with reduced stem lignin, and two derived lines with reduced stem lignin and altered S/G (Table 1). Given its extensive distribution in North America (Barnes and Wagner 2004), *P. tremuloides* is an ideal organism with which to approach the question of how genetic alterations interact with soil type (Tilston et al. 2004). Specifically, we tested how the indirect effects of lignin modifications in four related lines of *P. tremuloides* can affect microbial communities and their functioning across three soil types that included loam, silt loam, and sandy loam soils. We quantified the soil microbial community compositional, physiological and functional responses using: (1) phospholipids fatty acid (PLFA) analysis (assess soil microbial community composition); (2) neutral lipid fatty acid (NLFA) analysis (assess physiological responses of soil organisms; higher ratios of NLFA/PLFA indicate a build up of energy storage compounds); and (3) extracellular enzyme assays (functional response of the soil microorganisms).

Materials and methods

Soils

In May 2005, approximately 250 kg of mineral soil was collected from each of three different grassland sites: the Colorado Central Plains Experimental Range (CPER) northeast of Fort Collins; the Konza Prairie Biological Station, Kansas; and the Blackland Prairie at the USDA Grassland Station in Temple, Texas. Though all three sites are ''grasslands'', the climatic conditions and dominant grass species differed between the three sites (Table 2). The Colorado soil is an Olney fine sandy loam with 0–6% slopes (fine-loamy, mixed, superactive, mesic Ustic Haplargrid). Konza soil is a Reading silt loam with 1–3% slopes (fine-silty, mixed superactive, mesic Pachic Argiudoll). Texas soil is an Austin silty clay with 3–5% slopes (fine-silty, carbonatic, thermic Udorthentic Haplustoll).

Hereafter, the soils will be referred to as Colorado (CO), Kansas (KS), and Texas (TX), representing sandy loam, silt loam, and clay loam soils, respectively (Table 2). These soils were selected for their large texture differences. One randomly chosen area was sampled at each site. The top 5 cm of surface were removed with a shovel to reduce the amount of plant roots and litter collected. The 5–30 cm depth soils were then sampled and immediately shipped (<1d) to Houghton, MI. Upon arrival to Houghton, soil types were individually sieved twice through a 4 mm mesh screen to remove roots and rocks, and were then homogenized through repeated mixing.

Table 1 Aspen (*Populus tremuloides*) stem tissue chemistry of transgenic and control plants used in the greenhouse trials

	Line 72	Line 141	Line 23	Control (Line 271)
Description	40% decrease in lignin, 60% increase in S/G	50% decrease in lignin, 20% increase in S/G	35% decrease in lignin, normal S/G	Normal lignin, normal S/G
Stem % lignin	13.7	10.7	14.4	22.2
Syringyl/guaicyl monomers in lignin (S/G ratio)	3.6	2.7	2.2	2.2
Stem % cellulose	49.2	53.3	44.8	41.4

Table 2 Summary of climatic characteristics, dominant plant species, and soil properties of the three soils used in this study*

	Central Plains Experimental Range, Colorado	Konza Prairie, Kansas	Blackland Prairie, Temple, Texas
Latitude (N)	40°48′	39°05′	31°6′
Longitude (W)	104°46′	96°35′	97°21′
Ecosystem	Shortgrass steppe	Tallgrass prairie	Tallgrass prairie
Mean Annual Precipitation	307 mm	835 mm	877 mm
Mean Annual Temperature	8.9°C	12.8°C	25.8°C
Dominant perennial grasses	*Bouteloua gracilis*	*Andropogon gerardii*	*Bothriochloa ischemum*
	Buchloe dactyloides	*Schizachyrium scoparium*	
Percent sand by mass	73 [c]	27 [a]	30 [b]
Percent silt by mass	16 [a]	52 [c]	35 [b]
Percent clay by mass	11 [a]	21 [b]	35 [c]
pH in water	6.4 [a]	7.7 [b]	7.9 [c]
Percent C by mass	0.9[a]	3.4 [c]	2.4 [b]
Percent N by mass	0.1 [a]	0.3 [c]	0.2 [b]
Available P (g kg^{-1})	0.23 [a]	0.53 [c]	0.48 [b]
Available K (g kg^{-1})	2.33 [a]	7.77 [c]	3.97 [b]
Available Ca (g kg^{-1})	2.20 [a]	13.98 [b]	141.44 [c]
Available Mg (g kg^{-1})	1.95 [a]	7.06 [c]	3.97 [b]

The soils were from Colorado, Kansas and Texas. Means are given for soil physical and chemical properties. Values followed by different letters indicate significant differences by a LSD test ($P < 0.05$)

* Soil characteristics were determined approximately one month after micropropagates were planted in mist chambers

Transgenic aspen

Three transgenic trembling aspen (*Populus tremuloides*) lines were developed from a naturally occurring clone (Control) from the Great Lakes region (Karnosky et al. 1996) during the 1990s. The cuttings of the wildtype clone were originally vegetatively propagated from greenwood cuttings and maintained in a greenhouse. New copies were propagated as needed, so that control and transgenic individuals were the same age. The four lines varied in their stem lignin concentrations, stem cellulose concentrations, and in the ratio of syringyl (S) to guaiacyl (G) lignin monomers (Table 1). These transgenic lines were generated by *Agrobacterium*-mediated transformation (Tsai et al. 1994).

Greenhouse trials

Micropropagates of each line were raised in sterile culture for 3 months and then transferred to small pots in May (approx. 230 g soil). Pots contained one of the three soil types, so that the micropropagates were planted in a full factorial design (4 genetic lines × 3 soil types). These plants were maintained in mist chambers for three weeks and then transferred to larger pots (2.5 l). Each pot contained one tree, and pots were transferred to greenhouse benches in June 2005.

The greenhouse trial was a full factorial experiment (4 genetic lines × 3 soil types), with 15 individual trees per genetic line planted in each soil type. Sub-samples of soil were collected from each pot before the trees were transplanted. One sub-sample was oven dried (50°C) for pH, texture, total C, total N, available P, available K, available Ca, and available Mg analyses. A second sub-sample was collected for lipid analysis; these were frozen upon collection (−20°C) and then freeze dried (Labconco, Freezone 4.5, Kansas City, MO). Sub-samples for enzyme analysis were kept in cold storage (4°C) until roots could be picked out using tweezers and 1 g of root free soil could be weighed out for each sample. These were pre-treatment soil samples.

Through July, aspen were grown under natural light conditions, after which artificial light supplemented natural light to maintain a 16-h photoperiod until harvest in November. Plants were watered daily to field capacity with an automated irrigation system.

Complete fertilizer (nitrogen (N) + phosphorus (P) + potassium (K) plus micronutrients: 0.02% B, 0.07% Cu, 0.15% Fe, 0.05% Mn, 0.00005% Mo, 0.06% Zn) was applied at a rate of 0.06 g N, 0.09 g P, and 0.06 g K per plant once a week. In November 2005, eight pots per genetic line per soil type ($N = 96$) were harvested for root biomass and soil physical and chemical analyses.

The entire soil volume of each harvested pot was sub-sampled for C, N, lipid and enzyme analyses. The sub-sample for C and N analyses was oven dried (50°C) and then ground. The sub-sample for lipid analysis was frozen upon collection (−20°C) and then freeze dried (Labconco, Freezone 4.5, Kansas City, MO). The sub-samples for enzyme analysis were kept in cold storage (4°C) until roots could be picked out using tweezers and ~2 g of root free soil could be weighed out for each sample. One gram was used for the enzyme assays and the other gram was used to measure gravimetric water content, which was determined as the mass of water (g) per gram of dry soil.

Physical and chemical soil analyses

Soil pH was determined using a 1:1.3 soil:water ratio using a combination reference glass electrode. The hydrometer method was used to determine soil texture (Bouyoucos 1962). Total C and N were determined from ground samples using Costech 4010 elemental analyzer at Michigan Technological University. Available P and K were extracted simultaneously using 0.03 N NH_4F in 0.025 N HC1 (Bray P1 extract), but they were analyzed separately. The P concentration was determined colorimetrically, while extracted K was measured using atomic absorption. Available Ca and Mg were measured by ICP-OES (Thermo Jarrell Ash IRIS Advantage Inductively Coupled Plasma Optical Emission Spectrometry).

Lipid extraction

Lipids were extracted from freeze-dried, root free soils. Preliminary lipid analyses were performed to determine the amount of soil needed for extraction (KS and TX = 1.5 g soil; CO = 3 g soil). Soils were vortexed in a mixture of methanol, chloroform and phosphate buffer (pH 7.4) with a volume ratio of 2:1:0.8 using a modified Bligh and Dryer method (Bligh and Dryer 1959; Frostegård et al. 1991). Silicic acid column chromatography was used to separate the whole lipid extract into neutral lipid, glycolipid, and phospholipid fractions. The neutral and phospholipid fractions were methylated to free fatty acids methyl esters (FAME's). C19:0 (Matreya Inc., State College, PA) was added as an internal standard.

FAMEs were analyzed by gas chromatography (GC) and identified using the Sherlock Microbial Identification System. It consisted of a 6890N Series GC (Agilent Technologies, Palo Alto, CA), a flame ionization detector equipped with an Ultra 2 column (30 m, 0.2 m ID, 0.33 μm film) and a computer and associated software (Sherlock Pattern Recognitition Software, MIDI, Newark, DE). FAMEs were identified on the basis of retention times. A eukaryotic mix of fatty acids (MIDI Inc., Newark, DE) and series individual fatty acids (Matreya Inc., State College, PA) were employed as qualitative standards.

Fatty acid taxonomy

Lipid composition was determined using phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) analyses. Fatty acids (FAs) were assigned to taxonomic groups following literature (Frostegård and Bååth 1996; Ruess et al. 2005; Zelles 1999).

Terminally branched saturated FAs (i14:0, i15:0, i16:0, i17:0, a17:0) were designated as gram-positive bacterial markers. Some monosaturated FAs (17:1ω8c, 18:1ω5c) and cyclopropyl saturated FAs (cy17:0, cy19:0) were designated as gram-negative bacterial markers. 16:1ω5 has been used as a gram-negative marker for the PLFA fraction (Hassett and Zak 2005), but in the NLFA fraction, it is a known marker for arbuscular mycorrhizal fungi (Balser et al. 2005; van Aarle and Olsson 2003). Saprophytic fungi were associated with 18:1ω9c and 18:2ω6,9, while 16:1ω7c was considered a general bacterial marker. Common saturated FAs (16:0, 18:0) were not assigned to a taxonomic group, but 16:0 correlates well with total microbial biomass (Zelles et al. 1992). Bacterial to fungal ratios were calculated using the nmol percentages (relative abundance) following Frostegård and Bååth (1996). We used total PLFA as a measure of total microbial biomass (Frostegård et al. 1991). The NLFA/PLFA ratio was used as an indicator of physiological state (C surplus or C deficient) in soil fungi (Bååth 2003).

For each sample, the abundance of individual PLFAs and NLFAs were reported in absolute amounts (nmol C g^{-1} soil) and then converted to nmol percent (individual peak area/total sample peak area). Ratios less than 0.02 were omitted from the data set. PLFAs and NLFAs with retention times less than C14:0 and greater than C20:0 were also eliminated from the dataset. A total of 14 PLFAs and 8 NLFAs were extracted from all samples of the KS and TX soils. Two PLFAs (iC14:0 and 18:1ω5c) and three NLFAs (i16:0, 17:1ω8c, and cy19:0) were not detected in any of the CO samples, but were in all of the KS and TS samples. For statistical analyses, CO was excluded from the ANOVAs of these five lipids.

Soil enzyme assays

We measured activities of phenol oxidase, peroxidase, cellubiohydrolase, 1,4-β-glucosidase, and 1,4-α-glucosidase, using a microplate approach (DeForest et al. 2004; Sinsabaugh et al. 2005). Phenol oxidase and peroxidase are enzymes that oxidize C substrates like lignin by removing a pair of electrons and the accompanying hydrogen atoms. Cellubiohydrolase breaks off cellubiose molecules from a chain of cellulose, while β-glucosidase splits cellubiose molecules into two glucose molecules. The enzyme α-glucosidase catalyzes the hydrolysis of terminal 1,4-linked α-glucoses from the non-reducing ends of polysaccharide chains like starch. For each enzyme, there were eight replicate samples. To prepare soils, 60 ml of 50 mM sodium acetate buffer (pH 5) were added to 1 g of root free soil from each composite sample. A pH of 5 was chosen here because it approximates the bulk pH of many soils, and our three soils ranged from slightly acidic to slightly basic (Table 2). The mixture was blended using a tissue homogenizer (Polytron Devices Inc. Paterson, NJ) to generate a soil suspension. The remaining 65 ml of buffer was added and samples were kept in 125 ml screw top Nalgene bottles for up to 45 min prior to analysis. The same soil suspension was used to perform all five assays.

Colorimetric assays were used to determine the activities of phenol oxidase and peroxidase (DeForest et al. 2004; Sinsabaugh et al. 2005). A 25 mM solution of L-3,4-dihydroxyphenyalanine (L-DOPA) in acetate buffer was the colormetric reagent. We used clear 96 well microplates. For each assay there were eight blank wells (buffer only), eight reference standard wells (substrate + buffer), eight sample blank wells (soil + buffer), and sixteen replicate assay wells per sample. To reduce absorbance by soil particles, we used 100 µl of soil sample versus the typical 200 µl and increased the amount of buffer in each well (R. Sinsabaugh, personal communication). For the peroxidase assays, 25 µl of H_2O_2 was added to each well. Plates were incubated for 24 h. Absorbance was read at 460 nm on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA). Activity was calculated as µmol L-DOPA g^{-1} soil hr^{-1} expressed on a dry mass basis. Activity for the peroxidase enzyme was the increment in activity between samples incubated with and without hydrogen peroxide.

Fluorogenic assays were used to determine the activities of cellubiohydrolase, 1,4-β-glucosidase, and 1,4-α-glucosidase (DeForest et al. 2004; Sinsabaugh et al. 2005). These assays involved the use of methylumbelliferyl (MUB), a substrate that has side groups linked to a fluorgenic moiety. Black 96 well microplates were used. For each assay there were sixteen replicate assay wells per sample plus eight plate blanks, 4-MUB standards, negative controls (buffer + MUB linked substrate), quench controls (4-MUB standard + soil), and eight sample blanks. Plates were incubated for 2 h and then 10 µl of 0.5 M NaOH was added to each well to stop the enzymatic reactions. Fluorescence was measured using a SpectraMax M2 (Molecular Devices, Sunnyvale, CA), with excitation energy at 365 nm and emission at 450 nm. Activities were calculated as nmol 4-MUB g^{-1} soil hr^{-1}.

Statistical analysis

Statistical analyses were performed using SAS 9.1 and SPSS 10.0. Soil chemical properties, soil physical properties, and root data were analyzed using one-way ANOVA. For significant effects in the soil data, a protected Least Significant Difference test was used for post hoc comparisons (LSD) (Littell et al. 2002). Two-tailed *t*-tests were used to assess changes in root biomass production in the three transgenic lines relative to the control line, where the test value was zero. Two-way ANOVAs were used to test for differences in total PLFA, total

NLFA, the abundance of individual PLFAs (both absolute and relative abundance), the abundance of individual NLFAs (both absolute and relative abundance), and potential activity of all five enzymes. Soil type and genetic line were designated as fixed effects. For significant effects, a protected LSD test was used for post hoc comparisons (Littell et al. 2002). We controlled for comparisonwise type I error inflation by using the Tukey-Kramer adjustment (Littell et al. 2002). Linear correlations were used to explore relationships between different enzymes (Zar 1999).

Soil type strongly altered the quantity of most PLFA and NLFA markers. The large differences that existed between the lipid abundances of the three soil types potentially masked subtler genetic line effects, particularly if one lipid responded positively to a treatment, while the same lipid exhibited a negative response in a different soil. We were interested in the effects of genetic line, and the variation in genetic effects across soil types, on soil microbial community composition and function. To investigate effects of genetic line on the relative abundance of each PLFA and NLFA within each soil type, we performed one-way ANOVAs. When genetic line was significant, a protected LSD test was used (Littell et al. 2002). The overall type I error rate was addressed by only conducting pairwise comparisons when there was overall significance of an F test for genetic line. We used the Tukey-Kramer adjustment to control for comparisonwise type I error inflation (Littell et al. 2002).

To test for soil type and genetic line effects on microbial community composition, stepwise and canonical discriminant analysis were used on the 12 PLFAs (in nmol percents) that were present in all samples (SAS 1999). Stepwise discriminant analysis was used to select the most significant combination of variables that maximally separated the groups. Canonical discriminant analysis is essentially a graphical version of MANOVA, and its purpose is to determine a linear combination of variables that maximize separation between groups (SAS 1999). These linear combinations, known as canonical variables, summarize between-class variation; they can show large treatment differences even if none of the original variables do (SAS 1999).

Results

Soil effects on root biomass and PLFAs

The three soils significantly differed from each other in all of the chemical and physical properties measured (Table 2). Soil type significantly affected root biomass (Fig. 1a) across all genetic lines. The sandy CO soils had significantly higher root biomass than the clay rich TX soil (Fig. 1a). Root biomass in the KS soils did not significantly differ from CO or TX (Fig. 1a). Soil type also strongly affected total PLFA (two-way ANOVA: $F = 227$, 2 df, $P < 0.0001$, $R^2 = 0.82$), but the genetic line and line by soil type interactions were not significant. KS soils had significantly higher total PLFA (160 ± 4.1 nmol C g^{-1} soil) than both TX and CO; TX soil (PLFA = 131 ± 4.8 nmol C g^{-1} soil) had significantly higher total PLFA than CO soil (PLFA = 48 ± 2.5 nmol C g^{-1} soil). Soil type also significantly affected the abundance of individual PLFAs (highly significant for all PLFAs except 14:0) as well as their relative abundances.

Twelve of PLFAs were used to discriminate between soils and genetic line, but samples could only be grouped by soil type (Fig. 2a). The canonical discriminant analysis identified three significant canonical variates (Fig. 2; $P < 0.001$ for each CV). The first canonical variate (CV1) separated the CO soil from the TX and KS soils. The second canonical variate (CV2) separated the KS and TX soils from each other. Four PLFAs, a17:0, 16:1ω5c, 18:1ω9c, and cy19:0, were strongly and positively correlated with the first canonical axis, while 16:0 was negatively correlated with it (Fig. 2b). i16:0 and 18:0 were most positively correlated with the CV2 and a15:0 and cy17:0 were negatively correlated (Fig. 2b). It was not possible to separate the genetic lines using discriminant analysis.

The CO soil had the lowest relative bacterial abundance (Fig. 3a) and highest relative fungal abundance overall (Fig. 3b). Using relative PLFA abundances (Fig. 3a, b), we calculated the fungal:bacterial (F:B) ratios for each soil, which differed significantly by soil type ($F = 11.8$, 2 df, $P < 0.0001$, $R^2 = 0.16$), but not by genetic line. Overall, the CO soil had a significantly higher F:B ratio than the TX and KS soils, which did not significantly differ from each other (Fig. 3c).

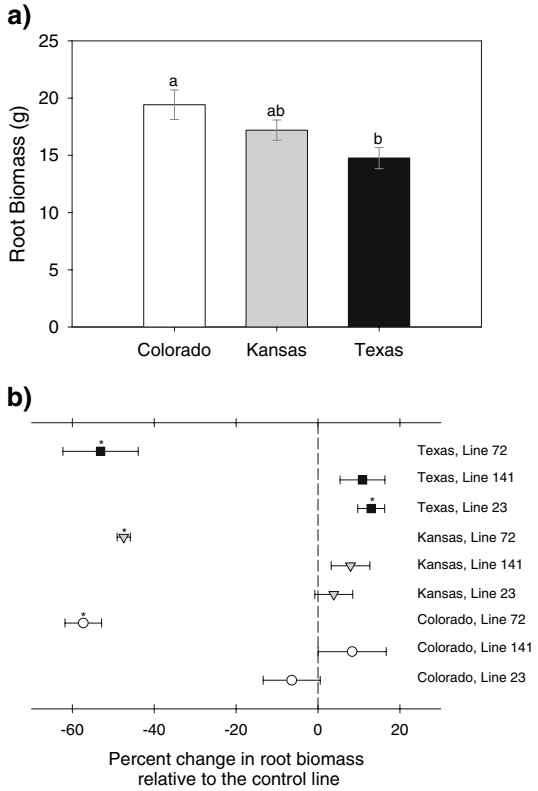

Fig. 1 The effect of (**a**) soil type on total root biomass, and (**b**) the effect of the three transgenic lines (23, 141, and 72) on root biomass production relative to the control line. Presented are means ± 1 SE. Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons. Stars indicate values are significantly different from zero by two-tailed *t*-tests. White circles represent Colorado soil, gray triangles Kansas soil, and black squares Texas soil

Genetic line effects on root biomass and PLFAs

The genetic modification in Line 72, which was designed to increase the S/G ratio in stemwood lignin, resulted in significant reductions in total root biomass across all three soils (Fig. 1b; CO $t = -12.6$, df 7, $P < 0.001$; KS $t = -27.2$, df 7, $P < 0.001$; TX $t = -6.6$, df 7, $P < 0.001$). In the TX soil, Line 23 had relatively more root biomass than the control line ($t = -3.8$, df 7, p = 0.006). In Line 141, root biomass was slightly increased relative to the control line across all three soils, but not significantly.

Due to large differences in total lipids, F:B ratios (Fig. 3c) and soil C (Table 2) between the soil types, we performed one-way ANOVAs to test for any genetic effects on the relative abundance of the PLFAs within a soil type. We observed responses across different groups of soil organisms (as indicated by individual PLFAs), but these responses were not consistent across the three soils. For the CO soil, there were no significant differences between the genetic lines in any of the PLFAs. In the KS soil, the relative abundance of elaidic acid (18:1ω9t) was significantly higher ($F = 3.29$, 3 df, $P = 0.034$, $R^2 = 0.17$) in Line 141 than Line 72. In the TX soil, Line 72 had the lowest proportion of a saprophytic fungal marker, 18:2ω6,9 ($F = 3.29$, 3 df, $P = 0.034$, $R^2 = 0.18$), although it was only significantly different from Line 23. There were also significant responses of four PLFAs in the TX soil. For all four PLFAs, their concentrations were highest in the control, but significant reductions only occurred in Line 23. These included i15:0 ($F = 3.75$, 3 df, $P = 0.022$, $R^2 = 0.21$), 16:1ω5c ($F = 3.09$, 3 df, $P = 0.042$, $R^2 = 0.15$), 18:1ω5c ($F = 5.19$, 3 df, $P = 0.005$, $R^2 = 0.28$), and cy17:0 ($F = 3.53$, 3 df, $P = 0.027$, $R^2 = 0.19$).

Soil and genetic line effects on NLFAs

Soil type significantly affected total NLFA (Table 3), explaining 80% of the variance. The KS (107 nmol C g^{-1} soil ± 4.6) and TX (NLFA = 101 nmol C g^{-1} soil ± 3.7) soils had significantly more NLFA than the CO soil (NLFA = 15 nmol C g^{-1} soil ± 1.0; LSD tests: CO vs. KS, $P < 0.05$; CO vs. TX, $P < 0.05$). However, in contrast to PLFAs, there was also a significant soil type by genetic line interaction for total NLFAs (Table 3), explaining another 3% of the variance. In the CO soils there were no differences among the lines (Fig. 4a). In the KS soil, total NLFA was significantly higher in Line 141 compared to Line 72 (Fig. 4b). In the TX soil, total NLFA was significantly higher in Line 141 than in the control (Fig. 4c).

The absolute quantities of the eight individual NLFAs (nmol C g^{-1} soil) were significantly affected by soil type. TX soils had the highest amounts of bacterial NLFAs (i16:0, 17:1ω8c; LSD tests $P < 0.05$) and KS soils had significantly more of 18:1ω9c than TX or CO (LSD tests $P < 0.05$). There was also a significant effect of genetic line on the total amount of 18:2ω6, 9 ($F = 6.61$, 3 df, $P = 0.0004$) and 18:1ω9c ($F = 3.65$, 3 df, $P = 0.016$), but genetic line accounted for only 10% of the variance for

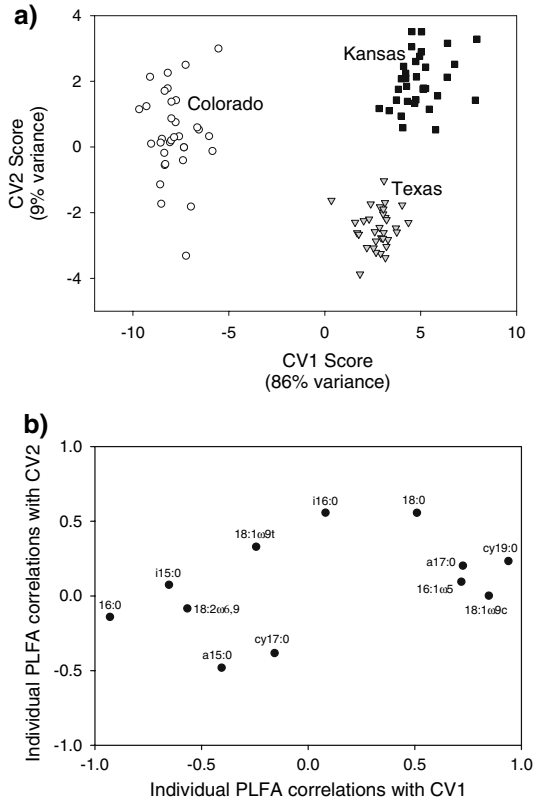

Fig. 2 Canonical discriminant analysis of the relative PLFA abundances across soil types and genetic lines. (**a**) Canonical scores for each soil are plotted for the first two significant canonical variates (CV1 and CV2). Genetic line was not a significant factor in the analysis. (**b**) Correlations of individual PLFAs with each canonical variate

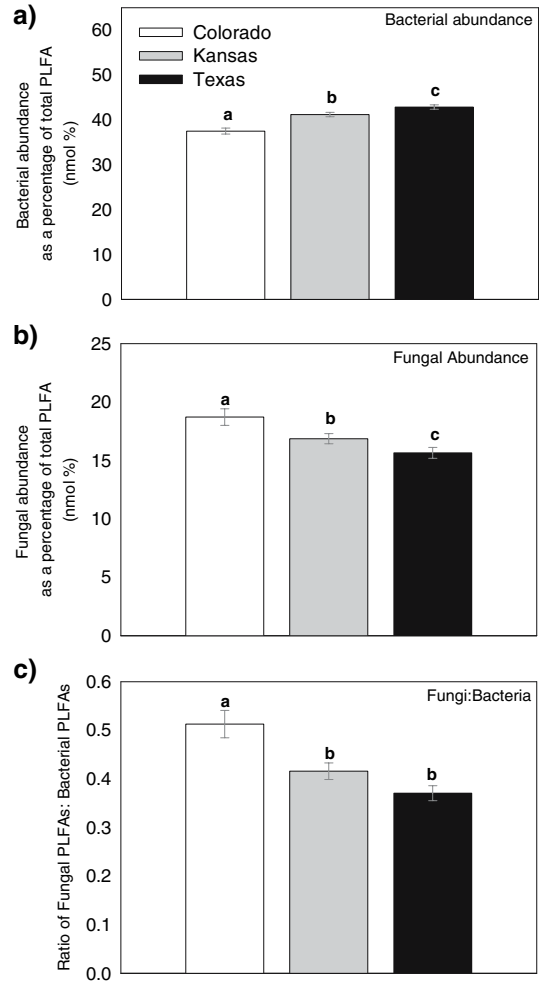

Fig. 3 The effect of soil type on the estimated (**a**) bacterial abundance (PLFA in nmol %), (**b**) fungal abundance (PLFA in nmol %), and (**c**) the fungal:bacterial PLFA. Bars represent adjusted means ± 1 SE (n = 8). Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons

18:2ω6,9 and only 1% of the variance for 18:1ω9c in the two-way ANOVAs, whereas soil explained 45% and 84% respectively. For 18:1ω9c, there was a significant soil type by genetic line interaction (F = 3.29, 6 df, P = 0.006), which explained another 3% of the variance. This interaction can be explained by the post hoc tested which showed that in the CO and TX soils, there were no significant genetic effects (Fig. 5a, c), but in the KS soil, Line 72 had the lowest amount of 18:1ω9c (Fig. 5b).

Relative abundances of individual NLFAs were significantly different across the three soil types (Table 3), and soil type explained 30–90% of the variance. There were significant genetic line effects on the relative abundance of four NLFAs as well as significant soil by genetic line interactions (Table 3), which accounted for another 2–11% of the variance. Across the three soil types, the same individual NLFAs were not responding similarly to the genetic lines (Table 3). This may have been related to the changes in total NLFA associated with different genetic lines in the KS and TX soils (Fig. 4).

Soil and genetic effects on NLFA/PLFA ratios

The NLFA/PLFA ratios for three fatty acids, 16:1ω5c, 18:1ω9c and 18:2ω6,9 were determined to assess the physiological status of the soil arbuscular mycorrhizal and other fungi (saprophytic

Table 3 Summary of results from fixed effect ANOVAs table, which tested genetic line, soil type, and the genetic line by soil type interaction for each identified NLFA (relative abundance in nmol%) and the total NLFA extracted from soil (in nmol C g^{-1} soil)

Fatty acids	Between Subject Effects		Within Subject Effects				LSD test
	Genetic Line Num df = 3		Soil Type Num df = 2		Genetic Line × Soil Type Num df = 6		Soil Type
	F	*P*	*F*	*P*	*F*	*P*	
Saturated							
i16:0*	0.11	0.95	206	<0.0001	0.48	0.70	TX > KS
16:0	1.46	0.23	14.6	<0.0001	1.30	0.27	CO > TX = KS
Monoenoic							
16:1ω5c	0.69	0.56	260	<0.0001	0.13	0.99	CO > KS = TX
16:1ω7c	4.08	0.009	25.2	<0.0001	2.61	0.023	CO > KS = TX
17:1ω8c*	3.56	0.019	235	<0.0001	1.14	0.34	TX > KS
18:1ω9c	3.06	0.032	107	<0.0001	3.18	0.0071	KS > CO > TX
19:1ω11c *	0.46	0.71	13	0.0006	1.21	0.31	TX > KS
Polyenoic							
18:2ω6,9c	10.2	<0.0001	286	<0.0001	1.52	0.18	CO > KS > TX
NLFA total (nmol C g^{-1} soil)	2.22	0.091	240	<0.0001	3.43	0.0043	KS = TX > CO

The post hoc tests indicate which soil type (Colorado = CO; Kansas = KS; Texas = TX) had the highest relative abundance of each identified NLFA. *Three NLFAs, i16:0, 17:1ω8c, and 19:1ω11c, were not extracted from any Colorado samples. Thus, for these three NLFAs, the numerator df equal 3, 1, and 3 for genetic line, soil type and the interaction term respectively

and ectomycorrhizal). NLFA/PLFA ratios differed significantly between soil types (Fig. 6a) and between genetic lines (Fig. 6b). There were no soil by genetic line interactions. The CO soil had the highest NLFA/PLFA ratio of the AM marker, 16:1ω5c, while the KS soil had the highest ratios for the two fungal markers (Fig. 6a). There were no genetic line effects on the NLFA/PLFA ratio of 16:1ω5c. The ratios of 18:1ω9c and 18:2ω6,9 were significantly smaller for Line 72 as compared to the control and Line 141.

Soil and genetic line effects on enzyme activity

Cellubiohydrolase was the only enzyme whose activity was significantly different between soil types (Table 4). The CO soil had significantly higher cellubiohydrolase activity (30.59 nmol/g soil/hour ± 2.50) than the (21.88 nmol/g soil/hour ± 1.94) KS soil, but did not differ from the TX (26.47 nmol/g soil/hour ± 2.03). The TX and KS soils did not differ from each other. There was a significant soil by genetic line interaction for cellubiohydrolase (Table 4). In the KS soil, the control had the highest cellubiohydrolase activity (Fig. 7a), but there were no differences between genetic lines in either the CO or TX soils. There was a significant soil by genetic line interaction for β-glucosidase (Table 4). Overall, β-glucosidase was highest in the control and lowest in Line 72. In the KS soil, the control and Line 23 had significantly higher β-glucosidase activity than Lines 141 and 72 (Fig. 7b). There were no differences in β-glucosidase activity between the genetic lines in the CO or TX soils.

Correlations were performed to assess the relationship between cellubiohydrolase and β-glucosidase activities; these two enzymes are sequentially involved in the degradation of cellulose, thus we expected that changes in the activity of cellubiohydrolase, which acts first, would affect β-glucosidase activity. Simple linear correlations demonstrated that β-glucosidase activity and cellubiohydrolase activity were positively related to each other. The correlation coefficients for each soil type did not significantly differ from each other ($X^2_{0.05,2} = 5.99$, calculated $X^2 = 2.29$) and the common correlation coefficient was 0.70. Neither cellubiohydrolase ($R^2 = 0.05$, $P = 0.65$) nor β-glucosidase ($R^2 = 0.13$, $P = 0.20$) activities were significantly related to root biomass.

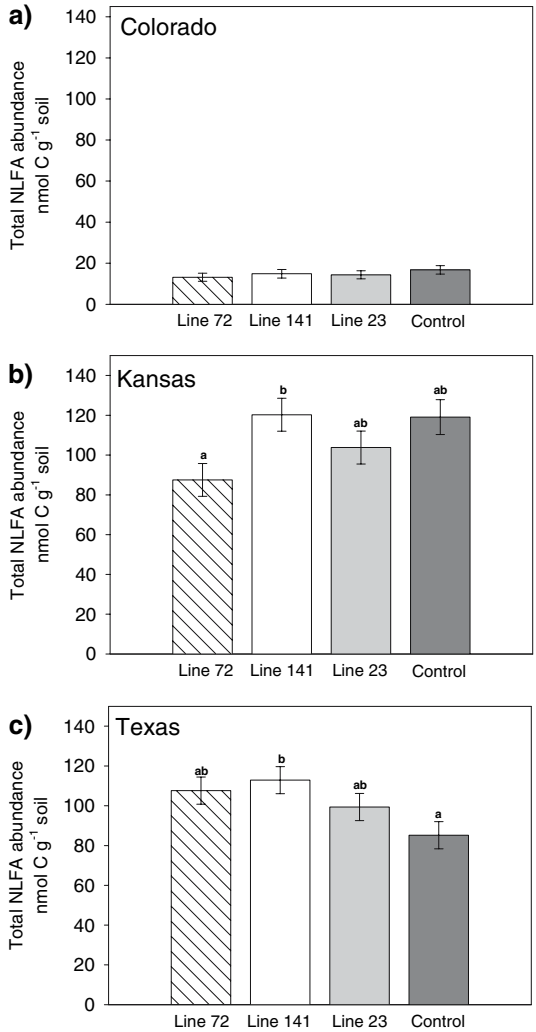

Fig. 4 The effect of genetic line on total NLFA abundance in (**a**) the Colorado soil, (**b**) the Kansas soil and (**c**) the Texas soil. Bars represent the absolute of amount of NLFAs extracted from the soil (nmol C g^{-1} soil) and are presented as adjusted means ± 1 SE (n = 8). Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons

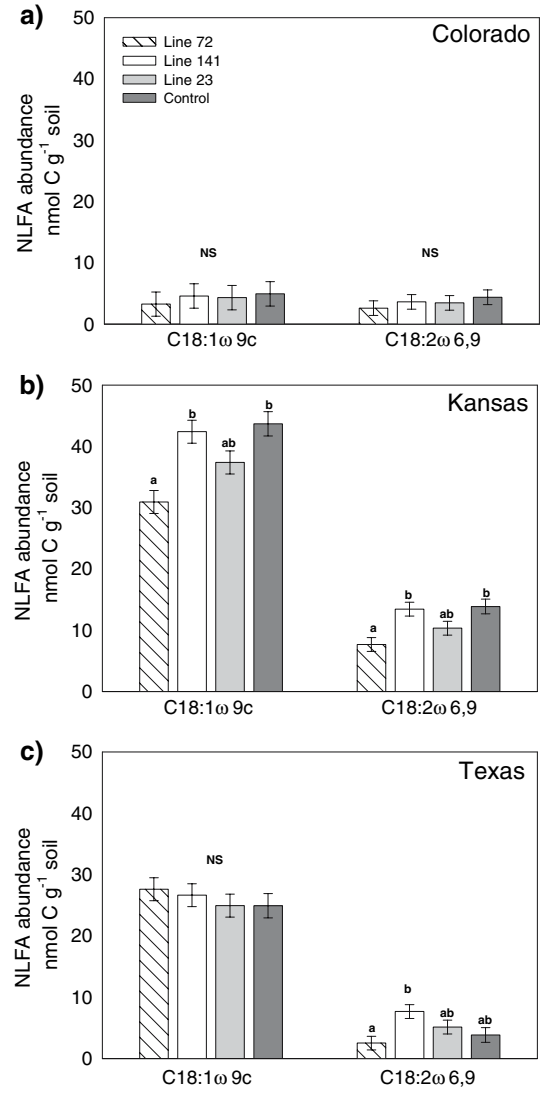

Fig. 5 The effect of genetic line on selected neutral fatty acids (NLFAs) within the (**a**) Colorado soil, (**b**) Kansas soil, and (**c**) Texas soil. The taxonomic association for each NLFA are given in the methods. Bars represent the abundance of individual NLFAs (nmol C g^{-1} soil) and are presented as adjusted means ± 1 SE (n = 8). Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons

Discussion

PLFA responses to soil type and genetic line

Our measurements of aspen growth indicated that belowground biomass varied significantly between the soils (Fig. 1a). Our multivariate analyses showed that microbial community composition significantly differed between the three soils (Fig. 2) but was not affected by genetic line. Soil texture, pH and the availability of soil C may explain differences in soil microbial community composition as they strongly influence the distribution of bacteria and fungi. The CO soil was characterized by low total microbial biomass and had the highest proportion of fungi relative to bacteria (Fig. 3c). Although low soil C

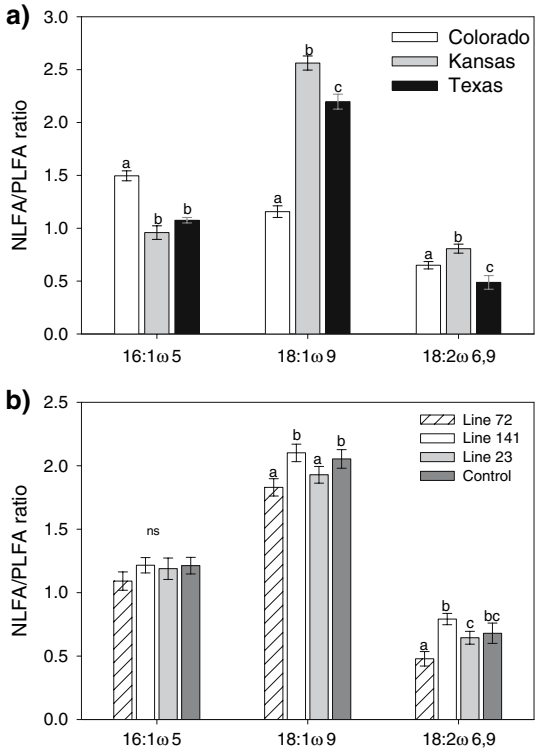

Fig. 6 The NLFA/PLFA ratios for three fatty acids of fungal origin for each (**a**) soil type and (**b**) genetic line. Bars represent the means ± 1 SE of each fatty acid ratio. Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons

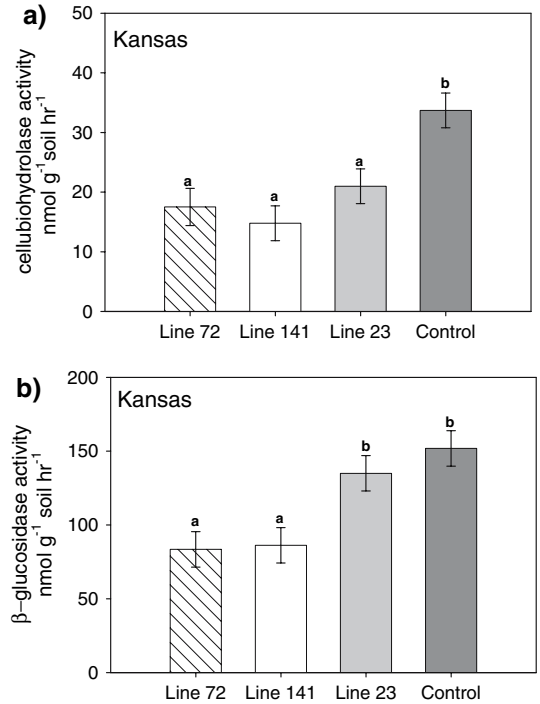

Fig. 7 The effect of genetic line on (**a**) cellubiohydrolase activity and (**b**) β-glucosidase activity in the Kansas soil. Bars represent potential enzyme activities and are presented as adjusted means ± 1 SE ($n = 8$). Different letters indicate significant differences at $P < 0.05$ by the Tukey-Kramer adjustment for multiple comparisons

likely limits microbial biomass, the CO soil has a slightly acidic pH and a sandy texture, conditions which favor fungal growth (Coyne 1999). In contrast, the KS and TX soils had high total biomass and low F:B ratios (Fig. 3c), but higher total soil C and more basic pH. The lower F:B ratios in the KS and TX soils also may have been related to differences in soil texture, as the KS and TX soils had 2 to 3 times higher clay content than the CO soil and as a result, they drained much more slowly than the sandy CO soil (personal observation), potentially creating conditions that favor bacteria over fungi (Coyne 1999).

Table 4 Summary fixed effect ANOVA table testing genetic line, soil type and the genetic line by soil type interaction for the activity of each extracellular enzyme in the Colorado, Kansas, and Texas soils

Soil Enzyme	Between Subject Effects		Within Subject Effects			
	Genetic Line Num df = 3		Soil Type Num df = 2		Genetic × Soil Num df = 6	
	F	*P*	*F*	*P*	*F*	*P*
1,4-α-glucosidase	0.23	0.88	0.26	0.77	1.93	0.08
1,4-β-glucosidase	4.73	0.004	2.35	0.10	2.20	0.05
Cellubiohydrolase	0.82	0.48	4.53	0.014	2.46	0.03
Phenol Oxidase	0.17	0.92	0.86	0.43	0.99	0.43
Peroxidase	0.26	0.86	1.78	0.17	0.58	0.74

Differences in soil texture, chemistry, and vegetation appeared to strongly influence the composition of the microbial communities (Fig. 2), making it difficult to detect any responses to the different transgenic aspen across all three soils. Variation in soil chemical and physical properties combined with differences in root production between the soil types, led us to test for compositional responses (PLFAs) to the genetic modifications in each soil type separately. Recent work with transgenic poplars has shown that microbial responses to altered plant genetics can vary between soil types (Tilston et al. 2004), but in our two-way ANOVAs, soil type generally explained 60–90% of the variance.

Although the transgenic aspen studied here had modifications aimed at reducing lignin content and/or changing S/G ratios of lignin monomers in the stem wood, these genetic modifications clearly affected root biomass production across all three soils, particularly in Line 72 (Fig. 1b). In the CO and KS soils, there were no major differences in PLFAs in response to the four genetic lines. However, in the TX soil, there were some significant differences in four different PLFAs between the aspen lines. These changes may have resulted from differences in root biomass in this soil. Root biomass was significantly lower in Line 72 relative to the control, while Line 23 had significantly more root biomass (Fig. 1b). Increases or decreases in root biomass could have affected the abundances of one or more groups of organisms (fungi, gram positive bacteria, etc.). A single shift in the abundance of one PLFA associated with one organism, would affect the relative abundances (calculated as individual PLFA/total sample PLFA) of all the measured PLFAs.

The three soils utilized in this study were from undisturbed grassland sites, and differed significantly with respect to chemical and physical properties, climatic conditions and dominant grasses (Table 2). The soil microbial communities had developed under contrasting vegetation types, soil, and climatic conditions, factors that have all been shown to affect microbial community composition (Bardgett et al. 1999b; Hassett and Zak 2005; Waldrop and Firestone 2006; Weltzin et al. 2003) and responded little to the differences in root production between the genetic lines. The speed and size of microbial community responses may depend on how much the ''new'' soil environmental conditions differ from the conditions to which they were acclimatized and the magnitude of natural variation they had previously experienced (Waldrop and Firestone 2006). Microbial communities from an open grassland, regularly experienced frequent extremes in soil temperature and moisture, and were non-responsive 2 years after being transplanted from grassland sites to spaces under oak trees (Waldrop and Firestone 2006). Similarly, the three soils in this study were from grassland sites that regularly experience large fluctuations in temperature and precipitation (Anderson et al. 2001; Zak et al. 1994); their respective microbial communities exhibited few differences between the control and three transgenic lines in the six-month greenhouse trial.

Soil and genetic effects on NLFAs and NLFA/PLFA ratios

We quantified changes in NLFAs to understand physiological responses of soil organisms to differences in soil C inputs among the four genetic lines. Neutral lipids are used as storage compounds in Eukaryotic organisms like fungi, while bacteria do not use lipids for energy storage (Bååth 2003). Generally, the neutral lipid fraction is composed of triacylglycerols, the main fungal storage compound (Bååth 2003). However, bacterial fatty acids originating from the phospholipid fraction can end up in the neutral lipid fraction as the phospholipids are degraded (Bååth 2003), thus serving as bacterial NLFAs.

The KS soil had the highest total NLFA (Fig. 4) also had the highest soil C (Table 2). However, there were soil by genetic line interactions with total NLFA (Fig. 4) as well as the abundance of individual NLFAs (Fig. 5). Total NLFA did not differ between the genetic lines in the CO soil (Fig. 4a), but within this soil the relative abundance of a bacterial NLFA increased (16:1ω7c), while the relative abundances of two fungal NLFAs (18:1ω9c and 18:2ω6,9) declined under Line 72. These changes in different groups of organisms in the CO soil seemed to offset each other, resulting in no difference in the total NLFA.

In the KS and TX soils, there were genetic line effects on total NLFA, but these effects differed between the two soils (Fig. 4b, c). Differences in total NLFA as well as differences in the relative abundances of individual NLFAs, appear to be driven by the responses of two saprophytic fungal biomarkers,

18:1ω9c and 18:2ω6,9 (Fig. 5). Across soil types there were smaller NLFA/PLFA ratios in Line 72 for both 18:1ω9c and 18:2ω6,9 (Fig. 5b) compared to the control. Lower ratios indicate reduced allocation of C to storage compounds and potentially increased physiological stress (Bååth 2003). Similarly, 18:2ω6,9 has been shown to respond differentially to decomposing lignin-modified tobacco tissue versus tissue from control plants in a brown forest soil from Scotland (Balrownie series, silty-clay loam, organic C 87 g/kg), but the addition of reduced CAD tobacco residues favored increases in relative abundance of 18:2ω6,9 (Hénault et al. 2006). These results suggested that fungi were able to more easily colonize lignin-modified plant residues and decompose them more quickly (Hénault et al. 2006).

Across all three soils we did observe decreases in the relative abundance (CO, TX) and absolute abundance (KS, TX; Fig. 5) of fungal NLFA markers in Line 72. These reductions were accompanied by increases in the relative abundance of some bacterial NLFAs (CO, KS). Changes in the relative dominance of fungi and bacteria may affect the stabilization of soil organic matter (Six et al. 2002, 2006). Previously we demonstrated that aspen with increased S/G ratios had reduced soil microbial biomass C and soil C formation (Hancock et al. 2007) although we did not characterize the composition of the microbial community. Given the results of the present study, it seems that reductions in fungally derived organic compounds (Six et al. 2006) could partially explain the decreases in soil C formation observed by Hancock et al. (2007).

Alternatively, decreased soil C formation could result from changes in tissue recalcitrance and plant C inputs (roots). Work with lignin-modified tobacco has shown that changes in the composition (ie: S/G ratio) and abundance of stem lignin could alter tissue decomposition rates (Webster et al. 2005). Specifically, C fractions from tobacco lines with antisense or partial sense transgenes for cinnamoyl CoA reductase (CCR) were more rapidly degraded in a soil incubation, likely due to the combined influence of decreased lignin content and increased access to labile C compounds (Webster et al. 2005). In our previous experiment (Hancock et al. 2007), the concentrations of lignin in root biomass were not affected by the modification to stem lignin, but we did not characterize the decomposability of the different C fractions (soluble, insoluble, lignin rich) as did Webster et al. (2005). Decreases in root production (Fig. 1b) combined with potential increases in root decomposability (decreased lignin and/or increased S/G), could lead to lower rates of soil C formation under some lignin-modified aspen.

The magnitude of the fungal biomarker (NLFA) responses to the different lines of plants appeared to increase with increasing levels of initial soil C (Table 2). There were no significant genetic effects on fungal NLFA abundance in the CO soil, but in the KS soil, which had the highest soil C levels, fungal stores of neutral lipids were 30% (18:1ω9c) and 55% (18:2ω6,9) lower in Line 72 relative to the control. While not measured, reductions in available C have been shown to favor fungi over bacteria (Hart et al. 1994), and the lines may have exerted a larger influence on C supply in high C soils compared with low C soils. Specifically, declines in root C (Fig. 1b) more negatively impacted fungal energy storage in C-rich soil environments than in C-poor soil environments.

The mechanisms driving these observations are unknown. Fungi in a low C soil already may be tolerant of C deficient conditions, so a reduction in root C input does little to alter their energy strategy. However, fungi adapted to systems like the Kansas tall-grass prairies, which are characterized by high C inputs, may have to rely on their energy stores to maintain their normal rates of metabolism during periods of low inputs. Alternatively, high C content may not translate into high C availability as was demonstrated in comparison of C mineralization rates between *P. tremuloides* and *Pinus contorta* (Giardina et al. 2001). Whatever the explanation, these results highlight that microbial community responses are often soil dependent (Bardgett et al. 1999a; Frostegård et al. 1991; Hassett and Zak 2005), which makes it difficult to generalize about the effects of plant genetic modification on soil communities.

Effects of soil type and genetic line on extracellular enzyme activity

We quantified the activities of five important extracellular enzymes involved in the degradation of common C compounds. Only cellubiohydrolase activity was significantly different between soil types (Table 4). Production and activity of extracellular enzymes is dependent on available substrate concen-

trations (Larson et al. 2002). Root biomass differed between the soil types (Fig. 1a). It was highest in the CO soil, which is also the soil with the highest cellubiohydrolase activity. There were two significant genetic line by soil type interactions for cellubiohydrolase and β-glucosidase, two enzymes involved in the decomposition of cellulose chains. Both β-glucosidase and cellubiohydrolase activity decreased in the KS soil in Lines 72 and 141 (Fig. 6a, b). Genetic modifications reduced the activity of these enzymes, indicating that across other soil types, planting of aspen with modifications to lignin biosynthesis may reduce enzyme activity.

These decreases in enzyme activity could have resulted from reduced cellulose (root) inputs or from reduced enzyme production. Although root biomass decreased significantly in Line 72 in all three soils (Fig. 1b), there was no significant relationship between root biomass and enzyme activity. Further, reductions in lignin concentrations in Lines 72 and 141 result in higher cellulose concentrations in stems (Table 1). Alternatively, decreases in the abundance of the organisms that produce cellubiohydrolase and β-glucosidase may explain why the activity of these enzymes was highest in the control treatment. Our data offer some support for this explanation. Abundance of the two fungal NLFAs associated with decomposers in the KS soil was highest in the control and Line 141 and lowest in Line 72 (Fig. 5b). The low moisture content of soil and the lignified nature of terrestrial plant residues (Lynd et al. 2002) often makes fungi the most important cellulose cleaving organisms in the soil. Declines in fungi in the KS soil could have contributed to declines in the production of extracellular cellulytic enzymes.

Conclusions

The chemical and physical properties of a soil strongly influence the composition, quantity and activity of soil microbial communities. To a lesser extent, they influence the composition, quantity and activity of extracellular enzymes. Although soil type explained much of the variance in both PLFA and NLFA data, soil microorganisms, particularly fungi, were significantly affected by the differences between the genetic lines, a result that is consistent with studies involving lignin-modified tobacco decomposition (Hénault et al. 2006; Webster et al. 2005). Genetically based traits are gaining more recognition as drivers of ecosystem processes (Madritch et al. 2006; Schweitzer et al. 2004). However, the effects of transgenic aspen on soil microbes were not consistent across three widely varying soils. The interactive effects of soil and genetic line suggest that the ecological impacts of transgenic aspen lines will be largely soil specific, but lignin modifications in tobacco have also been shown to affect fungal abundance in a silty-clay loam soil (Hénault et al. 2006). These results are consistent with the small but growing body of research on lignin-modified plants, which suggests that environmental variation has more influence on soil microbial communities than genetic modifications (Halpin et al. 2007; Tilston et al. 2004). Before transgenic aspen are released for commercial planting, field trials should be run across the range of soils in which they will be planted in order to evaluate their influence on soil organisms and belowground processes.

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