Anatomy and Chemistry of *Populus trichocarpa* with Genetically Modified Lignin Content

Zachary D. Miller,[a] Perry N. Peralta,[a] Phil Mitchell,[a] Vincent L. Chiang,[a,b] Stephen S. Kelley,[a] Charles W. Edmunds,[a] and Ilona M. Peszlen [a,*]

Vessel and fiber properties, diameter growth, and chemical compositions were investigated for transgenic *Populus trichocarpa* (black cottonwood) trees harvested after six months of growth in a greenhouse. Genetic modifications were cinnamyl alcohol dehydrogenase (*CAD*), cinnamate 3-hydroxylase (*C3H*), or cinnamate 4-hydroxylase (*C4H*), which resulted in modified lignin composition or content, and changed the syringyl-to-guaiacyl ratio. Comparing the genetic groups to the wild-type as the control, trees with reduced lignin content showed different results for vessel and fiber properties measured. Genetic groups with reduced *PtrC3H3* and *PtrC4H1&2* (with lower lignin content and higher S/G ratio than the control) exhibited splitting perpendicular to the rays, yet had the same fiber lumen diameter and the same fiber cell wall thickness as the control. Changes in lignin structure from modifications to *PtrCAD* resulted in reductions to the number of vessels, increases in vessel and fiber diameters, and had no consistent impact on stem diameter.

Keywords: Populus trichocarpa; Lignin content; Syringyl-to-guaiacyl (S/G) ratio; Stem diameter; CAD; C3H; C4H

*Contact information: a: Department of Forest Biomaterials, North Carolina State University, Box 8005, Raleigh, NC 27695-8005 USA; b: Forest Biotechnology Group, North Carolina State University, Box 7247, Raleigh, NC 27695-8005 USA; *Corresponding author: impeszle@ncsu.edu*

INTRODUCTION

Wood is a composite material made up of cellulose, hemicelluloses, lignin, and extractives. The chemical composition of wood has been well known for decades (Pettersen 1984); however the development of the tools that allow for some control over wood composition is a recent development (Van Doorsselaere *et al.* 1995; Baucher *et al.* 1996). The potential benefits, both economic and environmental, of using genetically-engineered trees for reducing the amount of chemicals and energy costs associated with pulp, paper, and bioethanol manufacturing are immense (Dinus *et al.* 2001; Chiang 2002; Talukder 2006). Pulp and paper manufacturing prefers longer fibers that are easier to separate but also have good strength properties. Previous work had examined the effects of changing lignin structure and content on the growth (Hancock *et al.* 2008), as well as the chemical (Li *et al.* 2003), morphological (Horvath *et al.* 2010a), and mechanical (Horvath *et al.* 2010b) properties of transgenic trees. An increase in knowledge of how chemical composition, with a particular focus on lignin (Hu *et al.* 1999), affects anatomical properties of wood could result in more efficient processing and more sustainable utilization of plantation-grown trees.

Lignin occurs within the cell wall of plants and in the middle lamella between the cell walls. It is formed from lignin subunits that are polymerized from their respective monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Whetten *et al.* 1998).

The monolignol biosynthetic pathways are complex, and research has recently focused on mapping these pathways (Shi *et al.* 2010; Shuford *et al.* 2012). The main steps have been investigated previously; however, modifications have generally been conducted with a focus on a single genetic modification for each tree. Cinnamate 3-hydroxylase (C3H) has been shown to significantly increase the proportion of H monolignols and increase the S/G ratio in alfalfa (Ralph *et al.* 2006) and in poplar (Coleman *et al.* 2008). Cinnamate 4-hydroxylase (C4H) has also been found to decrease the S/G ratio in sense or antisense expression (Sewalt *et al.* 1997), as well as to lower total lignin content (Blount *et al.* 2000). Cinnamyl alcohol dehydrogenase (CAD) is involved in the last step of monolignol biosynthesis; it reduces aldehyde compounds into their corresponding alcohols before polymerization (Sibout *et al.* 2005). In previous studies by Halpin *et al.* (1994), Baucher *et al.* (1996), and Lapierre *et al.* (1999), CAD-reduced trees showed no difference in tree growth, and had increased aldehyde content. These changes resulted in a pink-red color in the xylem.

In poplar trees, two major longitudinal cell types can be found: fibers and vessel elements. Fibers are responsible for the strength of wood, while vessels are utilized for water transport (Bailey 1953). Many studies have looked at the importance of anatomical properties on wood quality, wherein fiber wall thickness, fiber lumen diameter, and fiber length have been recognized as important traits (Kayama 1968; O'Neill *et al.* 1996). In general, cell wall thickness and cellulose microfibril angle are the determining factors of axial fiber properties (Salmén 2004; Salmén and de Ruvo 2007; Salmén *et al.* 2012). Several studies have quantitatively investigated anatomical elements in *Populus* spp. (Peszlen 1994; Mátyás and Peszlen 1997; Pliura *et al.* 2007; Huda *et al.* 2011, 2012); however, limited studies have been conducted on genetically modified trees.

Morphological changes are thought to occur frequently in transgenic trees, even though few studies have investigated this relationship. Grunwald *et al.* (2001) found stunted tree growth and thinner fiber cell walls in 35S-rolC transgenic aspen. Horvath *et al.* (2010a) reported reduced S/G ratio with aspen trees, which had smaller and more numerous vessels. Voelker *et al.* (2011) observed similar size but less numerous vessels in 4CL transgenic aspen. Li *et al.* (2011) described brittle stems with similar sized fibers and larger vessels in xylan reduced transgenic black cottonwood. Joshi *et al.* (2011) reported abnormal growth of cells when cellulose biosynthesis was targeted. Awad *et al.* (2012) observed that the vessel diameters were unchanged in lignin-modified poplars. Resistance to vessel cavitation is thought to be governed by wood density and by the strength of fibers surrounding the vessels (Hacke *et al.* 2001). It has been hypothesized that there is a trade-off between cavitation resistance and mechanical properties (Jacobsen *et al.* 2005). This was later disputed by Awad *et al.* (2012), who found little to no evidence of a trade-off between these phenomena.

In order to explore the relationship between the fiber cell wall anatomy and its chemical properties, a wider array of transgenic trees with specific modifications to the monolignol biosynthetic pathways are needed. The objective of this study was to investigate chemical composition, vessel and fiber properties, diameter growth, and relationships between these parameters in wild-type and transgenic six-month-old black cottonwood trees grown in a greenhouse.

EXPERIMENTAL

Materials

Young *Populus trichocarpa* (Nisqually-1) trees were used for this investigation. *Populus trichocarpa* is a model tree species because of its sequenced genome, fast growth, and ease of propagation. One non-transformed wild-type line (PtrWT-1) was used as a control. Seven different transgenic groups as described by Wang *et al.* (2018) were used in this study. Specifically, 12 transgenic lines were prepared with varying levels of expression denoted as low (L), medium (M) and high (H), namely:

- *PtrC3H3-05(L)*: Reduced PtrC3H3
- *PtrC3H3-10(M)*: Reduced PtrC3H3

- *PtrCAD1-05(L)*: Reduced PtrCAD1

- *PtrCAD2-19(H)*: Reduced PtrCAD2

- *PtrCAD1&2-01(L)*: Reduced both PtrCAD1 and PtrCAD2
- *PtrCAD1&2-07(M)*: Reduced both PtrCAD1 and PtrCAD2

- *PtrCAD1/CAD2/OMT-11(M)*: Reduced PtrCAD1 and increased PtrCAD2 (using a stem-regulated promoter)
- *PtrCAD1/CAD2/OMT-16(L)*: Reduced PtrCAD1 and increased PtrCAD2 (using a stem-regulated promoter)

- *PtrCAD1/CAD2/4CL-07(H)*: Reduced PtrCAD1 and increased PtrCAD2 (using a xylem specific promoter)
- *PtrCAD1/CAD2/4CL-21(M)*: Reduced PtrCAD1 and increased PtrCAD2 (using a xylem specific promoter)

- *PtrC3H3/C4H1&2-04(L)*: Reduced PtrC3H3, PtrC4H1 and PtrC4H2
- *PtrC3H3/C4H1&2-13(M)*: Reduced PtrC3H3, PtrC4H1 and PtrC4H2

Sample trees were propagated through rooted cuttings and grown in the greenhouse of the Forest Biotechnology Group at North Carolina State University (Song *et al.* 2006). Nine stems from each line were harvested as biological replicates after 6 months of growth between January and July 2012, and the lower 20 to 30 cm cut from the base of the stem was used. Stem diameter and height were measured at the time of harvest and stem slenderness was calculated as stem height/stem diameter (mm/mm). To maintain the green condition and to prevent fungal degradation, the specimens were placed in plastic bags and kept in a freezer until analyzed.

Quantitative Wood Anatomy

Key vessel characteristics that were measured included: vessel number (number per area measured), vessel lumen diameter (μm), and vessel lumen area as a percentage of the whole area (%). Key fiber properties were measured between two rays containing only fibers, and included fiber lumen diameter (μm) and fiber double cell wall thickness (μm).

For this part of the study, one to two representative stems from each line were selected for analysis for a total of 15 stems. Selection was based on the stem that was most representative of the mean modulus of elasticity for that line, as measured in a previous study (Miller 2013). All transgenic lines selected had severely reduced modulus of elasticity (MOE) (Miller *et al.* 2018). From each stem, five transverse microtome sections with a thickness of 20 μm were used for image analysis. Samples of *PtrC3H3-05(L)*, *PtrC3H3-10(M)*, *PtrC3H3/C4H1&2-04(L)*, and *PtrC3H3/C4H1&2-13(M)* were observed to be spongy and disintegrated when sections were cut.

The sections were stained with 1% aqueous safranin solution to enhance contrast and washed with deionized water. Sections were placed on separate glass slides, and anatomical properties were measured using an image analyzer system, which consisted of a light microscope (Nikon E200; Nikon Instruments Inc., Melville, NY), 3CCD color video camera (Sony DXC-390; Sony Electronics, San Diego, CA), and Image-Pro Plus 4.5 software (version 2009; Media Cybernetics, Rockville, MD). From different sections, five to eight 546 μm x 410 μm size images at 100x magnification for vessel measurements (~100 measured vessel/image), and five to eight 273 μm x 205 μm size images at 400x magnification for fiber analysis (~30 fibers/image measured) were taken randomly at 1280 x 960 pixel resolution. Images were inspected to be free of tension wood and other growth irregularities, and all intact cells were measured per image.

Chemical Composition Analysis

Lignin, glucose, and xylose contents were determined by Wang *et al.* (2018). Wood specimens were extracted with 90% acetone for 48 h, followed by three additional extractions (each 48 h) using 100% acetone, and air-dried. From each line, nine stems were used to create three biological replicates with three stems in each. Stems of each biological replicate were milled together to create a single sample, which was then partitioned into three technical replicate samples. Each sample was subsequently screened through 40 to 60 mesh sieves and vacuum dried under P_2O_5. In all, 0.1 g of extracted sawdust was hydrolyzed with 1.5 mL of 72% (w/w) H_2SO_4 at room temperature for 1.5 h. The mixture was then diluted with 56 mL of deionized water and autoclaved at 121 °C for 1.5 h. The hydrolysate was filtered through a fine coarseness crucible, and the acid-insoluble lignin was determined gravimetrically (Sluiter *et al.* 2011). The filtrate was used to determine the acid-soluble lignin content by UV-VIS absorption at 205 nm wavelength (HP 8453E UV-VIS spectrophotometer; Agilent Technologies, Palo Alto, CA) using a lignin absorptivity value of 110 L/(g•cm) (Sluiter *et al.* 2011). The concentration of sugars (glucose and xylose) in the filtrate was quantified by HPLC, which was equipped with a refractive index (RI) detector (Sluiter *et al.* 2011). Prior to injection, samples were filtered through 0.2 μm nylon Millipore filters; an injected volume of 20 μL was used. Milli-Q purified water was used as the mobile phase, at an elution flowrate of 0.5 mL/min.

Experimental Data Analysis

Analysis of variance (ANOVA) was used to test the effect of the transgenic modifications on the chemical and anatomical properties of transgenic black cottonwood. The analysis was performed using SAS software (SAS 2009) and Dunnett's multiple range test. An alpha (α) of 0.05 was used to compare the chemical and anatomical properties of each transgenic line to those of the wild-type.

NMR Spectroscopy

The S/G ratio of lignin was determined (Wang *et al.* 2018). 2D NMR spectra were acquired with a Bruker DRX-360 (Bruker AXS, Inc., Madison, WI) instrument. Acetylated lignins were dissolved in acetone; unacetylated lignins were dissolved in acetone and deuterium oxide. The central acetone solvent peak was used as the internal reference.

RESULTS AND DISCUSSION

Anatomical Properties

Anatomical differences were seen for a majority of the genetic groups when they were compared to the wild-type. The use of image analysis software allowed for a large number of cells to be measured on sample cross sections with high accuracy. The differences in wood anatomy were sometimes small but were statistically significant between the wild-type and the transgenics.

Mean vessels per measured area ranged from 60.8 for *PtrCAD1/CAD2/OMT-11(M)* to 119.5 for the wild-type, which are equivalent to 105.7 and 207.7 vessels per mm^2, respectively (Fig. 1). Microscopic images of wild-type and several transgenics also showed differences in vessel properties (Fig. 2 a to d). All of the transgenic lines had significantly fewer vessels per area measured except for *PtrC3H3-05(L)*. The vessel numbers measured in the present study were higher than what Horvath *et al.* (2010a) reported for one-year old wild-type (72.1) and transgenic (58.8 to 82.5) aspen trees. Xiang (2011) observed lower vessel numbers of 93 in the wild-type, as well as a wide range of 70.1 to 198.8 for 8-month-old cellulose-modified *P. trichocarpa* trees. Mean vessel lumen diameter values ranged from 32.2 µm for the wild-type to 49.3 µm for *PtrCAD2-19(H)* (Fig. 1).

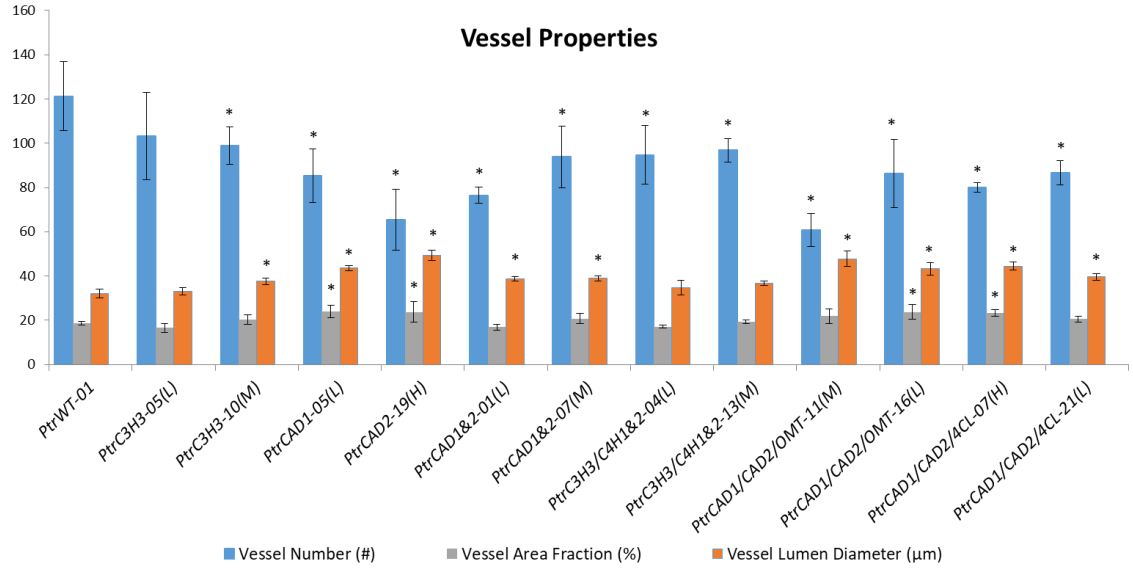

Fig. 1. Mean number of vessels measured per unit area, mean vessel lumen diameter and mean vessel lumen area fraction of wild-type (PtrWT-01) and transgenic black cottonwood trees with different genetic modifications to lignin. Measurements were taken on images with 100x magnification with 546 µm x 410 µm image area. Whiskers represent the standard deviation measured from five images. Asterisks (*) represent significant differences in properties of transgenic lines compared to the wild-type using Dunnett's comparison test at $\alpha = 0.05$ significance level.

Most of the transgenic lines had significantly larger vessel diameters than the wild-type, except for *PtrC3H3-05(L)*, *PtrC3H3/C4H1&2-04(L)*, and *PtrC3H3/C4H1&2-13(M)*. These observations are consistent with the work of Horvath *et al.* (2010a) for one-year-old *P. tremuloides* with modifications to lignin, and the work of Li *et al.* (2011) for 5-month-old xylose-modified *P. trichocarpa*. Voelker *et al.* (2011) reported larger vessel diameters for the wild-type (39.3 µm) than this study, but a smaller range (34.2 µm to 43.0 µm) for lignin-modified two-year-old staked *P. tremula* x *P. alba* trees. Xiang (2011) observed larger mean vessel diameters for young wild-type *P. trichocarpa* (47.7 µm). In cellulose modified transgenic trees, Xiang (2011) found that vessel diameters were either smaller (27.9 µm) or much larger (57.1 µm) than those found in this study. Wang *et al.* (2016) measured larger vessel diameters in 2-year old hybrid *P. deltoides* x *P. trichocarpa* (51.6 µm) compared to pure *P. deltoides* (64.8 µm). Irregularly shaped and collapsed vessels were reported by both Xiang (2011) and Joshi *et al.* (2011); however, abnormal vessels were not observed in this study.

Mean vessel lumen area fraction of the wild-type was about 18.7% and ranged from 16.5% for *PtrC3H3-05(L)* to 24.0% for *PtrCAD1-05(L)* (Fig. 1). Most of the transgenic lines had similar vessel lumen area fractions to the wild-type, except for *PtrCAD1-05(L)*, *PtrCAD2-19(H)*, *PtrCAD1/CAD2/OMT-16(L)*, and *PtrCAD1/CAD2/4CL-07(H)*, which all had significantly greater vessel lumen area fractions. Similarly, Horvath *et al.* (2010a) described vessel lumen area fractions that were larger in the transgenic trees when compared to the wild-type. On the other hand, Xiang (2011) found complex trends of both smaller and larger area fractions for transgenics and Li *et al.* (2011) observed no difference in vessel lumen area fractions between wild-type (19.6%) and xylose-reduced specimens (20.3%).

Mean fiber lumen diameter values ranged from 7.8 µm for *PtrC3H3/C4H1&2-04(L)* to 13.6 µm for *PtrCAD1/CAD2/OMT-11(M)* (Fig. 3). Microscopic images of wild-type and transgenics showed differences in fiber properties (Fig. 4a to d). Most of the transgenic lines had similar fiber diameters to the wild-type, except *PtrCAD2-19(H)*, *PtrCAD1/CAD2/OMT-11(M)*, *PtrCAD1/CAD2/OMT-16(L)*, and *PtrCAD1/CAD2/4CL-21(M)* all had significantly larger, and *PtrC3H3/C4H1&2-04(L)* had significantly smaller fiber diameters when compared to the wild-type. Transgenics with either similar or larger fiber diameters when compared to the wild-type were reported by Horvath *et al.* (2010a) and by Xiang (2011). Jourez *et al.* (2001) analyzed the fiber diameters of 4-month old poplar stems and found much larger fiber diameters (14.5 µm to 15.2 µm) than observed in this study (7.8 µm to 13.6 µm). Fiber widths of fast-growing *Populus* x *Euramericana* were found to be 21.1 µm for the first growth ring, which increased with tree age (Zhong *et al.* 2014). Wang *et al.* (2016) reported hybrid *P. deltoides* x *P. trichocarpa* with smaller fiber lumen diameters (13.0 µm) when compared to pure *P. deltoides* (16.7 µm).

Mean fiber double cell wall thickness ranged from 2.6 µm for *PtrCAD1&2-07(M)* to 4.3 µm for *PtrCAD2-19(H)* (Fig. 3) with 3.9 µm in the wild-type. Transgenic lines with thinner fiber double cell wall thicknesses included *PtrCAD1-05(L)*, *PtrCAD1&2-07(M)*, *PtrCAD1/CAD2/OMT-11(M)*, *PtrCAD1/CAD2/OMT-16(L)*, and *PtrC3H3/C4H1&2-04(L)*. Interestingly, *PtrCAD2-19(H)* had significantly greater fiber double cell wall thickness when compared to the wild-type. Both Xiang (2011) and Li *et al.* (2011) measured larger double cell wall thicknesses for wild-type (4.4 µm and 1.5 µm, respectively) than for transgenic specimens (1.4 µm and 0.9 µm, respectively). Zhong *et al.* (2014) analyzed fiber cell wall thicknesses in the first year of growth of *Populus* x *euramericana* to be 5.0 µm.

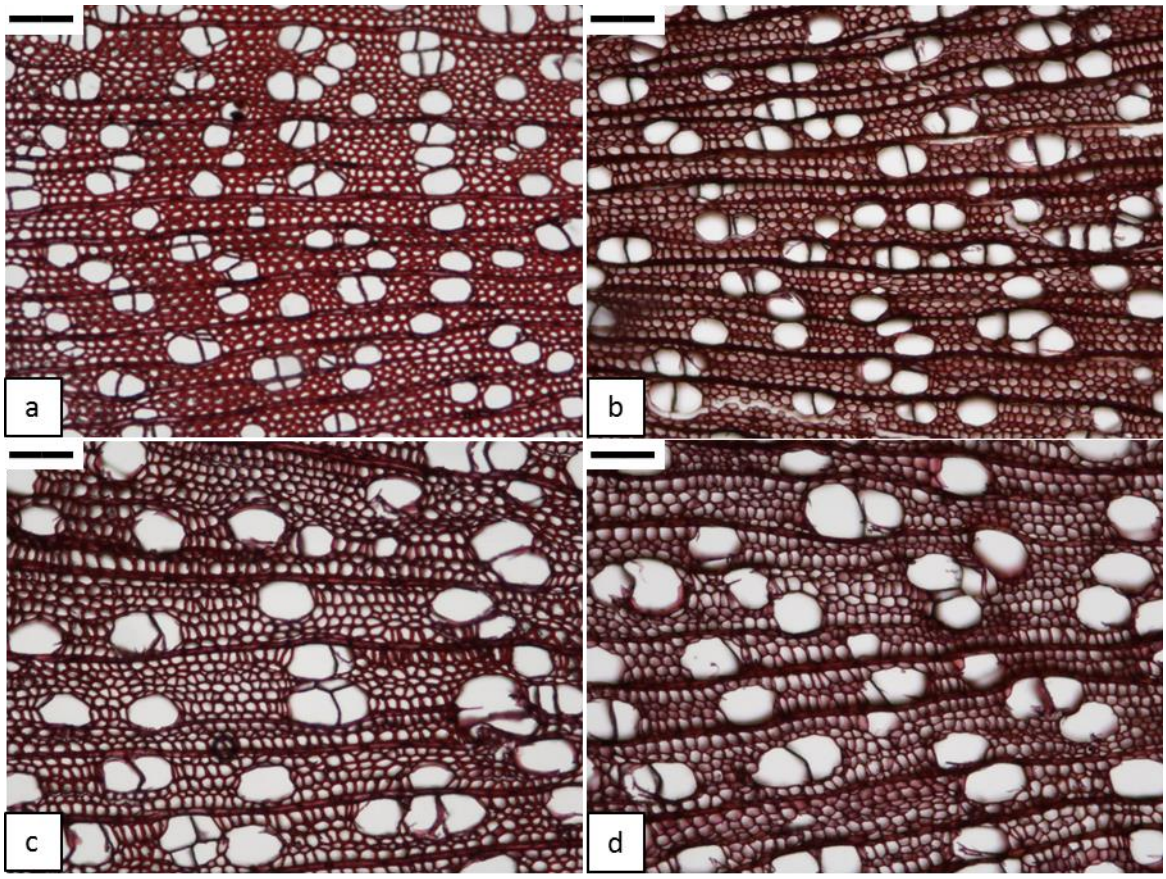

Fig. 2. Examples of transverse sections of wild-type (a) PtrWT-01 and transgenic trees (b) *PtrC3H3/C4H1&2-04(L)* having similar vessel lumen diameter and vessel number. (c) *PtrCAD2-19(H)* and (d) *PtrCAD1/CAD2/OMT-11(M)* have significantly larger vessel lumen diameters, lower vessel numbers, and smaller vessel lumen area fractions. Scale bars = 100 µm.

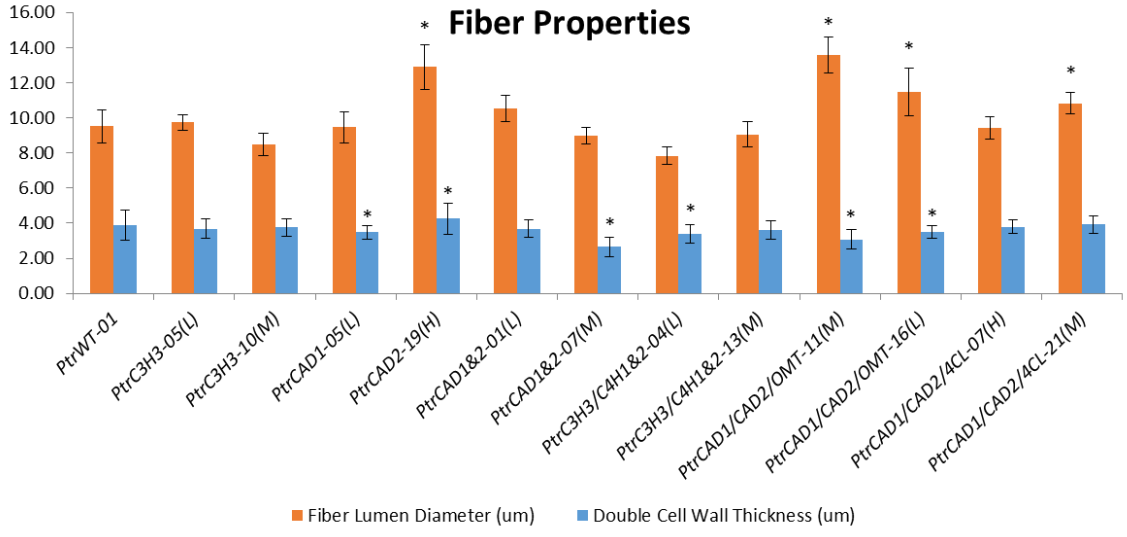

Fig. 3. Mean fiber diameter values and mean fiber double cell wall thickness values of wild-type (*PtrWT-01*) and transgenic black cottonwood trees with different genetic modifications to lignin. Measurements were taken on images with 400x magnification with 273 µm x 205 µm image area. Whiskers represent the standard deviation. Asterisks (*) represent significant differences of the expression levels compared to the wild-type using Dunnett's comparison test at $\alpha = 0.05$ significance level.

Sandquist *et al.* (2015) measured both radial and tangential double cell wall thickness of 3-month-old hybrid aspen and reported thinner cell walls (1.4 µm and 2.5 µm) than those found in this study. Wang *et al.* (2016) observed larger double cell wall thicknesses in hybrid *P. deltoides* x *P. trichocarpa* (5.2 µm) when compared to pure *P. deltoides*.

In this study, tension wood (*i.e.*, the reaction wood found in angiosperms that can be identified by a thick, loosely attached gelatinous layer in the inner cell walls) was found in almost all specimens. In general, tension wood forms on the upper side of leaning stems or branches, and it exhibits eccentric growth in response to the reorientation of the stem axis from its original position (Timell 1986). However, in some species, particularly in *Populus* spp., tension wood has been observed even in non-leaning stems (Kaeiser 1955), such as in this study. Tension wood can also be associated with fast growth (Berlyn 1961). It has been suggested that in transgenic trees, decreases in lignin are associated with increases in tension wood by as much as threefold in field-grown poplar (Voelker 2009).

Stem height values ranged from 168 cm for *PtrCAD1&2-07(M)* to 327 cm for *PtrCAD1/CAD2/4CL-21(M)*. Most of the transgenic stems exhibited stunted axial growth except for Stem diameter values of individual trees ranged from 8.3 mm for *PtrCAD1&2-07(M)* to 12.1 mm for *PtrCAD1/CAD2/4CL-21(M)* (Table 1).

Table 1. Mean (± COV) Stem Height, Stem Diameter and Stem Slenderness Ratios (Height/Diameter) of Wild-type (PtrWT-01) and Transgenic Black Cottonwood Trees with Different Genetic Modifications to Lignin. COV represents the coefficient of variation (%).

Sample name	Stem Height (mm)	Stem Diameter (mm)	Stem Slenderness ratio (mm/mm)
PtrWT-01	320.00 (± 4.10)	11.04 (± 0.83)	289 (± 8)
PtrC3H3-05(L)	200.33 (± 6.47)	9.51 (± 1.41)	211 (± 21)
PtrC3H3-10(M)	285.56 (± 3.74)	10.89 (± 2.07)	238 (± 5)
PtrCAD1-05(L)	290.56 (± 9.28)	11.53 (± 1.24)	244 (± 8)
PtrCAD2-19(H)	249.50 (± 14.72)	10.66 (± 1.12)	235 (± 11)
PtrCAD1&2-01(L)	234.67 (± 34.64)	9.51 (± 2.39)	240 (± 14)
PtrCAD1&2-07(M)	168.33 (± 5.71)	8.26 (± 0.96)	177 (± 11)
PtrC3H3/C4H1&2-04(L)	219.33 (± 10.89)	10.60 (± 1.08)	209 (± 21)
PtrC3H3/C4H1&2-13(M)	227.11 (± 5.07)	11.54 (± 0.60)	197 (± 3)
PtrCAD1/CAD2/OMT-11(M)	283.00 (± 17.43)	11.08 (± 1.78)	229 (± 14)
PtrCAD1/CAD2/OMT-16(L)	293.33 (± 9.03)	10.77 (± 0.87)	267 (± 8)
PtrCAD1/CAD2/4CL-07(H)	306.67 (± 3.31)	12.02 (± 0.38)	255 (± 3)
PtrCAD1/CAD2/4CL-21(M)	327.11 (± 2.38)	12.06 (± 0.85)	267 (± 6)

Most of the transgenic specimens exhibited the same diameter growth except for *PtrCAD1&2-07(M)*, *PtrCAD1&2-01(L)* and *PtrC3H3-05(L)*. Stem slenderness ratios ranged from 177 (mm/mm) for *PtrCAD1&2-07(M)* to 289 (mm/mm) for the wild-type. Transgenic line *PtrCAD1&2-07(M)* had the smallest stem height, diameter, and slenderness ratio and it also had poor survival rate in the greenhouse. Trees can be regarded as upright and free-standing columns that are fixed at the base, and those with stem slenderness values greater than 200 (mm/mm) are considered unstable (Wang *et al.* 1998). Most of the lines including the wild-type had slenderness ratios above 200 (mm/mm) other than *PtrCAD1&2-07(M)* and *PtrC3H3/C4H1&2-13(M)* indicating slender growth. However, these stems were grown in the greenhouse and supported with stakes, which could explain their slenderness. In comparison, Debell *et al*. (1997) analyzed two-years-

old *Populus* clones grown in a seed orchard and found smaller slenderness ratios ranging from 117 to 146 (mm/mm). In one of the first studies on transgenic poplars, Hu *et al.* (1999) reported larger diameter growth for reduced lignin transgenics; however, this was not observed by others (Pilate *et al.* 2002; Li *et al.* 2003; Hancock *et al.* 2007; Horvath *et al.* 2010a). Xiang (2011) studied cellulose-reduced transgenics and noted decreased diameter growth when compared with wild-type, while xylose-reduced transgenics were observed to have no change in diameter. Some works (Hancock *et al.* 2007, Horvath *et al.* 2010a) have also showed that increasing the S/G ratio in transgenics decreased the stem diameter growth when compared to the wild-type.

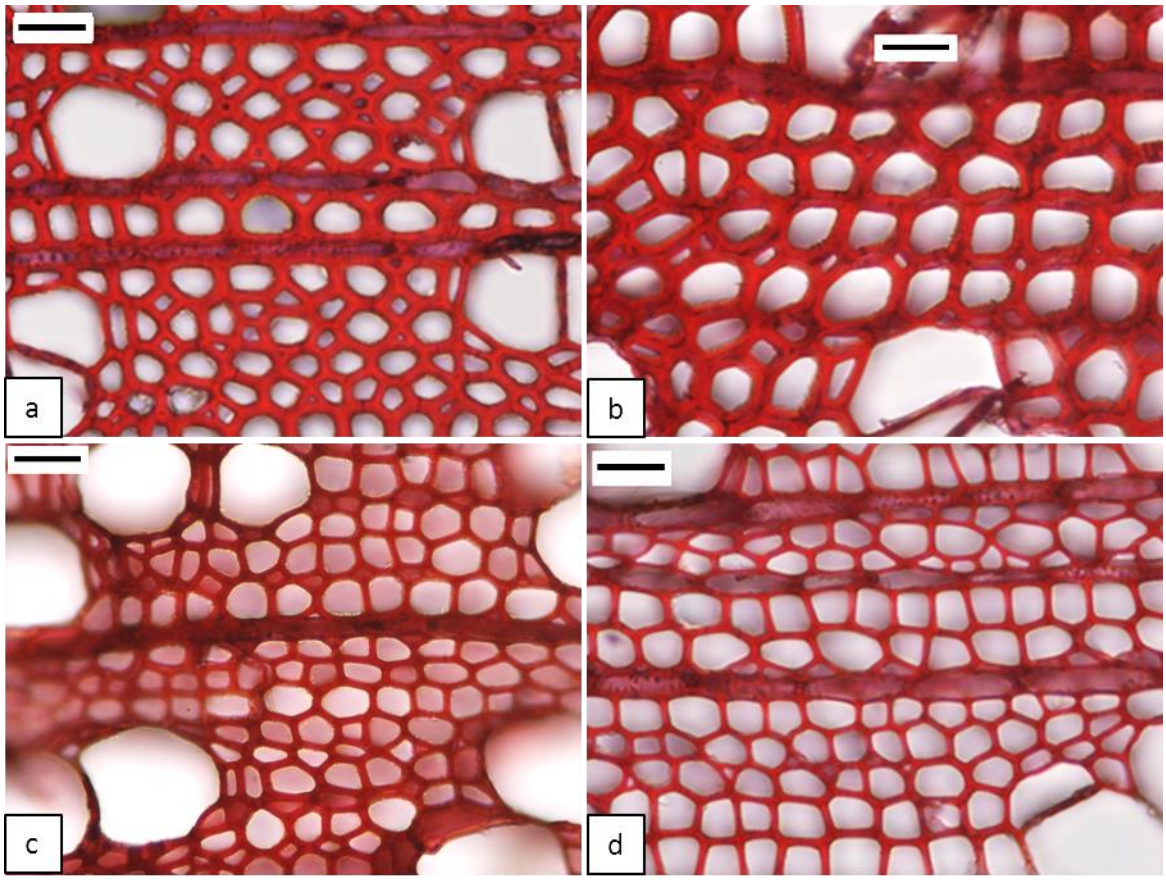

Fig. 4. Examples for transverse sections of wild-type (a) *PtrWT-01* and transgenic trees (b) *PtrCAD2-19(H)* with larger fiber lumen diameter and thicker double cell wall, (c) *PtrCAD1&2-07(M)* with similar fiber diameters but thinner cell walls, and (d) *PtrCAD1/CAD2/OMT-11(M)* with significantly larger fiber diameters and thinner double cell walls. Scale bars = 25 µm.

Chemical Properties

The results of the chemical analyses are shown in Fig. 5.

Lignin content

Across all the samples examined, the lignin contents ranged from 9.9% for *PtrC3H3-05(L)* to 23.2% for wild-type. Lignin levels were significantly lower in *PtrC3H3-05(L)* (9.9%) and *PtrC3H3-10(M)* (13.5%), *PtrC3H3/C4H1&2-04(L)* (11.7%), and *PtrC3H3/C4H1&2-13(M)* (13.4%), and *PtrCAD1&2-07(M)* (15.8%), which represented 58%, 42%, 50%, 43% and 32% decreases in lignin content when compared to the wild-

type, respectively. Genetic lines with low expression level of *PtrC3H3* and *PtrC3H3/C4H1&2* had lower lignin levels than the medium expression for the same transformation. *PtrCAD1&2-07(M)* had lower lignin content than *PtrCAD1&2-01(L)* even though it was anticipated that the lower expression level of *PtrCAD1&2* would have yielded lower lignin levels.

Lignin S/G ratios

The S/G ratios for the lignin ranged from 1.9 for *PtrCAD1/CAD2/OMT-16(L)* to 9.9 for *PtrC3H3-05(L)* (Fig. 6), with the wild-type having a S/G ratio of 2.6. The S/G ratio values were larger for both lines with the PtrC3H3 transformation.

Glucose content

The glucose content ranged between 41% in *PtrCAD1-05(L)* to 54% in *PtrC3H3-05(L)* with the wild-type having glucose values near 46% (Fig. 6). Glucose was significantly higher in the *PtrC3H3-05(L)*, and *PtrC3H3/C4H1&2-04(L)*; however, no lines had significant reductions in glucose when compared to the wild-type.

Xylose content

The xylose content ranged between 14% in *PtrCAD2-19(H)* to 20% in *PtrC3H3-05(L)* with the wild-type having values of 16% (Fig. 6). Xylose values were significantly higher in genetic groups *PtrC3H3-05(L)*, *PtrCAD1&2-01(L)*, *PtrCAD1&2-07(M)*, and *PtrCAD1/CAD2/4CL-21(M)* when compared to the wild-type. There were no lines with significant reductions in xylose.

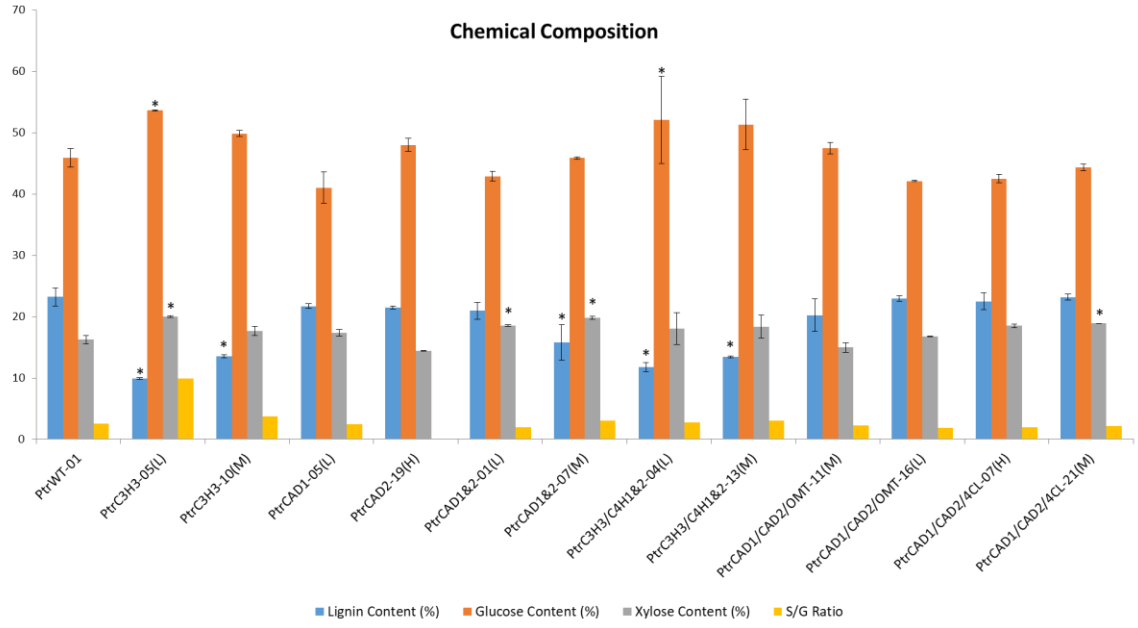

Fig. 5. Mean values for lignin content, glucose content, xylose content and S/G ratio of wild-type PtrWT and transgenic black cottonwood trees. Whiskers represent the standard deviation of three technical replicates. Asterisks (*) represent significant differences of the expression levels compared to the wild-type using Dunnett's comparison test at $\alpha = 0.05$ significance level.

Discussion of chemical properties

Swan and Kellogg (1986) reported lignin content values for 30 non-transformed *P. trichocarpa* trees harvested from three separate sites in British Columbia, which were between 21.8% to 23.2%. Studer *et al.* (2011) analyzed 1,100 natural *P. trichocarpa* trees in a population from a large geographical distribution and found lignin contents ranging from 16% to 28%. For transgenic trees, Li *et al.* (2011) studied *P. trichocarpa* trees with significantly decreased xylose values of 9 to 11% and increased lignin of up to 30% when compared to the wild-type trees, which had lignin values around 21% to 24%, glucose values ranging from 43% to 45%, and xylose values around 16%. Lu *et al.* (2013) explored the effects of a laccase gene, which was thought to be involved in monolignol polymerization, and they observed lignin contents of 20.1% with increases in xylose contents of 18.1%. Porth *et al.* (2013) investigated natural variation in 9-year-old *Populus trichocarpa* and found large ranges in lignin (14.7% to 25.7%), glucose content (40.7% to 61.7%) and xylose content (13.9% to 24.2%). Min *et al.* (2014) tested wild-type *P. trichocarpa*, which had lignin content of 21%, glucose content of 40% and xylose content of 15%. For field grown *P. trichocarpa*, Xiang *et al.* (2015) examined transgenic trees that had increased lignin content when compared with earlier measurements when the trees were grown in the greenhouse. Upregulation of lignin has been observed for transgenic *P. trichocarpa* grown in the field in response to environmental stresses (Stout *et al.* 2014).

Changes in chemical properties either resulted in no changes or induced modifications in the vessel and fiber properties of the transgenic lines. When compared to the wild-type, the three genetic lines, *PtrC3H3-05(L)*, *PtrC3H3/C4H1&2-04(L)*, and *PtrC3H3/C4H1&2-13(M)*, had significantly reduced lignin contents and had no changes in vessel and fiber diameter. Besides the lower lignin levels, *PtrC3H3-05(L)* and *PtrC3H3/C4H1&2-04(L)* also had increases in glucose levels. Two other lines also with reduced lignin contents, *PtrC3H3-10(M)* and *PtrCAD1&2-01(L)*, had larger vessel diameters, but no changes in fiber diameter. Genetic lines with modifications to *PtrCAD1*, *PtrCAD2*, and *PtrCAD1&2*, had no change in lignin contents, except for *PtrCAD1&2-07*, which had lower lignin levels and had larger vessel diameters. In addition to larger vessel diameters, *PtrCAD2-19(H)* and *PtrCAD1/CAD2/OMT-11(M)* also had larger fiber diameters with no significant chemical changes when compared to the wild-type. Lo Gullo *et al.* (1995) showed that water transport in 1 to 3-year-old twigs of oak with smaller diameter vessels (~20 µm) is much less efficient than with plants containing large diameter vessels (~64 µm); however, large vessels are more vulnerable to embolism, which negatively impacts survival rate (Tyree and Sperry 1989). In fact, survival rates of some genetic groups were very low, possibly due to lack of protection against embolization. Awad *et al.* (2012) hypothesized that alterations may reduce resistance to cavitation due to modifications in the lignification of vessel pit structure. Kitin *et al.* (2010) found a reduction in xylem-specific conductivity, which was attributed to tyloses and phenolic deposits in vessels of transgenic poplars with 40% reduction in lignin when compared to the wild-type. There is some evidence that carbon availability during the growth processes is affected by modifications to lignin biosynthesis, which can compromise vascular integrity (Hu *et al.* 1999, Kitin *et al.* 2010). Indeed, specific morphological and anatomical properties may relate to vascular efficiency and water transport; thus, any structural alterations could affect whole plant-water interactions (De Micco and Aronne 2009).

Genetic line *PtrC3H3-05(L)* had a very high S/G ratio (9.9), but no changes to vessel diameter or vessel number values, which is in contrast with the observations reported by Horvath *et al.* (2010a), who noted more numerous vessels but similar vessel diameter

for transgenic aspen with high S/G ratio (5.2) although the authors used a different sense CAld5H transformation. The type of lignin in vessel cell walls has been characterized and been found to be abundant in guaiacyl content (Musha and Goring 1975); hence, adjustments in the biosynthesis of guaiacyl lignin could result in smaller vessel lumen diameter.

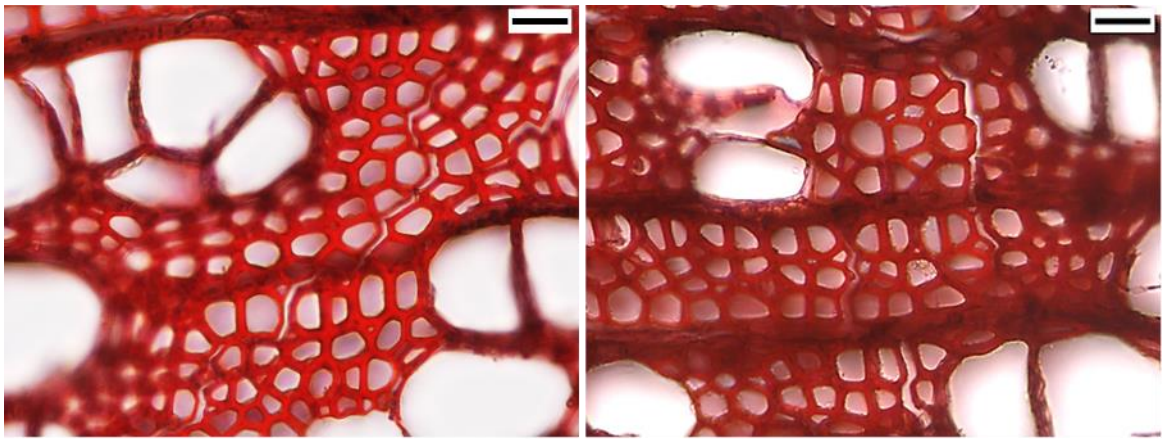

Fig. 6. Transverse sections taken with light microscope displaying cell walls splitting along middle lamella perpendicular to the rays in *PtrC3H3/C4H1&2-04(L)*. Scale bar = 20 µm.

Genetic lines *PtrC3H3-05(L)*, *PtrC3H3-10(M)*, *PtrC3H3/C4H1&2-04(L)*, and *PtrC3H3/C4H1&2-13(M)* with low lignin content exhibited splitting perpendicular to the rays (Fig. 6). Splitting on the tangential cell walls could be an indication of the possible weakness of the middle lamella that may be related to the severe reductions of 45 to 58% in lignin content, increases in S/G ratio, and increases in glucose content in these transgenics. However, fiber properties in the transverse direction are thought to be most influenced by hemicelluloses and lignin (Bergander and Salmén 2002). Resistance to splitting of cells is thought to be influenced by the lignification of the pectin-rich middle lamella (Hafren *et al.* 2000). In the current study, six transgenic trees had no change in cell wall thickness.

CONCLUSIONS

In this study, chemical composition, vessel and fiber properties, and diameter growth of several transgenic lines with modifications to the lignin pathways through altering CAD, C3H, and C4H were investigated for six-month-old poplar trees grown in a greenhouse. The following conclusions were drawn from the observations made in this work:

1. All PtrC3H3 transformations (with lower lignin content and higher S/G ratio than the control) had spongy phenotype, were difficult to cut, and exhibited splitting perpendicular to the rays, yet had the same fiber lumen diameter and the same fiber cell wall thickness as the control.
2. Changes in lignin structure through modification of the CAD genes corresponded to an increase in vessel and fiber lumen diameter. This was especially true when simultaneous modifications were made to CAD1 and CAD2. The stem diameter

trend was unclear; trees with CAD modification had the lowest growth and the highest growth. PtrCAD1&2-07 (M) had lower lignin content, high S/G ratio, but it was the only one that had lower cell wall thickness than the control.

3. There was a complex relationship between changes in the chemical composition of the transgenic wood and the vessel and fiber properties. More research is needed to elucidate these relationships in genetically modified trees.

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