Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees

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Because lignin limits the use of wood for fiber, chemical, and energy production, strategies for its downregulation are of considerable interest. We have produced transgenic aspen (*Populus tremuloides* Michx.) trees in which expression of a lignin biosynthetic pathway gene *Pt4CL1* encoding 4-coumarate:coenzyme A ligase (4CL) has been downregulated by antisense inhibition. Trees with suppressed *Pt4CL1* expression exhibited up to a 45% reduction of lignin, but this was compensated for by a 15% increase in cellulose. As a result, the total lignin–cellulose mass remained essentially unchanged. Leaf, root, and stem growth were substantially enhanced, and structural integrity was maintained both at the cellular and whole-plant levels in the transgenic lines. Our results indicate that lignin and cellulose deposition could be regulated in a compensatory fashion, which may contribute to metabolic flexibility and a growth advantage to sustain the long-term structural integrity of woody perennials.

Keywords: plant genetic engineering, lignin biosynthesis, 4CL, transgenic, *Populus tremuloides*

Many of society∏s fiber, chemical, and energy demands are met through the costly industrial-scale processing of wood. Secondary xylem (wood) of trees, from which pulp is derived, is composed of cellulose (β-1,4-glucan), lignin (phenolic polymer), and hemicelluloses (heterogeneous polysaccharides) in approximate proportions of 2:1:1[1]. During tree growth, cellulose microfibrils give cell walls tensile strength, and lignin encasing the cellulose microfibrils imparts rigidity to cell walls. Regardless of its importance during growth, lignin becomes problematic to postharvest, cellulose-based wood processing, because it must be separated from cellulose at enormous energy, chemical, and environmental expense. As a result, there has been long-standing incentive to develop healthy trees that accumulate less lignin and/or more extractable lignin to facilitate pulping.

Significant progress has been made in recent years toward an understanding of lignin biosynthesis (Fig. 1) through characterization of lignin biosynthetic pathway enzymes and genes from both herbaceous and tree crops[2]. Past efforts to reduce tree lignin content by downregulating genes encoding caffeate *O*-methyltransferase[3,4] or cinnamyl alcohol dehydrogenase[5] did not succeed but instead modified the lignin structure. This is in accordance with the results of downregulating these genes in transgenic tobacco plants[6–9], suggesting that neither enzyme limits lignin accumulation. However, reduction of lignin in plants was demonstrated in transgenic tobacco by suppression of phenylalanine ammonia-lyase[10,11], an enzyme that catalyzes the entry step to the phenylpropanoid pathway upstream of lignin biosynthesis. Suppressing its activity restricts overall phenylpropanoid biosynthesis, resulting in a wide range of abnormal growth phenotypes as well as reduced thioglycolate-extractable lignin. Reduced lignin content was also achieved in transgenic tobacco and *Arabidopsis* by downregulating 4CL[12–14] or cinnamoyl-coenzyme A reductase[15], but collapsed cell walls and stunted growth were reported in plants with the most severe lignin reductions (>25%)[12], (~40%)[15]. These herbaceous systems clearly illustrated the possibilities as well as the possible drawbacks of modifying secondary metabolism and lignin quantity in plants. Thus, the question from the perspective of wood biotechnology is whether lignin content can indeed be reduced without compromising the structural integrity and growth of trees.

We recently isolated two structurally and functionally distinct aspen 4CL genes, *Pt4CL1* and *Pt4CL2*, whose proteins catalyze the CoA ligation of hydroxycinnamic acids, generating activated phenolic precursors for lignin and flavonoid biosynthesis[16]. While *Pt4CL2* is expressed in stem and leaf epidermal cells and is thought to be involved in the synthesis of flavonoids, *Pt4CL1* is devoted to lignin biosynthesis in developing xylem tissue. By antisense downregulation of the lignin-specific *Pt4CL1* expression, we generated transgenic aspen trees that accumulated structurally normal lignin at substantially reduced levels. The reduced lignin was compensated for by a concomitant increase in cellulose. Our findings indicate that the deposition of these two cell wall structural components in trees may be regulated in a compensatory fashion not reported in herbaceous plants. Furthermore, cell walls were not collapsed—in fact, growth of transgenic plants was substantially enhanced.

Results

Characterization of transgenic aspen. Twenty-five transgenic aspen lines were obtained through *Agrobacterium*-mediated transformation under the selection of hygromycin. The presence of T-DNA in the genome of transgenic plants was confirmed by PCR and Southern

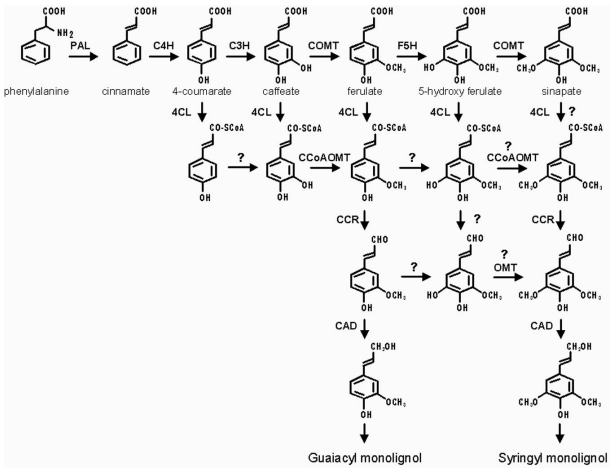

Figure 1. Biosynthetic pathways to guaiacyl and syringyl monolignols for the formation of guaiacyl:syringyl lignin in woody angiosperms. Enzymes are indicated for each reaction step. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeate *O*-methyltransferase; F5H, ferulate 5-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase. Angiosperm 4CL converts 4-coumaric, caffeic, ferulic, 5-hydroxyferulic, and sinapic acids into their corresponding thioesters for the formation of guaiacyl or syringyl monolignols. Question marks indicate hypothesized or unclarified alternative enzymatic steps. In many angiosperm species, sinapate is a poor in vitro substrate of 4CL; thus, it is hypothesized that in vivo synthesis of sinapoyl-CoA may proceed by alternative pathways[2].

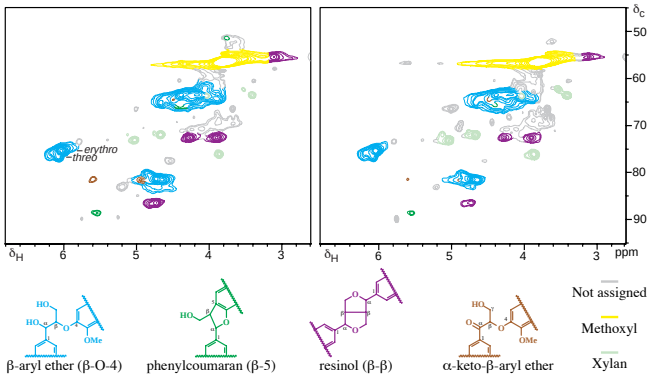

Figure 3. HSQC spectra of isolated milled wood lignins from (A) control and (B) transgenic line A6 indicate the similarity of major lignin structural units in both samples. The *erythro*- ($\delta_{C\alpha}/\delta_{H\alpha}$: 75.4/6.05) and *threo*- ($\delta_{C\alpha}/\delta_{H\alpha}$: 76.6/6.08) isomers of β-aryl ethers are indicated. Yellow contours are from intense methoxyl signals and light-green contours from xylan residues. Gray contours indicate irrelevant or unidentified compounds that are commonly seen in angiosperm lignin preparations. Structure similarity was also observed in the HSQC-TOCSY counterparts and HMQC spectra (not shown).

hybridization (data not shown). Transgenic trees grown in a greenhouse were harvested at the age of 10 months during the growing season to collect developing xylem and stem wood tissues for molecular, biochemical, and chemical analyses. The effect of antisense inhibition on xylem *Pt4CL1* gene expression is shown in Figure 2A. Steady-state *Pt4CL1* mRNA levels were drastically reduced in four lines (A4, 5, 6, and 8), leading to a >90% reduction in xylem Pt4CL1 enzyme activity with various hydroxycinnamic acids (Fig. 2B), and a 40–45% reduction in stem lignin (Fig. 2C). A smaller reduction in lignin content was found in those lines (A3 and 9) with less drastic suppression of Pt4CL1 activity. Fluorescence microscopy was used to examine the tissue distribution of lignin reduction in stem transverse hand-sections of transgenic line A6. As shown by the pattern of attenuated lignin autofluorescence following UV irradiation, lignin reduction occurred in woody xylem but not in phloem fibers of the transgenic lines (Fig. 2D and E). This observation is in agreement with our previous findings that *Pt4CL1* expression is confined to developing xylem[16] and that lignin metabolism may be independently regulated in xylem and phloem tissues in aspen[4].

Structural analysis of lignin and wall-bound phenolics. Thioacidolysis analysis of stem woody tissue revealed that the syringyl:guaiacyl (S:G) lignin ratio was essentially the same in transgenic (1.8–2.2) and control (2.3) plants. To conduct an in-depth study on the determination of lignin structural components and linkages, milled wood lignin was isolated from stem wood of control and transgenic line A6 and subjected to nuclear magnetic resonance (NMR) spectroscopic analysis. Common lignin structural linkages, including *erythro*- and *threo*-isomers of β-aryl ethers (β-*O*–4), phenylcoumaran (β-5) units, resinol (β-β) units, and α-keto-β–aryl ethers (derivatives from oxidation of syringyl β-aryl ethers), are all similarly represented in control and transgenic lignin samples (Fig. 3). Pt4CL1 substrates, such as *p*-coumaric, ferulic, and possibly sinapic acids (Fig. 1), did not accumulate in transgenic lignin based on the long-range ^{13}C-^{1}H-correlation heteronuclear multiple-quantum coherence (HMQC) NMR analysis. However, these acids were found to accumulate as nonlignin cell wall (NLCW) constituents by gas chromatography–mass spectrometry analysis. Transgenic A6 stem cell walls contained alkaline-extractable *p*-coumaric, ferulic, and sinapic acids at levels of 2,145 ± 93, 2,431 ± 120, and 2,452 ± 119

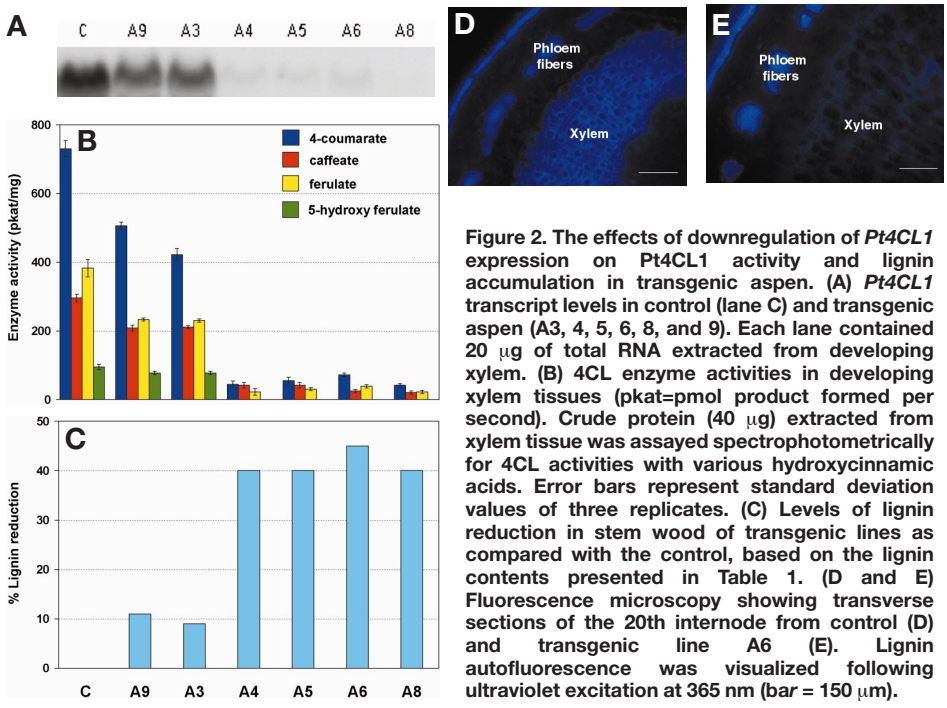

Figure 2. The effects of downregulation of *Pt4CL1* expression on Pt4CL1 activity and lignin accumulation in transgenic aspen. (A) *Pt4CL1* transcript levels in control (lane C) and transgenic aspen (A3, 4, 5, 6, 8, and 9). Each lane contained 20 μg of total RNA extracted from developing xylem. (B) 4CL enzyme activities in developing xylem tissues (pkat=pmol product formed per second). Crude protein (40 μg) extracted from xylem tissue was assayed spectrophotometrically for 4CL activities with various hydroxycinnamic acids. Error bars represent standard deviation values of three replicates. (C) Levels of lignin reduction in stem wood of transgenic lines as compared with the control, based on the lignin contents presented in Table 1. (D and E) Fluorescence microscopy showing transverse sections of the 20th internode from control (D) and transgenic line A6 (E). Lignin autofluorescence was visualized following ultraviolet excitation at 365 nm (bar = 150 μm).

Table 1. Lignin and cellulose contents in stem wood of control and transgenic aspen.

Plant	Lignin Content*	Cellulose Content*	Cellulose-to Lignin Ratio
Control	21.62 ± 0.30 (100)	44.23 ± 0.43 (100)	2.0
A4	12.83 ± 0.28 (60)	48.35 ± 0.60 (109)	3.8
A5	13.02 ± 0.28 (60)	49.74 ± 0.45 (112)	3.7
A6	11.84 ± 0.08 (55)	50.83 ± 0.26 (115)	4.3
A8	12.90 ± 0.04 (60)	48.14 ± 0.29 (109)	3.8
A15	18.60 ± 0.18 (86)	45.98 ± 0.83 (104)	2.5
A9	19.40 ± 0.27 (89)	47.49 ± 0.30 (107)	2.4
A11	20.40 ± 0.10 (94)	45.95 ± 0.28 (104)	2.2
A12	20.60 ± 0.08 (95)	46.55 ± 0.04 (105)	2.3

Data are the means ± SD of three independent experiments. Normalized values relative to control are shown in parentheses. Lines A4, 5, 6, 8 exhibit less than 7% residual 4CL activity with 4-coumaric acid, while A15 had 30%, A9 60% and A11 and A12 90% residual 4CL activity compared to the control level. *% of dry wood weight.

Table 2. Hemicellulose composition in control and transgenic lines.

Plant	% DWW* Arabinan	% DWW Galactan	%DWW Rhamnan
Control	0.47 ± 0.05 (100)	0.79 ± 0.01 (100)	0.37 ± 0.02 (100)
A4	0.58 ± 0.03 (123)	1.20 ± 0.03 (152)	0.43 ± 0.04 (116)
A5	0.56 ± 0.04 (119)	1.12 ± 0.05 (142)	0.43 ± 0.03 (116)
A6	0.55 ± 0.03 (117)	1.24 ± 0.04 (157)	0.48 ± 0.03 (130)
A8	0.56 ± 0.03 (119)	1.07 ± 0.01 (135)	0.48 ± 0.04 (130)
A15	0.48 ± 0.02 (102)	1.00 ± 0.04 (127)	0.42 ± 0.03 (114)
A9	0.43 ± 0.02 (91)	0.99 ± 0.03 (125)	0.40 ± 0.04 (108)
A11	0.41 ± 0.03 (87)	0.80 ± 0.03 (101)	0.37 ± 0.01 (100)
A12	0.34 ± 0.00 (72)	0.82 ± 0.02 (104)	0.37 ± 0.01 (100)

Data are the means ± SD of three independent experiments. Normalized values relative to control are shown in parentheses. DWW: dry wood weight.

nmol/g dry wood, respectively, as compared with the control values of 199 ± 13, 510 ± 9, and 0, respectively (n = 3). Levels of alkaline-extractable hydroxycinnamic acids in the NLCW of four other transgenic lines were also elevated above those of the control (data not shown). Together, the analyses show that lignin structure was preserved, but that the phenolic content of the NLCW fraction increased in the transgenic lines.

Carbohydrate analysis. Total carbohydrates in stem wood were analyzed to identify any effects that lignin reduction might have on other cell wall structural constituents. The four most severely lignin-deficient transgenic lines exhibited a 9–15% increase in cellulose (Table 1), identified as β-1,4-glucan by methylation-based linkage analysis[17,18] and enzymatic hydrolysis[19]. Because of the increase in stem woody biomass (see below), the increase in calculated percentage of cellulose represents an absolute increase in cellulose content. β-1,3-linked glucan (callose), reportedly deposited in plant cell walls as a result of perturbed secondary metabolism[20], was not detected in transgenic or control wood. The increased cellulose content together with reduced lignin quantity resulted in a cellulose:lignin ratio of 4 in the most severely downregulated lines, compared with 2 in the control (Table 1). There was a smaller increase in cellulose content and cellulose:lignin ratio in transgenic lines with less severe 4CL and lignin reductions (Table 1). The relative abundance of the major hemicellulose component xylan was unaffected in the transgenic lines (data not shown), but increases in the relative abundance of arabinan, galactan, and rhamnan averaging 20, 46, and 23% over control were observed in the four most severely 4CL-suppressed lines (Table 2). Smaller increases were measured in transgenic lines exhibiting less severe reductions in lignin. Characteristic of every transgenic line was a higher galactan:arabinan ratio ($\times_{8\ \text{lines}}$ 2.1 ± 0.2) than in the control (1.7).

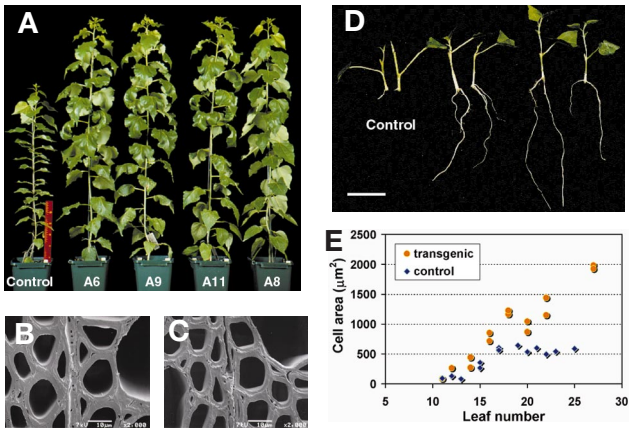

Figure 4. Enhanced growth in transgenic aspen. (A) Ten-week-old plants of control and four transgenic aspen grown in a greenhouse (ruler = 25 cm). (B and C) Scanning electron micrographs of stem transverse sections of control (B) and transgenic line A6 (C). Stem segments were sampled from 10-month-old trees at a point 15 cm above soil level (bar = 10 μm). Light microscope examination of the entire cross-sectional area of both samples revealed a similar stem cell wall morphology (not shown). A similar result was seen in additional sections of line A6 as well as line A8. (D) Two-week-old ex vitro rooted stem cuttings from control and transgenic aspen A5 and A6. Two cuttings from each line are shown (bar = 5 cm). Similar results have been obtained in five experiments using six independent transgenic lines. (E) Leaf upper epidermal cell area. The exposed outer surface area of leaf adaxial epidermal cells was measured from nail polish casts of the leaf surface using Optimas 6.2 image analysis software (Optimas Corp., Bothell, WA). Values represent the mean of at least 100 determinations per leaf. Sample standard deviation was 15–20% of the mean for all determinations. Similar results have been obtained in three experiments with three independent transgenic lines.

Downregulation of *Pt4CL1* expression causes enhanced growth phenotypes in transgenic aspen. Trees with downregulated Pt4CL1 had thicker stems, longer internodes, and larger (frequently epinastic) leaves than controls (Fig. 4A). Scanning electron microscopy revealed that the shape and size of stem xylem fiber and vessel cells were similar to those of controls (Fig. 4B and C). Root growth rates also increased in these lines, resulting in greater length (15-fold) and fresh weight gains (20-fold) than in controls over 14-day ex vitro rooting experiments (Fig. 4D). Cell size distribution in the meristematical and elongation zones of root tips and on the stem transverse sections was similar in control and transgenic plants (data not shown). Thus, the enhanced stem and root growth in these transgenic lines might be caused by increased cell proliferation activity rather than increased cell size. Leaf morphometric analysis indicated that adaxial epidermal cells stopped expansion at leaf number 15 below the first emerging leaf in control plants, but continued to expand as far down as leaf number 28 in the transgenics (Fig. 4E). Thus, the increased leaf size in transgenic aspen was likely due to a prolonged duration of epidermal cell expansion.

Discussion

In this study, we found that lignin content in a tree species can be reduced without the negative side effects on growth and structural integrity reported in herbaceous systems[10,12,15]. Two interrelated phenomena, conserved lignin structure and increased cellulose content, may account for the preservation of plant structural integrity we observed in the transgenic aspen trees. Lignin structural preservation may be due to the key position that Pt4CL1 occupies for controlling

the flow of its hydroxycinnamic acid substrates into the guaiacyl- and syringyl-monolignol pathways (Fig. 1). Suppression of Pt4CL1 activity did not alter the lignin S:G ratio or promote the incorporation of unusual monomeric units into lignin that have been reported in many other studies[6–15,21]. However, it led to an increase of hydroxycinnamic esters in the NLCW fraction that may provide clues pertinent to 4CL modulation of S:G ratio, as well as 4CL modulation of lignin content (and indirectly, cell wall strength). First, saponifiable *p*-coumaric, ferulic, and sinapic acids occur at similar elevated levels in the NLCW of the transgenics. This may reflect similar residual in vivo activities of 4CL with respect to its various hydroxycinnamic acid substrates, including sinapic acid, interesting in view of questions surrounding 4CL substrate preference and the control of the S:G ratio of lignin[2]. Second, our finding that these hydroxycinnamoyl-CoAs are more abundant in the NLCW fraction of 4CL-suppressed plants indicates that lignin is not the only sink for these compounds, and reducing the rate of lignin biosynthesis by suppressing 4CL may lead to incorporation of 4CL product phenolics into the NLCW, where they can contribute to cross-linkages that increase cell wall strength[22]. Alternatively, the increased phenolic acid content of the NLCW fraction may be due to a 4CL-independent phenolic acid activation pathway involving glucosyltransferases. By this alternative, glycosidic esters formed by the action of UDP-5′-diphosphoglucose–hydroxycinnamate D-glucosyltransferase become potential donors of acyl groups for esterification to the NLCW fraction[23]. Suppression of 4CL activity may lead to increased hydroxycinnamic acid incorporation into the NLCW by the glucosyltransferase pathway. By this scenario, 4CL, with its control of the flux of phenolics into lignin biosynthesis, competes with other activities that can divert the same substrates into other subcellular fractions, including NLCW. Whether 4CL products or substrates incorporate into the NLCW fraction, a role for 4CL with regard to plant cell wall strength deriving from its role in lignin biosynthesis is implied. From the standpoint of the tree biotechnologist, manipulating the flow of monolignol biosynthesis at the CoA activation step may be critical for downregulating lignin biosynthesis while maintaining normal lignin structure and cell wall strength.

The second of the two interrelated phenomena was a concomitant increase in cellulose content and a doubled cellulose:lignin ratio in the most severely lignin-reduced transgenic trees (Table 1). Such compensatory regulation of lignin and cellulose deposition could represent a tree-specific adaptation to sustain mechanical strength in lignin-deficient xylem. This finding is consistent with the manner in which angiosperm trees naturally regulate lignin and cellulose deposition during the formation of tension wood in response to gravitational and mechanical stimuli[24]. The formation of tension wood involves a coordinated regulation of lignin and cellulose deposition with increased cellulose accumulation occurring in a localized zone.

The molecular basis for this compensatory cellulose deposition is not known. Transcript levels of an aspen *celA* homolog (L. Wu, C.P. Joshi, and V.L.C., GenBank accession no. AF072131 and unpublished data) encoding a catalytic subunit of cellulose synthase[25] remained unchanged in trees with increased cellulose content. This argues against increased transcription of cellulose synthase in transgenic trees. The observation may instead suggest that cellulose synthesis is normally substrate limited and that reducing the flow of carbon into the lignin pathway at the Pt4CL1 step increases the availability of carbon for cellulose deposition. Indeed, the demand placed by lignin biosynthesis on carbon supply is probably higher in woody than herbaceous species where such compensatory deposition has not been reported. This suggests, in turn, that suppressing lignin biosynthesis can be used as a strategy to increase cellulose deposition in woody species.

Along with the cellulose increase, the enhanced growth of transgenic trees was a surprising observation not reported in transgenic tobacco or *Arabidopsis* with downregulated lignin pathway enzymes[6–15,21]. Growth was similarly enhanced in all transgenic lines whereas Klason lignin content of these lines ranged from 55 to 97% of the wild-type control (Table 1 and data not shown). There was neither a growth enhancement nor a lignin reduction in the "transgenic control" plant expressing a *GUS* reporter gene (with a Klason lignin of 21.1%), arguing against the possibility that some artifact of transformation or tissue culture caused the growth enhancement in the Pt4CL1-suppressed lines. Taken together, this indicates that growth enhancement is probably due to the Pt4CL1 transgene-induced perturbation of phenylpropanoid metabolism but is not solely due to a reduction in lignin content. Although ascribing the phenomenon to a discrete causal mechanism is not yet warranted, several points of discussion can be offered. In the case of Pt4CL1 downregulated aspen, lignin composition and structure were conserved, eliminating the possibility that altered lignin constituents promoted growth. However, the quantities of certain cell wall hemicellulose constituents were altered, with increases in the percentage of arabinan, galactan, and rhamnan in lines with the most severe lignin reductions and increases in galactan:arabinan ratio in all transgenic lines. It is becoming increasingly evident that complex carbohydrates have numerous biological, including signaling and growth-regulating, properties[26]. Changes in signaling at key stages of development could help account for the fact that increases in leaf and stem growth were evident in all transgenic lines, even those exhibiting modest decreases in 4CL activity and lignin content. There is also some basis for suggesting that part of the growth enhancement was due to increased carbon supply. It has been shown that cell division decreases in cultured parsley cells following treatments that divert carbon flow away from primary metabolism into the phenylpropanoid (secondary) pathway[27]. Whether the growth enhancement we observed in the transgenic aspen resulted from the converse, a shift from secondary to primary metabolism is one possibility to consider.

The compensatory regulation of lignin and cellulose described earlier for tension wood formation is associated with changes in radial auxin gradient[24] and suggests cross-talk between auxin, lignin, and cellulose during certain growth responses in woody plants. In the context of growth effects associated with Pt4CL1 suppression, wall-bound phenolics present at elevated levels in the transgenic lines reflect changes in the metabolic flow of hydroxycinnamic acids. Several hydroxycinnamic acid substrates of Pt4CL1 exhibit growth-stimulating[28] and auxin-protecting activities[29,30]. Also related to Pt4CL1's role in modulating phenylpropanoid metabolism is the synthesis of flavonoids[31,32], which, through their role as potential auxin transport regulators[33], may affect growth[34]. It should also be noted that 4CL isoforms other than Pt4CL1 may regulate these pathways, and that the effect of antisense suppression of Pt4CL1 on these activities is not yet clear. The mechanism(s) by which lignin biosynthesis, carbohydrate properties, and altered phenylpropanoid flux contribute to the 4CL-mediated growth enhancement we observed is currently being investigated.

Lignin has long been viewed as an essential component of cell wall architecture and an impediment to plant biomass utilization. Our results indicate that genetic manipulation of the lignin-specific 4CL may be an efficient strategy for producing crops with improved pulping efficiency or digestibility. We further demonstrate that cellulose and hemicellulose deposition and, perhaps less directly, growth are sensitive to changes in carbon flow directed into lignin biosynthesis. That 4CL activity and lignin biosynthesis may be important regulators of plant growth and development should broaden the scope of lignin research to consider the mechanisms coordinating primary and secondary metabolism with growth.

Experimental protocol

Plant transformation. Wild-type aspen was transformed with *Agrobacterium tumefaciens* carrying an antisense *Pt4CL1* gene construct as described[35]. The construct was generated by fusing the *Pt4CL1* cDNA coding sequence in an

antisense orientation with respect to a duplicated-enhancer cauliflower mosaic virus 35S promoter[36]. Wild-type aspen plants derived from in vitro micropropagation were used as the control.

Northern hybridization and enzyme assay. Total RNA was isolated from developing xylem of 10-month-old trees grown in a greenhouse as described[37]. RNA gel electrophoresis, blotting, and hybridization were performed according to previously described methods[4]. Crude protein was extracted from developing xylem of 10-month-old trees and assayed for 4CL activity with various hydroxycinnamic acid substrates as described[4].

Lignin analysis. Lignin content was determined by the Klason method[38], and lignin structure was analyzed by thioacidolysis as described[39]. Milled wood lignin was purified from stem wood[38] and metal ions removed[40] for NMR analysis. NMR experiments were performed at 360 MHz on a Bruker DRX-360 (Bruker Instrument Inc., Billerica, MA) using a narrow-bore probe with inverse-coil geometry and gradients. Bruker implementations of gradient-selected inverse (^{1}H-detected) heteronuclear single-quantum coherence (HSQC)[41], HSQC–total correlation spectroscopy (HSQC-TOCSY)[42], and HMQC[43] along with the standard one-dimensional ^{13}C (proton-decoupled) and ^{1}H NMR experiments were conducted. TOCSY experiments used a 100 ms spin lock period.

Analysis of wall-bound phenolics. Alkaline hydrolysis of stem wood meal (80 mesh) was performed at room temperature for 24 h in 1 M NaOH (ref. 44). The hydrolysates were neutralized, extracted with ethyl acetate, and concentrated. The concentrated products were derivatized with N,O,-bis(trimethylsilyl)trifluoroacetimide and analyzed by gas chromatography–mass spectroscopy using a DB-5 column and selected-ion monitoring.

Carbohydrate analysis. Cellulose and hemicellulose contents were determined based on the total sugars after acid hydrolysis of these polysaccharides in stem wood[45]. Wood meal (80 mesh) was vacuum-dried at 45°C and hydrolyzed with H_2SO_4. Following high-pH anion-exchange chromatography with pulsed amperometric detection), glucan and other polysaccharides (hemicelluloses) were quantified based on hydrolysate composition[46].

Acknowledgments

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