MECHANICAL PROPERTIES OF GENETICALLY ENGINEERED YOUNG ASPEN WITH MODIFIED LIGNIN CONTENT AND/OR STRUCTURE

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Abstract. Reducing lignin content is a target for processes such as papermaking because lignin removal requires a tremendous amount of energy and chemicals. Recent advances in tree genetics permit modification of lignin content and structure. The consequences of lignin modifications on many wood properties are not known. The purpose of this study was to establish the effect of genetic modification of lignin on selected wood mechanical properties. In this study, genetically modified young quaking aspen trees with reduced lignin content and/or increased syringyl to guaiacyl (S/G) ratio were investigated and compared with the wild type. The modulus of elasticity in three-point bending and the compression strength parallel to the grain were measured using modified micromechanical tests. The results indicate that the genetic modification used in this study had a negative effect on these mechanical properties. The transgenic trees with reduced lignin content showed a severe reduction in modulus of elasticity and compression strength parallel to the grain, whereas the transgenic trees with increased S/G ratio had only a slight decrease in these properties compared with the wild type. The simultaneous modification of lignin content and S/G ratio shows inconsistent results and needs further investigation.

Keywords: Alpha-cellulose, aspen, compression strength, lignin, mechanical properties, modulus of elasticity, *Populus tremuloides*, transgenic tree.

INTRODUCTION

Wood is a complex organization of three major polymers: cellulose, lignin, and hemicelluloses. Lignin represents 20-30% of the dry weight of

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wood. It is a complex polymer of phenylpropanoid subunits of syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) types. These monomers are connected together by carbon–carbon or ether linkages (Chen et al 2001). Gymnosperms mainly contain G type, whereas angiosperms contain S and G type lignin in roughly a 2:1 ratio. Lignin rich in S units has fewer carbon—carbon bonds, thus having greater reactivity than lignin rich in G units (Baucher et al 2003). Lignin provides impermeability to the xylem vessels, protects the tree and wood against microbial attack, gives rigidity to the cell wall, and imparts compression strength to the wood (Moerschbacher et al 1990; Chen et al 2001).

Lignin has to be removed from the wood during the process of papermaking and bioethanol production. The removal of lignin makes the utilization of woody biomass highly energy- and chemical-intensive (Lee 1997; Chiang 2002). To reduce the energy and chemicals needed to remove lignin, trees with lower lignin content and/or higher lignin reactivity are preferred as biomass feedstocks. Genetic engineering provides an opportunity to alter the chemical and physical properties of wood (Whetten and Sederoff 1991). Researchers at North Carolina State University (Li et al 2003) have successfully reduced the lignin content of quaking aspen (*Populus tremuloides* Michx.) by as much as 55% with the overexpression of antisense 4-coumarate-CoA ligase gene (4CL) and have increased the S/G ratio 3-fold by the overexpression of sense coniferaldehyde 5-hydroxylase gene (CAld5H). *Populus* spp. was chosen as a model because it is the first species whose genome was sequenced, easy to propagate, fast growing, and the most widely distributed species in North America (Tuskan et al 2003). The effect of reduced lignin content on CO_2 emission and on the fitness of the trees was the subject of several recent reviews (James et al 1998; Pilate et al 2002; Talukder 2006). However, the effect of altered lignin content and composition on the mechanical properties of young trees with small diameter has not received enough attention. The integrity of wood and the ability of the tree to mechanically support a heavy crown are influenced by lignin content (Sederoff and Chang 1991; Gindl and Teischinger 2002) and thus are important from silvicultural and wood utilization points of view. The mechanical properties of transgenic trees need to be measured at an early age (1-3 yr old) to provide feedback to researchers on the genetic modification. Our group has developed testing techniques to measure these properties using micromechanical methods (Kasal et al 2007).

The objective of this study was to investigate the modulus of elasticity in bending and the compression strength parallel to the grain of genetically modified young aspen trees with reduced lignin content and/or increased S/G ratio. Based on a literature search, this is the first attempt to use transgenic trees to improve understanding of the mechanism of lignification as it relates to biomechanics of lignocellulosic materials.

MATERIALS AND METHODS

Young quaking aspen (*Populus tremuloides* Michx.) trees were used for this investigation, including one wild type line as a control (PtrWT-271), three lines of transgenics with reduced lignin content through transfer of the antisense 4CL gene (Ptr4CL-21, Ptr4CL-23, Ptr4CL-37), two lines of transgenics with increased syringyl/guaiacyl ratio (S/G) through insertion of the sense CAld5H gene (PtrCAld5H-94, PtrCAld5H-96), and two lines of transgenics with both reduced lignin content and increased S/G ratio through simultaneous insertion of 4CL and CAld5H genes (Ptr4CL/CAld5H-72, Ptr4CL/CAld5H-141). Sample trees were propagated in vitro as described by Li et al (2003) and grown in the greenhouse of the Forest Biotechnology Group at North Carolina State University.

A total of 94 stems was harvested; 33 sample trees were harvested after 1 yr (Age Group 1), 29 after 2 yr (Age Group 2), and 32 after 2.5 yr of growth (Age Group 3). Each sample tree was debarked and, depending on the stem length, was cut into 1-4 pieces of 250-mm-long stem sections. To maintain the green condition and to prevent fungal degradation, the specimens were placed in plastic bags and kept in a freezer. Just before the mechanical tests, specimens were removed from the freezer and the test conducted shortly after the samples were defrosted.

The modulus of elasticity (MOE) was determined using a micromechanical three-point bending test (Kasal et al 2007). Before the test, the stem sections were trimmed into different lengths, depending on the stem diameter, to keep a constant span/diameter ratio of 15 plus an additional 10 mm overhang at each end. The diameter of the pith was subtracted from the diameter of the specimen, which was measured in the direction of the load at the midspan of the specimen. A special bearing block was designed to reduce surface crushing of the specimen. The specimen was supported with fixed rollers, which allowed the observation of possible rotation of the specimen about its longitudinal axis. To observe the rotation, a 30-mm-long aluminum clip was attached close to the neutral axis of the specimen. A white board with a center mark was placed behind the clip (Fig 1). The specimen was repositioned until no rotation was observed. The bending test was conducted using an MTS Alliance RF/300 mechanical testing machine at a crosshead speed of 1.27 mm/min.

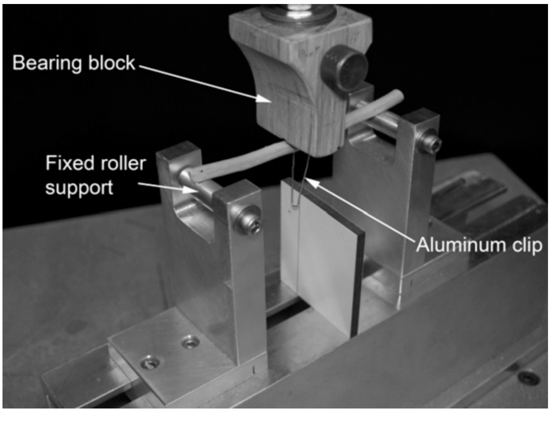

Figure 1. Three-point-bending experimental setup for testing young, small-diameter transgenic aspen using fixed roller support, a special bearing block to reduce surface crushing, and an aluminum clip to detect specimen rotation.

After the bending test, a compression specimen was cut from the straightest portion of the bending specimen. A micromechanical compression test (Kasal et al 2007) was modified and used to measure the compression strength parallel to the grain ($\sigma_{c,||}$). The modification included a reduction in length:diameter ratio from 6 to 3, the use of a fixed load head, and a reduction in crosshead speed from 0.635 to 0.4 mm/min^{-1}. An MTS Alliance RF/300 universal testing machine was used to conduct the compression test. The compression strength parallel to the grain was calculated based on the load at failure and the circular cross-sectional area without the pith.

To determine the chemical composition of the experimental material, lignin and alpha-cellulose contents were measured. From the mechanical test specimens, two specimens were selected from each genetic line. The acid-insoluble lignin content was measured using a modified Klason lignin method and the acid-soluble lignin was calculated from UV absorbance at 205 nm. The acid-insoluble and -soluble lignin contents were combined to obtain the total lignin content (Yeh et al 2005). Alpha-cellulose was prepared from holocellulose and the yield was calculated according to the description of Yokoyama et al (2002). The specific gravity of 1-, 2-, and 2.5-yr-old sample trees was measured using the standard water displacement method.

Analysis of variance (ANOVA) was used to test the effects of genetic modification, genetic lines within genetic groups, and age on the mechanical properties of transgenic aspen with specific gravity and diameter as covariates. The following linear model was used to test the effect of genetic modification on the response variables:

$$\begin{aligned} Y_{ijkl} = \mu + \beta_1 D_{ijkl} + \beta_2 S_{ijkl} + A_i + G_j \\ + L(G)_{k(j)} + A_i G_j + \varepsilon_{ijkl} \end{aligned} \quad (1)$$

where Y_{ijkl} = trait of interest (mechanical properties), μ = overall mean, β_1 = coefficient related to diameter, D_{ijkl} = stem diameter of l^{th} tree in k^{th} genetic line of j^{th} genetic group of i^{th} age, β_2 = coefficient related to specific gravity, S_{ijkl} = specific gravity of l^{th} tree in k^{th} genetic line of j^{th} genetic group of i^{th} age, A_i = effect of i^{th} age, G_j = effect of j^{th} genetic group, $L(G)_{k(j)}$ = effect of k^{th} genetic line within j^{th} genetic group, A_iG_j = interaction effect between i^{th} age group and j^{th} genetic group, and ε_{ijkl} = random error with expectations (0, σ^2). The analysis was performed using the GLM procedure of SAS software (SAS 2006).

To compare the properties of the genetic groups and lines, Tukey multiple comparison test at alpha = 0.05 was used. The assumptions of the general linear model were tested using Q-Q plots and the plots of residuals vs predicted values.

RESULTS AND DISCUSSION

The total lignin and alpha-cellulose content are presented in Table 1. The values were in agreement with those reported by Li et al (2003), except for genetic lines Ptr4CL/CAld5H-72 and Ptr4CL/CAld5H-141, which have significantly higher lignin content and lower cellulose content values. This could have been caused by the transgene instability of the tissue culture propagation method (Fladung 1999; Fladung and Kumar 2002). For statistical analysis on genetic groups, genetic lines Ptr4CL/CAld5H-72 and Ptr4CL/CAld5H-141 were handled as different genetic groups because of large chemical differences between them (Table 1).

The results of ANOVA are presented in Table 2. From among the covariates, the effect of specific gravity was found significant for $\sigma_{c,\parallel}$ but not for MOE. Diameter had no significant effect either on MOE or $\sigma_{c,\parallel}$. Age had a significant effect on MOE: Age Group 1 had significantly lower MOE than the other two groups; age did not affect $\sigma_{c,\parallel}$. Genetic group had a significant effect on both MOE and $\sigma_{c,\parallel}$. The effect of genetic lines within a genetic group was not significant. Age × genetic group interaction was found significant for MOE only, which resulted mainly from the crossover interaction between genetic groups PtrCAld5H and Ptr4CL/CAld5H-141 because their ranking changed between the first two age groups.

Table 1. Lignin content, alpha-cellulose content, and syringyl/guaiacy (S/G) ratio of wild type (WT) and transgenic aspen groups and of the different genetic lines within the genetic group.

	Genetic group	Ptr WT	Ptr 4CL (reduced lignin content)			Ptr CAld5H (increased S/G ratio)		Ptr 4CL/CAld5H (reduced lignin content and increased S/G ratio)	
	Genetic line	271	21	23	37	94	96	72	141
Lignin content[a] (%)	Genetic group	**21.6** ± 0.52		**14.8** ± 0.46		**19.9** ± 0.40		**16.7** ± 0.77	**19.1** ± 1.06
	Genetic line	21.6 ± 0.52	16.0 ± 0.79	14.4 ± 0.50	14.2 ± 1.06	19.9 ± 0.62	20.0 ± 0.68	16.7 ± 0.77	19.1 ± 1.06
Alpha-cellulose content[a] (%)	Genetic group	**41.3** ± 0.40		**43.3** ± 0.57		**40.1** ± 1.04		**42.3** ± 1.81	**38.6** ± 0.09
	Genetic line	41.3 ± 0.40	43.9 ± 1.02	43.0 ± 1.48	43.0 ± 1.03	39.2 ± 2.07	41.0 ± 0.76	42.3 ± 1.81	38.6 ± 0.09
S/G ratio[b]	Genetic group	**2.2**		**2.1**		**5.2**		**3.6**	**2.7**

[a] Determined by analytical chemistry for genetic lines and genetic groups. The values are means ± standard error of two to four samples from each genetic line. The values in bold are the mean chemical content values of the genetic groups.

[b] Published S/G ratio by Li et al (2003).

The individual MOE values at the green condition ranged 1422-6283 MPa. The mean MOE values for the different genetic groups (Table 3) were similar to values published for poplars by Bendtsen and Senft (1986) for the second year of growth in 30-yr-old trees (2590 MPa), Hernandez et al (1998) for 9-yr-old trees (5525 MPa), and by Coutand et al (2004) for 1-yr-old trees (2103 MPa). However, higher MOE values were reported in the Wood Handbook (FPL 1999) for mature quaking aspen (5900 MPa) and by Bjurhager et al (2008) for 15-mon-old European (6200 MPa) and hybrid aspen (5500 MPa) in microtension. Conversely, Peszlen (1998) measured lower values for three poplar clones in tension (1500, 1700, and 1800 MPa) and compression (1200, 1500, and 1600 MPa) for the first four growth rings. The coefficients of variation (COVs) of MOE for the different genetic groups ranged 11-17%, which were comparable to the 12% COV published by Bendtsen and Senft (1986) and the 23.7% COV published by Peszlen (1998).

The individual $\sigma_{c,\parallel}$ values for the green condition ranged 4.2-16.4 MPa. The mean values for the different genetic groups (Table 3) were lower than the 14.8 MPa published in the Wood Handbook (FPL 1999) for mature quaking aspen in the green condition. The results were similar to those reported for poplars by Bendtsen and Senft (1986) for the second year of growth in 30-yr-old trees (11.43 MPa), Hernandez et al (1998) for 9-yr-old trees (17.1 MPa), and Peszlen (1998) for the first four growth rings of three poplar clones (11.7, 11.9, and 12.3 MPa). The COVs of $\sigma_{c,\parallel}$ for the different genetic groups ranged 7-13%, which were comparable to the 11.1%

Table 2. Results of analysis of variance for modulus of elasticity and compression strength parallel to the grain of wild type and transgenic aspen.

Source	Modulus of elasticity df	MS	F	p	Compression strength parallel to the grain df	MS	F	p
Specific gravity	1	408002	0.25	0.6200	1	7.61	8.29	0.0047*
Diameter	1	318068	1.93	0.1675	1	1.02	1.12	0.2928
Age	2	1593148	9.65	0.0001*	2	2.21	2.41	0.0937
Genetic group	4	11547927	69.93	< 0.0001*	4	82.86	90.27	< 0.0001*
Genetic line (genetic group)	3	299419	1.81	0.1478	3	2.16	2.35	0.0751
Age × genetic group	8	1432570	8.67	< 0.0001*	8	0.91	0.98	0.4507
Error	134	165146			131	0.92		

* Statistically significant at the 5% significance level.

Table 3. Mean and coefficient of variation (COV) of the mechanical and physical properties of wild type (WT) and transgenic aspen groups.

	Genetic group	Ptr WT	Ptr 4CL (reduced lignin content)	Ptr CAld5H (increased S/G ratio)	Ptr 4CL/CAld5H (reduced lignin content and increased S/G ratio)	
	Genetic line	271	21 & 23 & 37	94 & 96	72	141
No. of specimens		23	56	39	14	22
Specific gravity[a]	Mean	0.38	0.35	0.37	0.32	0.37
	COV (%)	6	3	5	3	4
Diameter	Mean (mm)	8.7	9.0	6.0	7.6	8.6
	COV (%)	15	24	19	18	15
Modulus of elasticity	Mean (MPa)	4902 A	2892 B	4463 A	2085 C	4531 A
	COV (%)	12	12	13	22	20
Compression strength	Mean (MPa)	13.2 A	7.0 C	11.7 B	6.6 C	11.0 B
parallel to the grain[b]	COV (%)	8	12	12	15	9

[a] Specific gravity by Horvath (2009).
[b] Three samples less were used for compression test.
Note: The mean of genetic groups with common letters are not significantly different from each other as determined by Tukey multiple comparison test at $\alpha = 0.05$.

COV reported by Peszlen (1998) and the 9.72% COV reported by Bendtsen and Senft (1986).

All transgenic groups had lower mechanical properties than the wild type (Table 3). However, the MOE of genetic groups PtrCAld5H and Ptr4CL/CAld5H-141 were not significantly different from the wild type. Meanwhile, significant differences were found in MOE for genetic groups Ptr4CL and Ptr4CL/CAld5H-72. Similar to the MOE, significantly lower $\sigma_{c,\parallel}$ values were observed for genetic groups Ptr4CL and Ptr4CL/CAld5H-72 compared with the wild type. A statistically significant decrease was also observed in $\sigma_{c,\parallel}$ for genetic groups PtrCAld5H and Ptr4CL/CAld5H-141, but the decrease was smaller than for the other two transgenic groups. The simultaneous modification of lignin content and S/G ratio gave inconsistent results: Ptr4CL/CAld5H-72 always had the lowest mechanical properties, but genetic group Ptr4CL/CAld5H-141 had properties similar to the wild type aspen. This must be because of the differences in their chemical composition as discussed previously.

The results of this study are in agreement with the findings of Jones et al (2001) and Köhler and Spatz (2002) in which lower MOE was found for genetically modified *Arabidopsis thaliana* (L.) with reduced lignin content. However, the observed decrease in both mechanical properties for genetic groups with increased S/G ratio is in contradiction with Koehler and Telewski (2006) who reported an increase in MOE for poplar when the S/G ratio was increased.

MOE and $\sigma_{c,\parallel}$ were plotted against the total lignin content for samples that were included in both the mechanical tests and chemical analysis (Fig 2). There is a definite clustering in the data reflecting the genetic modification. A distinct pattern of increasing mechanical properties with increase in lignin content can be seen. The observed scatter in the data is lower for $\sigma_{c,\parallel}$ compared with MOE. This is in agreement with the widely known fact that lignin plays a significant role in the compression strength of the wood and standing tree (Wardrop 1971; Gindl 2001; Gindl and Teischinger 2002).

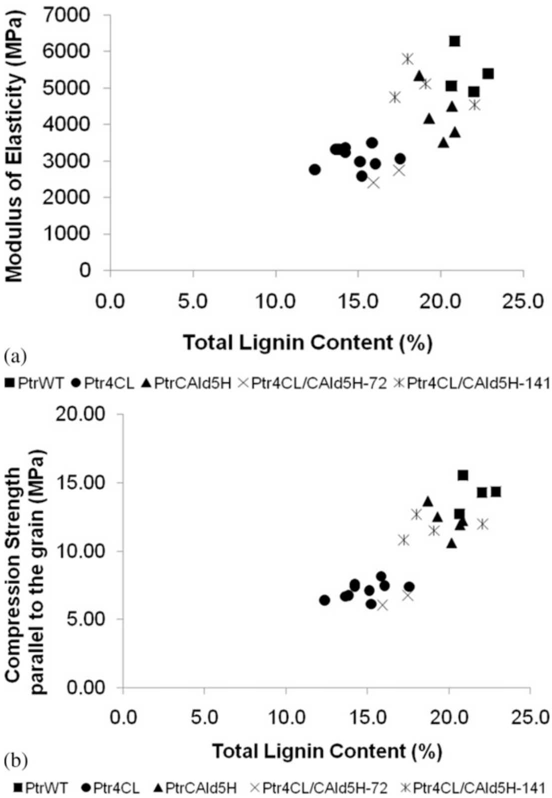

Figure 2. Modulus of elasticity (a) and compression strength parallel to the grain (b) vs total lignin content of wild type and transgenic aspen.

CONCLUSIONS

The genetic modifications used in this study have a negative effect on the mechanical properties of wood from young aspen trees. The reduction in lignin content causes a severe reduction in both MOE and compression strength; the increase in S/G ratio has only a minor effect. The simultaneous modification of lignin content and S/G ratio shows inconsistent results and needs further investigation. The observed scatter in the data is lower for compression strength parallel to the grain as opposed to MOE, indicating a stronger influence of lignin on the compression strength. The study underscores that genetic modification of the chemical structure has consequences on the mechanical properties of wood. Geneticists then must proceed with caution because these modifications can have serious implications on the tree's survival and in its utilization.

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