

# Plant growth, biomass partitioning and soil carbon formation in response to altered lignin biosynthesis in *Populus tremuloides*

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## Summary

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- We conducted a glasshouse mesocosm study that combined <sup>13</sup>C isotope techniques with wild-type and transgenic aspen (*Populus tremuloides*) in order to examine how altered lignin biosynthesis affects plant production and soil carbon formation.
- Our transgenic aspen lines expressed low stem lignin concentration but normal cellulose concentration, low lignin stem concentration with high cellulose concentration or an increased stem syringyl to guaiacyl lignin ratio.
- Large differences in stem lignin concentration observed across lines were not observed in leaves or fine roots. Nonetheless, low lignin lines accumulated 15–17% less root C and 33–43% less new soil C than the control line. Compared with the control line, transformed aspen expressing high syringyl lignin accumulated 30% less total plant C – a result of greatly reduced total leaf area – and 70% less new soil C.
- These findings suggest that altered stem lignin biosynthesis in *Populus* may have little effect on the chemistry of fine roots or leaves, but can still have large effects on plant growth, biomass partitioning and soil C formation.

**Key words:** carbon cycling, leaf area, lignin, photosynthesis, plant production, *Populus*, tissue quality, transgenic aspen.

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## Introduction

Plant species can have distinct effects on ecosystems, often linked conceptually to the quality of plant litter, with important implications for ecosystem function (Paul & Clark, 1996; Binkley & Giardina, 1998). Of particular interest are plant effects on soil carbon (C) process rates, which are mediated by the quality and quantity of detrital inputs and detrital losses resulting from microbial decomposition. While the biotic and abiotic controls on net primary production (NPP) and hence detritus production are well understood, and detailed models exist to describe the disappearance of surface detritus during decomposition, there is a poor understanding of how litter quality and quantity interact to

regulate soil C formation (Scott, 1998; Giardina *et al.*, 2001; Joffre *et al.*, 2001).

These knowledge gaps may be attributed to a reliance upon species differences in tissue quality (e.g. pine vs maple litter) to understand decomposition processes (Melillo *et al.*, 1982; Berg & Lundmark, 1985; McClaugherty & Berg, 1987; Aber *et al.*, 1990; Magill & Aber, 1998), and a greater focus on mass loss than on understanding the fate of detrital residues. Further, ecosystem models have focused on the quantity and dynamics of above-ground litter, but mineral soil C dynamics may be correlated most strongly with below-ground inputs (Zak & Pregitzer, 1998; Zak *et al.*, 2000; Giardina *et al.*, 2004), of which the quality is measured less frequently. In order to improve understanding of how plant inputs and tissue quality

influence soil C formation, model systems are needed that: simultaneously vary lignin quantity or quality without changing other plant properties that can influence decomposition rates; and allow for tracing the fate of C from plants into soils. The genetic alteration of lignin biosynthesis in aspen (Li *et al.*, 2003) coupled with  $^{13}\text{C}$  isotope techniques (Amundson *et al.*, 1998; Collins *et al.*, 1999; Liu *et al.*, 2003; Loya *et al.*, 2003; Pataki *et al.*, 2003; Giardina *et al.*, 2004; Hobbie *et al.*, 2004; Steinmann *et al.*, 2004; Heath *et al.*, 2005) offers a powerful approach for understanding how plants influence ecosystem processes.

Significant progress has been made in understanding lignin biosynthesis in both herbaceous and woody species through the characterization of the genes and enzymes regulating this complex process (Higuchi, 1997; Whetten *et al.*, 1998). Genetic transformation of lignin biosynthesis in trees could therefore be used to better understand how altering specific 'tissue quality' genes impacts ecosystem processes. In aspen trees, stem lignin is derived primarily from syringyl (S)–guaiacyl (G) monolignols, which are synthesized from coniferyl and sinapyl alcohols. By modifying genes that encode for enzymes along the lignin biosynthetic pathway, genetic alteration of both lignin quantity and S : G ratios can now be achieved in aspen. Specifically, transgenic aspen produced by antisense suppression of the gene encoding 4-coumarate:CoA ligase (4CL) results in up to a 45% reduction of stem lignin concentrations with an associated 15% increase in stem cellulose concentrations (Hu *et al.*, 1999), while overexpression of the gene encoding coniferaldehyde 5-hydroxylase (Cald5H) increases stem lignin S : G ratio. Simultaneous suppression of 4CL and overexpression of Cald5H can lead to a 38–52% reduction in stem lignin concentrations and a 22–64% increase in the stem lignin S : G ratio (Li *et al.*, 2003).

For our research, we used three transgenic lines of aspen (*Populus tremuloides*; Table 1) derived from a well-characterized, naturally occurring aspen clone from the northern Great Lakes region (line 271 – the control line in this study). Two of the modified lines express reduced stem lignin concentrations with or without increased cellulose concentrations or elevated S : G lignin ratios: (1) low lignin concentration (35%), normal cellulose concentration, normal S : G lignin ratio (line 23; 'low lignin/normal cellulose'); and (2) low lignin concentration (50%), high cellulose concentration (30%), slightly elevated (20%) S : G lignin ratio (line 141; 'low lignin/high cellulose'). Because syringyl-rich lignin is substantially easier to separate from cellulose during pulping for paper production than guaiacyl-rich lignin (Li *et al.*, 2000), with important economic and ecological benefits, we examined a third modified line that expresses normal lignin and cellulose concentrations but a 150% increase in the S : G ratio of lignin (line 93; 'high S : G lignin'; Table 1).

In order to understand the impacts of altered lignin biosynthesis on resource capture and growth we measured total plant leaf area, specific leaf area, leaf-level physiology and biomass accumulation for each aspen line over 8 months of growth in a glasshouse. After 8 months, we harvested plants and

**Table 1** Aspen (*Populus tremuloides*) stem tissue chemistry (Hu *et al.*, 1998) of transgenic and control plants used in glasshouse mesocosm experiment

	Plant line number			
	93	23	141	271
Gene integrated				
4CL		√	√	
Cald5H	√		√	
Stem lignin (%)	21.1	14.4	10.7	22.2
S : G of stem lignin	5.5	2.2	2.7	2.2
Stem cellulose (%)	44.7	44.8	53.3	41.4

4CL, 4-coumarate:CoA ligase; Cald5H, coniferaldehyde 5-hydroxylase; S : G, syringyl–guaiacyl ratio; Ticks in table body indicate which gene was manipulated for each transgenic aspen line.

measured above- and below-ground biomass in order to understand the impacts of altered lignin biosynthesis on biomass partitioning. To understand plant effects on soils, we measured soil microbial biomass at harvest, and, because aspen were grown in a  $\text{C}_4$  prairie soil, we used  $^{13}\text{C}/^{12}\text{C}$  isotope methods to measure how altered lignin biosynthesis in aspen affected soil C formation.

In designing this study, we developed several hypotheses about the effects of altered lignin biosynthesis on: the lignin content of leaves and fine roots (Hypothesis 1); plant growth and above- and below-ground allocation (Hypothesis 2); and given Hypotheses 1 and 2, below-ground C cycling and soil C formation (Hypothesis 3). For Hypothesis 1, we predicted that alteration of lignin quality or quantity in the stems of aspen plants would result in parallel changes in leaf and fine root lignin – the tissues that dominate detritus return to ecosystems in early stages of stand development in forests. For Hypothesis 2, we predicted that in low lignin lines, the reduced C cost of lower lignin production would result in more C being available for plant growth (Herms & Mattson, 1992) such that low lignin aspen (lines 23 and 141) would accumulate more biomass than control (271) and high S : G lignin (93) lines (Hypothesis 2a). We further predicted that high S : G lignin aspen (93) would grow more slowly than other lines (Hypothesis 2b) because of the higher C costs of syringyl production (Amthor, 2003). For Hypothesis 3, we predicted that the combination of lower lignin concentration in roots (Hypothesis 1), which would lead to faster root decomposition rates but greater root-derived detritus to soil (Hypothesis 2a), would lead to little difference in rates of soil C formation between low lignin (23 and 141) and control (271) lines (Hypothesis 3a). Further, because the syringyl component of plant lignin is more reactive than the guaiacyl component, high syringyl lignin should decompose faster than lignin with a normal structure (Hedges *et al.*, 1985; Chefetz *et al.*, 2000). As a result, we predicted that aspen with high S : G lignin (Hypothesis 1) combined with reduced below-

ground inputs (Hypothesis 2b) would result in the lowest formation rates of aspen-derived soil C (Hypothesis 3b).

## Materials and Methods

### Soil collection and mesocosm construction

Approximately 250 kg of mineral soil was collected from a  $C_4$ -dominated grassland site located at Konza Prairie Biological Station, KS, USA. Soils at Konza Prairie Biological Station are described by Zak *et al.* (1994) as silty clay loams. Before sampling in May 2004, plants and the surface mineral soil to 5 cm depth were removed. The underlying soils were sampled to a depth of 30 cm. Soils were immediately shipped by express mail to Houghton, MI, USA, where soils were twice sieved through a 4-mm mesh to remove roots and rocks and then homogenized by mixing.

### Transgenic aspen

An aspen xylem-specific promoter (Pt4CL1P)-GUS binary plasmid DNA was used as a module for creating transgenic constructs (Harding *et al.*, 2002). Antisense Pt4CL1 or sense LsCald5H cDNA replaced the GUS fragment of the Pt4CL promoter and were mobilized individually into *Agrobacterium* strain C58/PMP90 to create two engineered *Agrobacterium* strains for transforming aspen. Standard protocols for *Agrobacterium*-mediated plant transformation and regeneration were used (Tsai *et al.*, 1994; 1998; Ho *et al.*, 1998; Hu *et al.*, 1999). Individual plants expressing one of three transformations were distinguished using polymerase chain reaction (PCR) screening techniques (Li *et al.*, 2003): (1) antisense suppression of genes encoding 4-coumarate-CoA ligase (4CL; line 23); (2) overexpression of genes encoding coniferaldehyde 5-hydroxylase (Cald5H; line 93); or (3) suppression of 4CL and overexpression of Cald5H (line 141). Small differences in suppression/overexpression resulted in separate lines that were micropropagated and maintained in the glasshouse. In March 2004, approx. 30 individuals per line were micropropagated for this study.

Rooted micropropagates were transferred from sterile culture to Konza soils (approx. 230 g) in May 2004 and maintained in mist chambers for *c.* 3 wk. Fifteen plants per line were repotted in larger 2.5-l pots containing approx. 2080 g wet weight of Konza soil and then transferred to glasshouse benches in June 2004. Aspen were grown under natural light conditions throughout July, after which we supplemented light to maintain a 16-h photoperiod from August to December. Plants were watered daily to soil moisture capacity with an automated irrigation system. Complete fertilizer (nitrogen (N)–phosphorus (P)–potassium (K); macronutrient + micronutrient) was applied at a rate of 0.08 g N, 0.03 g P and 0.03 g K per plant every 2 wk. Eight to 10 of the best plants per line were selected for intensive measurements throughout the growing season. In December 2004, eight plants per line

were harvested for total biomass (leaves, stems and roots), total leaf area and tissue chemistry. Subsamples of soil were collected at harvest for soil C and microbial biomass.

### Tissue chemistry

Subsamples of lower stem, mid canopy leaves, and fine roots (< 1 mm) were ground with a ball mill grinder and analysed for C,  $\delta^{13}C$ , and N using an elemental analyzer (Costech 4010, Costech Analytical Technologies, Inc., Valencia, CA, USA) connected to the Isotope Ratio Mass Spectrometer (Delta<sup>PLUS</sup>, Finnigan MAT, Bremen, Germany) by a ConFlo III interface (Finnigan MAT). To determine tissue lignin concentrations, we used a modified Klason method based on work by Booker *et al.* (1996). Soluble C compounds and proteins were first removed by extracting 50–100 mg of tissue sample three times with 1 ml of 50% methanol solution, two times with 0.8 ml of methanol:chloroform–water (2 : 1 : 0.8), and two times with 0.8 ml phenol–acetic acid–water (2 : 1 : 0.9). Samples were incubated overnight at 4°C in the second phenol–acetic acid–water extract. After incubation, samples were centrifuged at 1415 g for 5 min and washed once with 0.8 ml of phenol–acetic acid–water (2 : 1 : 0.8) and five times with 1 ml of ethanol. Samples were then oven-dried at 70°C and stored in a desiccator. Dry samples were acid hydrolysed in 72%  $H_2SO_4$  for 1 h at room temperature, diluted to 3%  $H_2SO_4$  and then heat digested for 4 h. Digested samples were filtered, and the acid-insoluble residues were dried and weighed to determine lignin concentration.

### Photosynthesis

Maximum photosynthetic rates ( $A_{max}$ ) were measured on 10 plants during days 57–60 of the experiment, and on eight plants on days 74–76 and days 140–142. Measurements were made using a LiCor 6400 portable photosynthesis system (LiCor, Lincoln, NE, USA). At least two plants of each treatment were sampled each day and plants from different treatments were sampled randomly to minimize confounding treatment effects due to variations in weather conditions and diurnal patterns. The  $A_{max}$  value was measured on the youngest fully expanded leaf of each plant. Light was controlled with a red–blue light-emitting diode and light levels were set at a photosynthetic photon flux density of 1200  $\mu mol m^{-2} s^{-1}$ ; relative humidity was set at 55–65% by a combination of removing water vapor from incoming air and rehumidification during transpiration. Temperature was not regulated, with measured glasshouse temperatures ranging from 27 to 29°C on days 57–60, 24–29°C on days 74–76 and 24–29°C on days 140–142.

### Plant growth

Plant height and the length and width of every leaf of the plants selected for harvest were measured biweekly from June

2004 to September 2004. From September 2004 until harvest in December 2004, plant height and leaf lengths and widths were measured biweekly to monthly. In October 2004, 15 leaves per line were randomly harvested from top, middle and lower canopies for chemistry analyses, with no more than two leaves sampled per plant. The area of each harvested leaf was measured using a LiCor 3100C leaf area meter.

In December 2004, plants were harvested, and tissues were separated into leaves, roots and stems. At this time, the height and total leaf area of each plant was measured. For total plant leaf area, we measured the area of all leaves of each plant with a LiCor 3100C leaf area meter. Linear allometric equations were then developed for each transgenic line between leaf area of individual leaves and the product of leaf length and width ( $X$ ). The nondestructive leaf length and width measurements were used to reconstruct individual and whole-plant leaf areas over the 8-month study. The allometric equations used were:  $0.68 \times X =$  line 93 leaf area ( $R^2 = 0.96$ ),  $0.64 \times X =$  line 23 leaf area ( $R^2 = 0.98$ ),  $0.64 \times X =$  line 141 leaf area ( $R^2 = 0.97$ ) and  $0.64 \times X =$  line 271 leaf area ( $R^2 = 0.99$ ). Specific leaf area (SLA) of individual leaves at the time of harvest was also calculated from harvested leaves for each line as total leaf area divided by total leaf dry biomass. SLA is important because reduced SLA decreases the amount of leaf area available for light interception per unit of leaf mass, and in some plants, SLA may be positively correlated with relative growth rates (Poorter & Remkes, 1990; Reich *et al.*, 1998).

To quantify root biomass at harvest excess soil was shaken gently off of the root mass, and the root mass and any remaining soil were placed in a deionized water bath to completely remove all soil from roots. Most soil was removed from roots before washing, and from this soil, subsamples were taken for C and microbial biomass analyses. Roots were handpicked from soil subsamples to ensure that all visible fine roots were removed.

All tissues and soil subsamples were placed in a  $-80^\circ\text{C}$  freezer. Plant tissue samples were then lyophilized and weighed to obtain dry weights of each tissue component. Subsamples of roots from each individual plant were ashed in a countertop furnace at  $450^\circ\text{C}$  to determine mineral content. Thus, root biomass is expressed on a mineral-free basis.

### Soil microbial biomass

Microbial biomass C was determined by fumigation-extraction according to Vance *et al.* (1987). Briefly, approx. 25 g (wet weight) of root-free soil was extracted for 2 h with 100 ml of  $\text{K}_2\text{SO}_4$ , while a second sample of similar mass was fumigated with alcohol-free chloroform for 24 h before extraction. Total organic C (TOC) in the extracts was determined on a TOC 5000 (Shimadzu Corporation, Kyoto Japan). Microbial C was calculated as the difference between the TOC in fumigated samples minus TOC of unfumigated samples. For comparisons with other estimates of microbial biomass, microbial biomass

C was calculated using an extraction efficiency coefficient ( $K_{\text{ec}}$ ) of 0.35 (Sparling *et al.*, 1990).

### Soil carbon formation

Per cent soil C (%C) and  $\delta^{13}\text{C}$  were determined using ground subsamples of the initial grassland soil sampled from each mesocosm at the time of mesocosm construction and of soils collected at harvest from each mesocosm. The percentage soil C derived from aspen plants was estimated using the following equation:

$$\%C \text{ from } C_3 = (\delta - \delta_0 / \delta_1 - \delta_0) \times 100 \quad \text{Eqn 1}$$

( $\delta$  is the  $\delta^{13}\text{C}$  of the soil sample;  $\delta_0$  is the initial  $\delta^{13}\text{C}$  of soil from  $C_4$  grassland; and the  $\delta_1$  is the  $\delta^{13}\text{C}$  of  $C_3$  plant material (aspen roots and leaves)). No leaf litter accumulated in the pots during the experiment as all senesced leaf material was immediately collected to estimate above-ground plant production.

### Statistical analyses

We used a one-way univariate ANOVA with genetic line as the main fixed effect to assess treatment differences among lines in above- and below-ground C process rates. The effect of potting date was examined in all analyses and in no case was it found to be significant, and so was excluded from final models. All analyses were performed with the GLM univariate procedure in SPSS (SPSS, Inc., Chicago, IL, USA), and an  $\alpha = 0.05$  was used to protect against Type I errors. Because height and leaf area were measured for the same individuals across the experiment, we performed a repeated measures analysis of variance to examine height and leaf area differences among lines. For leaf photosynthesis measures, eight plants per line were randomly selected per date. These data were analysed using a univariate analysis of variance with date and genetic line as independent variables. We used a least significant difference *post hoc* test to determine significant differences among genetic lines.

## Results

### Lignin biosynthesis and tissue chemistry

Stem lignin concentrations were 17–24% lower in the low lignin aspen (line 23 and 141) compared with control and high S : G lignin aspen (271; Table 2). Stem lignin concentrations in high S : G lignin plants did not differ from the control (Table 2). The lignin concentrations of leaves and roots of the three transgenic aspen lines did not differ from each other or from control aspen (Table 2).

The C concentration of stem tissue among the four lines of aspen followed trends of lignin concentrations in stem tissue (Table 2) such that low lignin aspen (line 23 and 141) had

**Table 2** Mean carbon (C) and nitrogen (N) concentrations of leaves, stems, and roots found in three transgenic lines of aspen (*Populus tremuloides*) with altered lignin biosynthesis and one control line of aspen

	Lignin (%)			C (%)			N (%)		
	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf	Root
93 (high S : G lignin)	36.3 <sup>ab</sup> (1.8)	19.2 <sup>a</sup> (1.2)	38.9 <sup>b</sup> (1.7)	45.9 <sup>a</sup> (0.3)	49.3 <sup>a</sup> (0.2)	48.2 <sup>a</sup> (1.1)	1.1 <sup>b</sup> (0.13)	2.7 <sup>a</sup> (0.12)	1.8 <sup>a</sup> (0.12)
23 (low lignin/normal cellulose)	29.8 <sup>c</sup> (1.5)	21.4 <sup>a</sup> (0.6)	42.2 <sup>b</sup> (1.3)	44.9 <sup>b</sup> (0.1)	47.7 <sup>a</sup> (0.7)	47.9 <sup>a</sup> (0.3)	0.6 <sup>a</sup> (0.09)	2.4 <sup>b</sup> (0.28)	1.5 <sup>b</sup> (0.12)
141 (low lignin/ high cellulose)	32.5 <sup>bc</sup> (1.3)	21.9 <sup>a</sup> (1.6)	43.8 <sup>b</sup> (2.0)	45.2 <sup>b</sup> (0.3)	48.0 <sup>a</sup> (0.5)	44.1 <sup>a</sup> (2.5)	0.7 <sup>a</sup> (0.13)	2.3 <sup>b</sup> (0.26)	1.5 <sup>b</sup> (0.14)
271 (control)	39.2 <sup>a</sup> (1.8)	21.3 <sup>a</sup> (1.0)	42.3 <sup>b</sup> (1.2)	45.9 <sup>a</sup> (0.1)	47.7 <sup>a</sup> (1.2)	46.4 <sup>a</sup> (1.3)	0.6 <sup>a</sup> (0.04)	2.1 <sup>c</sup> (0.16)	1.5 <sup>b</sup> (0.09)

S : G, syringyl–guaiacyl ratio. Values in parenthesis are standard errors. Statistical *post hoc* comparisons were made among lines for each tissue quality characteristic (lignin, C and N) and significance was established at  $P < 0.05$ .

**Table 3** Mean light-saturated photosynthetic rates ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) of transgenic and control aspen (*Populus tremuloides*)

Growth day	93 (high S : G lignin)	23 (low lignin/normal cellulose)	141 (low lignin/high cellulose)	271 (control)
45	12.3 (0.7)	13.3 (0.6)	13.9 (0.5)	12.2 (0.7)
75	15.5 (0.6)	16.3 (0.4)	16.9 (0.4)	16.3 (0.5)
135	12.1 (1.6)	14.4 (0.6)	14.6 (0.9)	14.3 (0.9)

S : G, syringyl–guaiacyl ratio. Growth day refers to days since start of the experiment. Values in parenthesis are standard errors. Line 141 showed significantly higher rates of light saturated photosynthesis than line 93 in *post hoc* comparisons ( $P = 0.02$ ). No other comparisons were significant.

significantly lower C concentrations in stems compared with control and high S : G lignin plants. The C concentration of leaves and roots did not differ significantly among the four lines of aspen.

In contrast to C concentrations, N concentrations of high S : G lignin aspen (93) leaves and roots were 20% higher than those of control plants, while stem N concentrations were nearly double those of the other lines (Table 2). Low lignin lines (23 and 141) did not differ from control aspen (271) in stem and root N concentrations. However, low lignin aspen had 10% higher concentrations of N in leaf tissues.

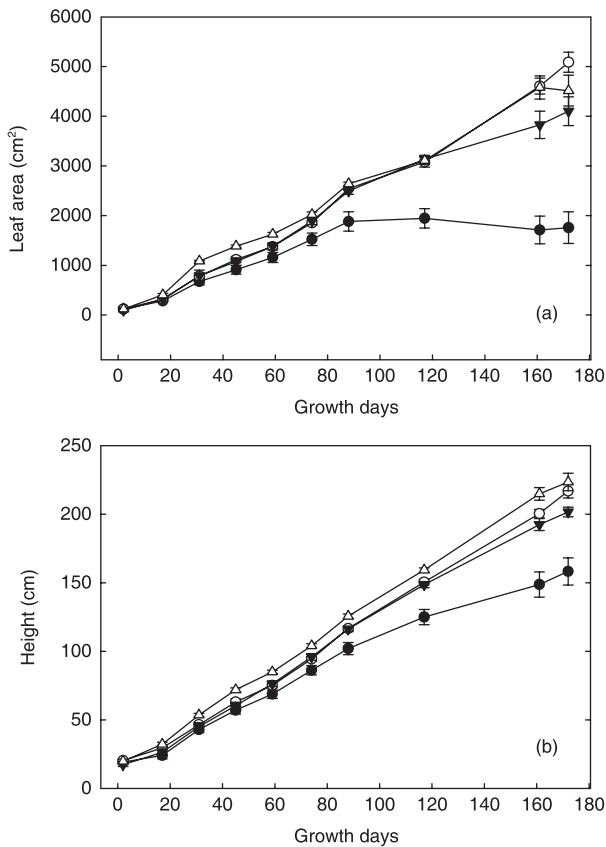
#### Plant growth – $A_{\text{max}}$ , specific leaf area, plant leaf area and height

Aspen with high S : G lignin (93) had the lowest rates of light-saturated photosynthesis ( $A_{\text{max}}$ ) throughout the experiment, but differences were significant only with low lignin/high cellulose (141) plants (Table 3,  $P = 0.02$ ), which maintained the highest rates of  $A_{\text{max}}$  throughout the experiment. No other  $A_{\text{max}}$  comparisons resulted in significant differences.

Specific leaf area varied widely across lines with the lowest mean value for high S : G lignin plants (93;  $122 \pm 6.5 \text{ cm}^2 \text{ g}^{-1}$ ) and the highest for aspen with low lignin/normal cellulose

(23;  $169 \pm 2.4 \text{ cm}^2 \text{ g}^{-1}$ ), followed by low lignin/high cellulose plants (141;  $158 \pm 3.6 \text{ cm}^2 \text{ g}^{-1}$ ) and control plants (271;  $160 \pm 1.9 \text{ cm}^2 \text{ g}^{-1}$ ). Mean SLA for high S : G lignin plants (93) was significantly lower than each of the other lines ( $P < 0.01$ ). There were strong trends towards higher SLA for low lignin/normal cellulose (23) compared with low lignin/high cellulose lines (141;  $P = 0.071$ ) and control plants (271;  $P = 0.12$ ). The two low lignin lines did not differ.

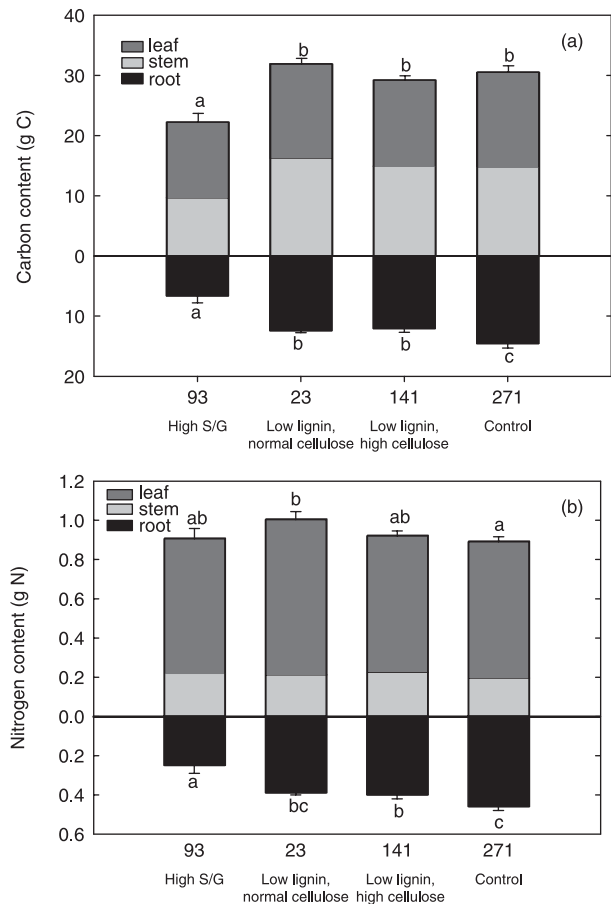
Total leaf area for the 8-month-old plants ranged from a mean of  $1759 \pm 901 \text{ cm}^2$  for high S : G lignin plants (93) to  $5087 \pm 572 \text{ cm}^2$  for low lignin/normal cellulose plants (23), with low lignin/high cellulose (141) and control (271) plants accumulating  $4102 \pm 814 \text{ cm}^2$  and  $4515 \pm 879 \text{ cm}^2$  of total leaf area, respectively. At harvest, total leaf area for plants with high S : G lignin (93) was significantly lower than the other three lines (Fig. 1a;  $P < 0.01$ ). Low lignin/normal cellulose (23) plants accumulated significantly more leaf area than low lignin/high cellulose (141) plants ( $P = 0.02$ ), while differences between control and low lignin (23 and 141) plants were not significant. Repeated measures analyses revealed a significant line–time interaction ( $P < 0.01$ ) for the experiment, and *post hoc* comparisons revealed that patterns of leaf area accumulation for high S : G lignin plants were significantly lower than the three other lines throughout the experiment.



**Fig. 1** (a) Plant total leaf area and (b) height of transgenic and control aspen (*Populus tremuloides*) throughout the experiment which started in May 2004 and ended in December 2004. Values are means  $\pm$  SE ( $n = 8$ ). Closed circles, high syringyl–guaiacyl (S : G) lignin (93); open circles, low lignin, normal cellulose (23); closed triangles, low lignin, high cellulose (141); open triangles, control (271).

Unlike the harvest results, repeated measures analyses showed that total leaf area for control plants was not significantly different from the low lignin lines.

Patterns for height growth generally matched those of leaf area accumulation (Fig. 1b), but there were subtle differences. At harvest, mean height was highest for control plants (271;  $224 \pm 18$  cm) and lowest for high S : G lignin plants (93;  $158 \pm 28$  cm). Low lignin/normal cellulose (23) plants averaged  $217 \pm 14$  cm, while low lignin/high cellulose (141) plants averaged  $202 \pm 10$  cm. In line with leaf area data, height growth for aspen with high S : G lignin (93) was, on average, 30% lower than the other three lines (Fig. 1b), with all differences being highly significant ( $P < 0.01$ ). At harvest, control plants were significantly taller than the low lignin/high cellulose (141) plants ( $P = 0.03$ ) but not low lignin/normal cellulose (23) plants. Growth in height did not differ between the two low lignin lines (23 and 141;  $P = 0.12$ ). Repeated measures analyses revealed that differences in height observed at the



**Fig. 2** Distribution of (a) total carbon (C) and (b) total nitrogen (N) in above-ground and below-ground pools in transgenic and control aspen (*Populus tremuloides*) after 8 months of glasshouse growth. Significance was established at  $P < 0.05$ , and *post hoc* comparisons are displayed for both above-ground (leaves plus stems) and root C and N content.

end of the 8-month study were largely present throughout the experiment. *Post hoc* comparisons following the repeated measures analyses showed that throughout the study high S : G lignin plants were significantly shorter than the other three lines ( $P < 0.01$ ), and that control line plants were taller than low lignin/high cellulose plants (141). The difference between low lignin lines (23 and 141) across the study period was not significant.

### Plant growth – accumulation and partitioning of biomass, carbon and nitrogen

Overall, high S : G lignin plants had 33% less total plant C than the other aspen lines (Fig. 2a;  $P < 0.01$ ), which did not differ from each other. Aspen with high S : G lignin (93) accumulated the lowest total stem, leaf and root C (Fig. 2a;  $P < 0.01$ ) compared with other lines of aspen. Low lignin aspen (23 and 141) accumulated similar total stem and total leaf C as control aspen. However, low lignin/normal cellulose (23)

aspen accumulated 15% less total root C than control aspen (Fig. 2a;  $P=0.056$ ), while low lignin/high cellulose (141) aspen accumulated 17% less total root C than control aspen (Fig. 2a;  $P=0.03$ ).

High S : G lignin plants (93) were significantly smaller than the other lines of aspen, such that despite higher tissue N concentration, total N content of high S : G lignin plants (93) were still significantly lower than control (271;  $P=0.02$ ), low lignin/normal cellulose (23;  $P<0.01$ ), and low lignin/high cellulose (141;  $P=0.04$ ) plants (Fig. 2b). Above-ground N content (leaves plus stems) did not differ significantly among the S : G lignin plants (93), low lignin/high cellulose plants (141) and control plants (271; Fig. 2b). However, low lignin/normal cellulose aspen (23) allocated roughly 5% more N to above-ground tissues compared with control (271) aspen (Fig. 2b;  $P<0.05$ ). High S : G lignin plants (93) had significantly lower total root N than the other three lines of aspen (Fig. 2b;  $P<0.01$ ). In line with lower root biomass, low lignin/normal cellulose plants (23) showed a near-significant reduction in root N content ( $P=0.06$ ) compared with control aspen (Fig. 2b) and there were also trends towards lower root N content for low lignin/high cellulose (141) plants ( $P=0.14$ ) compared with control plants.

#### Soil microbial biomass and soil carbon formation

Average soil microbial biomass C of the control aspen mesocosms ( $271, 5.0 \pm 0.4 \text{ mg C g}^{-1} \text{ soil C}$ ) was similar to that of low lignin aspen mesocosms (23,  $4.3 \pm 0.1 \text{ mg C g}^{-1} \text{ soil C}$  and 141,  $4.9 \pm 0.7 \text{ mg C g}^{-1} \text{ soil C}$ ). However, control soil microbial biomass C was nearly double that of high S : G lignin mesocosms (93;  $2.9 \pm 0.2 \text{ mg C g soil C}^{-1}$ ;  $P<0.05$ ).

We used the original  $\delta^{13}\text{C}$  signature of premesocosm soil ( $-14.9 \pm 0.04\text{‰}$ ), the  $\delta^{13}\text{C}$  signature of the soil at harvest (Fig. 3a) and the average  $\delta^{13}\text{C}$  signature of aspen roots ( $-28.6 \pm 0.2\text{‰}$ ) in a two-source isotope mixing model (Eqn 1) to quantify soil C formation rates. Differences among lines in the  $\delta^{13}\text{C}$  signature of bulk soils taken at the time of harvest (see Fig. 3a) indicate that altered stem lignin biosynthesis influenced soil C formation in our aspen mesocosms. In just over 8 months, mesocosms with unmodified control trees accumulated 50–75% more new soil C than low lignin lines (23 and 141;  $P<0.01$ ) and 200% more new soil C than plants with high S : G lignin (93;  $P<0.01$ ; see Fig. 3b).

#### Discussion

The role of individual genes in the regulation of plant–environment interactions is an emerging field within ecology (Jackson *et al.*, 2002). Important advances have included identification of genes regulating disease resistance (Bent *et al.*, 1994; Bent, 1996), ecophysiology (Martienssen, 1998; Byrne *et al.*, 2000; Jackson *et al.*, 2002; Bergmann *et al.*, 2004)

and plant nutrition (Gallais & Hirel, 2004; Franco-Zorilla *et al.*, 2004). While the focus of these studies has been on the genetic basis for plant adaptations to the environment, the effects of individual genes on ecosystem processes have received much less attention (Pilate *et al.*, 2002). By combining  $^{13}\text{C}$  isotope techniques with aspen trees expressing modifications to lignin biosynthesis, we were able to examine how alteration of individual genes affected plant growth and physiology, and in turn below-ground ecosystem processes.

#### Lignin biosynthesis and tissue chemistry

We hypothesized that alterations of stem lignin concentrations would result in changes in the lignin concentration of leaves and fine roots (Hypothesis 1). However, no differences among transgenic aspen in stem lignin concentrations were observed in fine roots and leaves (Table 2), indicating that transgenic alterations to plant stem tissue may not result in parallel changes to leaves and fine roots. It is well established that lignin formation and resulting structure varies widely across cell types within a plant (Whetten *et al.*, 1998) and that controls on lignin biosynthesis may be tissue specific (Lagrimini *et al.*, 1997). Further, lignin biosynthesis may occur along several pathways (Whetten & Sederoff, 1995; Whetten *et al.*, 1998), with molecular and enzymatic regulation of lignin biosynthesis in one tissue type (e.g. stem wood) potentially differing from that in another tissue (e.g. leaves and fine roots).

Stem lignin concentrations for the four lines examined here were higher than previously reported values for transgenic aspen with altered lignin biosynthesis (Hu *et al.*, 1998, Table 1). However, we did not remove bark tissue from our stem samples before lignin analysis, while Hu *et al.* (1998) removed bark before stem tissue analyses. Because bark is rich in polyphenolic compounds, our lignin numbers may be higher than if only wood had been examined.

Across lines, leaf lignin concentrations averaged  $21 \pm 0.5\%$ . This value is similar to lignin concentrations previously observed in field grown trees and now widely incorporated into ecosystem models simulating northern forests (Running & Gower, 1991, lignin concentration fixed at 22%). These values are slightly lower than lignin concentrations reported in leaf litter studies (Melillo *et al.*, 1982; Aber *et al.*, 1990; Berg & Ekbohm, 1991), though higher concentrations (25% to 30%) in leaf litter studies may relate to leaf mass loss resulting from retranslocation or leaching of labile compounds out of leaves during and after senescence (Pregitzer *et al.*, 2006).

Our root lignin concentrations averaged  $42\% \pm 0.9$ , which is on the high end of published estimates for broadleaved species. Camiré *et al.* (1991) reported fine root lignin concentrations of 25.4% for a hybrid poplar (*Populus nigra* L.  $\times$  *Populus trichocarpa* Torr & Gray), which matches mean root lignin concentrations identified in reviews of root tissue quality (Silver & Miya, 2001; broadleaf mean lignin concentration = 28%; Chen *et al.*, 2002, broadleaf mean = 22%; Gordon &

Jackson, 2000, temperate broadleaf mean = 26%). Other studies, however, have reported root lignin concentrations of 35% (Puttsepp, 2004) and 49% (Hendricks *et al.*, 2000). Differences across studies could result from differences in root age or size, growth environment, methodology, or a combination of these factors.

Lower C concentrations in the stem tissue of low lignin lines (23 and 141) compared with control aspen may relate to the fact that lignin has a high C content, such that a reduction in lignin concentrations in stem tissue could lead to a reduction in C concentrations. In line with the lack of difference in lignin concentrations of leaves and fine roots, we observed no differences in the C concentrations of leaves and fine roots among the four lines of aspen. Low lignin aspen (23 and 141) did have higher concentrations of N in leaf tissues compared with control aspen, and for low lignin/high cellulose (141) aspen, higher N concentration of leaves were associated with higher  $A_{\max}$  values (Table 3), perhaps indicating that higher N concentrations in low lignin lines were not caused by luxury consumption but rather by increases in chlorophyll. By contrast, the significantly higher N concentrations in leaves, roots, and stems of high S : G lignin plants compared with control plants was likely a result of luxury consumption of N in high S/G plants (93), as shown by overall low rates of light saturated photosynthesis compared with the other lines.

### Plant physiology, growth and carbon allocation

Across plants and lines,  $A_{\max}$  values of transgenic and control aspen from this study are within the range of  $A_{\max}$  rates reported for aspen in the Great Lake region (Whitehead & Gower, 2001), and they overlap with ambient CO<sub>2</sub> treatment  $A_{\max}$  values reported by Takeuchi *et al.* (2001) for field-grown, mid-canopy leaves of control (271) trees (10–20  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ) at the Aspen FACE facility in Rhinelander, WI, USA. The range of  $A_{\max}$  for upper canopy leaves for the 3-yr-old field-grown aspen were *c.* 3  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  higher than the mid-canopy leaves, but photosynthetic photon flux density (PPFD) reaching upper canopy leaves of field grown trees (maximum light of > 1500  $\mu\text{mol PPF} \text{ m}^{-2} \text{ s}^{-1}$ ) is higher than maximum PPFD encountered in our glasshouse (maximum light of *c.* 600  $\mu\text{moles PPF} \text{ m}^{-2} \text{ s}^{-1}$ ).

Our hypothesis that low lignin/normal cellulose (23) and especially the low lignin/high cellulose plants (141) would allocate more C to growth (Hypothesis 2a) was not supported by our results. There are a limited number of studies that have attempted to quantify the effects of altered lignin biosynthesis on the growth of aspen, but results have been conflicting. In an earlier glasshouse study, Hu *et al.* (1999) observed larger leaves and longer internodes for transgenic aspen expressing a 45% reduction in stem lignin concentration and a 15% increase in stem cellulose concentration compared with the unaltered control aspen. Notably, these authors did not examine below-ground biomass. By contrast, our results show that

significant reductions in stem lignin concentrations in *Populus* may not substantially or even positively alter leaf area, height growth, above-ground or total biomass accumulation, particularly if the S : G ratio of lignin is also increased. Pilate *et al.* (2002) found no differences in height growth between 4-yr-old field grown control aspen and aspen with slightly reduced stem lignin concentrations, though this study did not examine increased S : G ratio plants. Given the greatly reduced growth performance of high S : G lignin plants (93), the modest increase in S : G ratio in low lignin/high cellulose plants (141) may explain the reduced growth performance of this line.

In Hypothesis 2b, we predicted that high S : G lignin (93) aspen would show reduced growth compared with the other lines because of higher C costs for syringyl production (Amthor, 2003). The results of this study support our hypothesis as aspen with high S : G lignin had significantly reduced height, leaf area, and above and below-ground biomass. Syringyl monolignols have a 17% higher molecular mass than guaiacyl and contain 132 g C mol<sup>-1</sup> while guaiacyl monolignols contain 120 g C mol<sup>-1</sup>. Lignin biosynthesis from syringyl monolignols through the most efficient biosynthetic pathway has a higher glucose requirement than lignin biosynthesis from guaiacyl monolignols (Amthor, 2003). Overall, however, these differences are too small to account for the observed reduction in growth for high S : G plants. While genetic alteration of lignin quality in high S : G lignin plants (93) resulted in a large reduction in leaf area, SLA and photosynthetic rates, with impacts on growth, the molecular basis for these changes remain unclear.

Carbon allocation patterns to above and below-ground tissues differed across lines, though we are uncertain of the mechanism controlling these differences and whether changes represent the direct or indirect effects of altered lignin biosynthesis. Nonetheless, genetically related changes in above- and below-ground plant growth have important implications for industrial plantings of reduced lignin plants. In earlier studies, differences in root architecture in conifers (Coutts *et al.*, 1999) and reduced biomass partitioning to roots in *Populus* (Harrington & DeBell, 1996) increased susceptibility to wind throw damage. In our study, if observed reductions in biomass partitioning to roots in low lignin lines persists in field settings, the commercial benefits of reduced lignin in stem wood and higher stem wood production could be offset by the risk of increased loss to wind throw. We caution that C allocation to roots and root to shoot ratios can change with stand development, and C allocation to roots in pots may differ from allocation under field conditions. Clearly, longer-term studies are needed to confirm whether observed differences among control and low lignin lines persist, increase or disappear with time.

### Soil microbial biomass and carbon formation

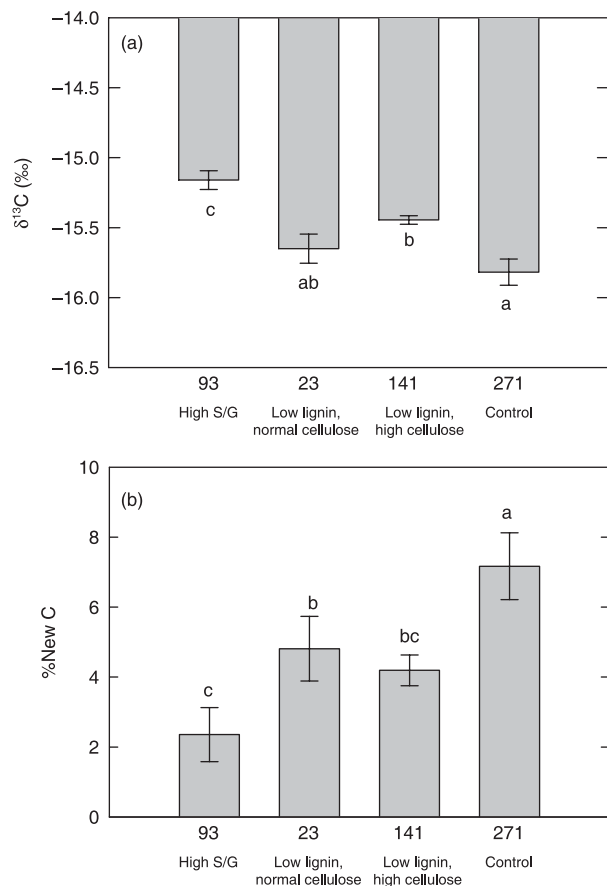
Across plants, the values of microbial biomass in transgenic and control aspen from this study are on the high end of the



range of published microbial biomass values for soils from the Konza Prairie where our soils were collected. Vinton & Burke (1997) reported microbial biomass values for field moist Konza soils of  $0.24 \text{ mg biomass C g}^{-1} \text{ soil}$ , while Zak *et al.* (1994) reported  $1.47 \text{ mg biomass C g}^{-1} \text{ soil}$ , for field moist soils. Following establishment of prairie vegetation, Baer *et al.* (2003) reported microbial biomass C-values that ranged from 1.78 to  $2.84 \text{ mg biomass C g}^{-1} \text{ soil}$  for field moist Konza prairie soils. Though differences or similarities are difficult to interpret, the mean values we observed for Konza soils in our glasshouse experiment may indicate that below-ground C supply was higher in our mesocosm pots than in field environments where moisture and nutrient availability are likely to be lower. Given the optimal growth conditions for plants and microbes, differences are not surprising. Overall, patterns of microbial biomass across lines could in part be explained by a significant relationship between total root C and microbial biomass C ( $\text{MB} = \text{Root C} \times 0.22 + 1.5$ ;  $R^2 = 0.25$ ,  $P = 0.04$ ), indicating that the mechanisms driving reduced microbial biomass may include reduced below-ground inputs that relate to reduced root biomass.

We originally hypothesized that low lignin aspen would have higher below-ground production than control aspen (Hypothesis 2a) but combined with high microbial respiration of labile low lignin roots (Hypothesis 1), these lines would accumulate similar amounts of new soil C (Hypothesis 3a). However, altering lignin biosynthesis in stem tissue did not affect the lignin concentrations of leaves and fine roots, and the low lignin lines did not allocate more biomass below-ground than control lines. Instead we observed modestly lower root biomass C in low lignin aspen (23 and 141), which may have contributed to lower accumulation rates of aspen-derived soil C relative to control aspen. The reason for the observed shift in biomass partitioning in low lignin lines is unclear. However, the changes we observed in biomass partitioning, below-ground allocation, and processing of below-ground inputs indicate that ecosystem responses to genetic alterations are more complex than could be predicted from information on the function of single genes. For example, in line with hypothesis 3b, increasing the S/G of lignin (line 93) reduced growth, leaf area and physiology, below-ground biomass allocation, and ultimately rates of soil C formation in soils (Figs 1–3).

Overall, the detrital decomposition pathways leading to soil C formation are very complex (Stevenson, 1994), with nonlignin-based pathways potentially exerting an important influence on formation rates. Variation in rates of soil C formation was most strongly related to total root C (soil C formation =  $0.44 \times (\text{total root C}) - 0.48$ ;  $R^2 = 0.36$ ;  $P < 0.01$ ), indicating that below-ground inputs (rhizodeposition, fine root and mycorrhizal turnover) can exert an important influence on soil C formation, independent of tissue quality effects. While most of the variation in this relationship is not explained, these results are in line with previous field studies



**Fig. 3** (a) Soil  $\delta^{13}\text{C}$  values and (b) estimates of percentage new carbon (C) derived from mixing models for transgenic and control aspen (*Populus tremuloides*) mesocosm soils after 8 months of glasshouse growth.

documenting positive correlations between soil C and root biomass (Jobbagy & Jackson, 2000), fine root turnover (Pregitzer *et al.*, 1995) or rhizosphere C flux (Giardina *et al.*, 2004).

## Conclusion

We combined  $^{13}\text{C}$  isotope-based techniques with modified aspen to examine, within a common genome, the effects of altered stem lignin concentration and/or altered lignin quality on plant performance, including leaf photosynthesis, above- and below-ground biomass growth, and leaf area growth. We also examined how changes in the plant tissue chemistry and performance led to changes in soil properties including microbial biomass, soil surface  $\text{CO}_2$  efflux and organic C formation in soil.

Changes in stem lignin concentration in the two low lignin lines (23 and 141) did not reduce lignin in fine roots or leaves, did not affect above-ground growth, but compared with the control line (271), did reduce biomass C in roots and formation rates of new soil C. By contrast, increasing stem lignin S : G ratio had a strong negative effect on leaf area, height

growth and biomass accumulation, and negatively affected soil C formation. Taken together, our results indicate that trees expressing reduced lignin content could simultaneously provide comparable growth rates while reducing the economic and environmental costs of lignin removal in the pulping process. By contrast, increasing the S : G ratio of lignin, while also of economic and environmental interest because of increased ease of lignin removal, appears to negatively affect growth of this species. Changes in susceptibility to pest and pathogens or climate events were not examined here. Clearly, any benefits of reduced lignin in wood will need to be weighed against potential reductions in rates of soil C formation and, because of changes in biomass partitioning to roots, potentially increased susceptibility to wind throw.

Overall, we show that these C<sub>4</sub> grassland soil mesocosms provide a powerful tool for assessing and screening above and below-ground plant performance and for testing basic questions in plant biology. We caution that our short-term results need to be validated in longer glasshouse studies and in field trials across a range of soil types.

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