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Growth under field conditions affects lignin content and productivity in transgenic *Populus trichocarpa* with altered lignin biosynthesis

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ABSTRACT

This study evaluated the potential of transgenic *Populus trichocarpa* with antisense 4CL for reduced total lignin and sense Cald5H for increased S/G ratio in a short rotation woody cropping (SRWC) system for bioethanol production in the Southeast USA. Trees produced from tissue-culture were planted in the Coastal Plain, Piedmont, and Mountain regions of North Carolina, USA. Trees were observed for growth differences and biomass recorded for two coppices. Insoluble lignin and S/G ratio were determined by molecular beam mass spectroscopy after the second coppice. Survival, growth form, and biomass were very consistent within construct lines. Higher total lignin content and S/G ratio were positively correlated with total aboveground biomass. The low-lignin phenotype was not completely maintained in the field, with total lignin content increasing on average more than 30.0% at all sites by the second coppice The capacity to upregulate lignin in the event of environmental stress may have helped some low-lignin lines to survive. More research focused on promising construct lines in appropriate environmental conditions is needed to clarify if a significant reduction in lignin can be achieved on a plantation scale, and whether that reduction will translate into increased efficiency of enzymatic hydrolysis.

1. Introduction

Reliance on fossil energy for transportation fuels contributes to greenhouse gas emissions and environmental pollution, jeopardizes energy security, and ultimately is not sustainable. Energy from cellulosic biomass, however, has potential to decrease greenhouse gas emissions [1,2], can support the growth of rural economies, and when used to produce biofuels can reduce the need for oil and gas imports if desired [3].

In the USA, bioethanol has traditionally been produced from non-cellulosic sources such as the starch in corn grain (*Zea mays* ssp. *mays*). More recently, considerable attention has turned to cellulosic, perennial crops, such as grasses and fast-growing trees, as bioenergy feedstocks [4]. The current USA Renewable Fuels Standards set out in the Energy Independence and Security Act of 2007 mandates the use of 36

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billion gallons (136 billion liters) per year of renewable fuels by 2022, with 16 billion gallons (61 billion liters) per year produced from advanced cellulosic biofuels [5]. Dedicated plantations of fast-growing, genetically improved planting material will play an important role in meeting rising cellulosic feedstock demands. Short rotation woody cropping systems (SRWC) often rely on hardwood trees that sprout vigorously following coppice, and are fast-growing, such as species of *Populus*, *Salix*, *Acacia*, and *Eucalyptus* [6–9]. SRWC hardwood tree plantations have the potential to produce a renewable feedstock for biofuels while simultaneously engendering a suite of environmental and social benefits [4].

The product of SRWC is not just the biomass in its raw state, but the energy yield or the yield of the chemicals to be produced from the biomass [6]. About 70% of lignocellulosic biomass is composed of cellulosic carbohydrates (45% cellulose and 25% hemicelluloses) in a matrix of lignin that composes about 25% of the cell wall [10,11]. In order to produce ethanol, the cellulose and other cell wall polysaccharides need to be broken down by enzymes into fermentable sugars [12]. However, lignin hinders the enzymatic hydrolysis of cellulose [13]. In order to gain access to the cellulose, pretreatment with hot acid to open the macroscopic cell wall structure and break the interactions between lignin and cellulose is necessary. Pretreatment is costly, energy intensive, and produces substances that subsequently inhibit fermentation [14].

Substantial research has gone into elucidating the pathway of lignin biosynthesis in plants in order to lower the amount of total lignin and/or change its relative monolignol subunits to enhance saccharification efficiency. Lignin is synthesized from monolignol precursors: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, referred to as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units upon incorporation into the lignin polymer [15,16]. Angiosperm lignin is composed almost entirely of S and G units, and their relative abundance in the lignin polymer is an important feedstock characteristic. A syringyl-rich lignin is substantially easier to separate from cellulose than a guaiacyl-rich lignin, therefore substrates with high S/G ratios have been shown to increase the efficiency of biochemical conversion [17–19]. Indeed, it has been shown that for every unit increase in lignin S/G ratio, the rate of lignin removal roughly doubles [20]. Average S/G ratio in angiosperms ranges from approximately 2–2.5 [21].

Results from several studies indicate that total lignin and S/G ratio are regulated independently during lignin biosynthesis in *Populus*. Increases in S/G ratio have been achieved by the overexpression of coniferaldehyde 5-hydroxylase (CAld5H) [19,22–24] in the phenylpropanoid pathway. Overexpression of the Cald5H enzyme alone results in plants with substantially elevated syringyl monomer concentrations, but no difference in total lignin content [23].

While many transformations to lower total lignin content have been studied, our study focused on the insertion of the antisense 4-coumarate:coenzyme A ligase (4CL) gene to decrease total lignin content and the sense CAld5H gene to increase S/G ratio. Transgenic down-regulation of the 4CL gene has been demonstrated to limit lignin content in *Nicotiana* [25], *Arabidopsis* [26], and *Populus* [17,27–29]. Studies of transgenic, greenhouse grown trees have shown that modification of 4CL in *Populus* can achieve stable reductions in lignin content while modification of CAld5H can increase S/G ratio [17,19,21,23,27,28,30]. However, the repercussions of such transformations on plant growth, especially under field conditions, are still poorly understood.

In a 10 month greenhouse study, Hu et al. (1999) [27] found that *Populus tremuloides* trees with antisense 4CL transgene insertion expressed a 40–45% reduction in stem lignin content and displayed enhanced growth compared to wildtype. The transformed trees showed no loss of structural integrity at the cellular or whole plant level and no change in S/G ratio.

Subsequently, in a two year greenhouse study, Huntley et al. (2003) [23] found that *Populus tremula* × *Populus alba* trees altered with the sense CAld5H transgene had increased S/G ratios of up to 14.2, compared to a wildtype S/G ratio of 1.9. The transgenic trees had no change in total lignin content and no observed phenotypic differences from wildtype.

To verify that total lignin content and S/G ratio are regulated independently, Li et al. (2003) [21] studied transgenic *P. tremuloides* trees with the antisense 4CL transgene alone, the sense CAld5H transgene alone, and a combination of both genes for 10 months in the greenhouse. The trees with the antisense 4CL gene had a 30–40% reduction in total lignin content but no change in S/G ratio, while the trees with sense CAld5H gene had a 2.5-fold increase in S/G ratio but no change in total lignin content. Trees with both transformations had a 38–52% reduction in total lignin content and a 22–64% increase in S/G ratio. However, in contrast to the Hu et al. (1999) [27] study, the antisense 4CL transgenics did not have increased growth over wildtype.

Similar to Li et al. (2003) [21], Hancock et al. (2007) [17] studied *P. tremuloides* with the antisense 4CL gene, sense CAld5H gene, and a combination of both genes in an eight month greenhouse study. Notably, and in contrast to Huntley et al. (2003) [23], researchers found that trees with increased S/G ratio had significantly decreased above-ground and below-ground biomass than the wildtype and antisense 4CL trees. In addition, in contrast to Hu et al. (1999) [27] and in corroboration with Li et al. (2003) [21], trees with the antisense 4CL transgene did not have higher biomass than wildtype.

Roque-Rivera et al. (2011) [30] also examined *P. tremuloides* with the antisense 4CL gene, sense CAld5H gene, and a combination of both genes grown in the greenhouse for four months. Trees with antisense 4CL alone expressed a 35% reduction in lignin content and had no difference in cumulative growth from wildtype. However, trees with high S/G ratio, a 100–150% increase of wildtype, produced significantly less biomass than antisense 4CL alone or wildtype.

The results of these studies suggest that significant reductions in total stem lignin content in *Populus* may not positively affect biomass accumulation as previously thought, especially if S/G ratio is simultaneously increased. However, few studies [29,31,32] have observed how these modifications affect growth under field conditions, and to the best of our knowledge, no studies have observed how growth, total lignin content and lignin composition are affected over several coppice cycles, across a range of field environments. This study aimed to evaluate the potential of trees with modified lignin biosynthesis to be grown in a SRWC system for bioethanol production in the Southeast USA. The objective of this investigation was to evaluate the growth and physiological

response of *Populus trichocarpa* with antisense 4CL for reduced total lignin content, and antisense 4CL and sense Cald5H for reduced total lignin content and increased S/G ratio, under a range environmental conditions common to the Southeast USA, pre- and post-coppice. We hypothesized that transgenic trees with antisense 4CL would have increased growth over wildtype trees because of the reduced cost of lignin biosynthesis. We also hypothesized that the transgenic and wildtype trees would grow best in the Mountain region of North Carolina as this region is most similar to the range of the native, wildtype trees.

2. Materials and methods

2.1. Plant material

Plant material was derived from a clone of *P. trichocarpa*, Nisqually-1, that originated from a female clone found near the Nisqually River in central Washington, USA, in 1995. The full genome of *P. trichocarpa* Nisqually-1 was sequenced in 2006 by the U.S. Department of Energy Joint Genome Initiative [33]. The *Populus* genus in general has diverse phenotypes, rapid growth, and early reproductive maturity, making it an excellent candidate for selective breeding and large-scale sustainable plantation forestry [33]. *P. trichocarpa* is the largest of the American poplars and the largest hardwood tree in western North America. It grows primarily on moist sites west of the Rocky Mountains, most productively in the bottom lands of major streams and rivers. Planted cuttings produce deep and widespread root systems if soil conditions allow. *P. trichocarpa* is classified as very shade intolerant and in its native range has rapid initial growth to outcompete slower growing associated species [34].

Plant material was received from Dr. Vincent Chiang (North Carolina State University Forest Biotechnology group) in the form of twelve transgenic lines (construct lines) within three constructs (Table 1), along with the untransformed clone as a control (wildtype).

The wildtype clone had an average lignin content of 22% and an S/G ratio of 2.5. Two of the constructs had the antisense 4CL transformation only, while the third had antisense 4CL and sense CAld5h. The first construct, As4CL, was transformed using the 35s promoter from cauliflower mosaic caulimovirus and 4CL from *P. trichocarpa* in antisense orientation. We studied six construct lines within the As4CL construct, with total lignin reductions of 12–46% of the control (total lignin contents of 11.8–21.0%), and S/G ratios from 2.0 to 3.7 in the original greenhouse grown plants.

The second construct, PT, was transformed using the 4CL promoter from *P. tremuloides* and 4CL from *P. tremuloides* in antisense orientation. We studied four construct lines within the PT construct, with total lignin reductions between 7 and 37% of the control (total lignin contents of 13.9–20.5%), and S/G ratios from 1.8 to 3.4 in the original greenhouse grown plants.

The third construct, CH, was transformed using the 4CL promoter from *P. trichocarpa*, 4CL from *P. trichocarpa* in antisense orientation, and CAld5H from *Liquidambar styraciflua* in sense orientation. We studied two construct lines within the CH construct, with total lignin reductions of 24–29% of the control (total lignin contents of 15.7–16.7%), and S/G ratios from 1.8 to 2.9 in the original greenhouse grown plants.

2.2. Sites and experimental design

Trees were planted in the Coastal Plain, Piedmont, and Mountain regions of North Carolina, USA. The Mountain site was located in Fletcher, Henderson County, NC. Mean annual precipitation at the Mountain site ranges from 114 to 178 cm, mean annual temperature 7.8–13.8 °C, frost free period 130–180 days, and elevation 647 m. The soil is Hayesville loam, a well-drained loam. The Piedmont site was located in Oxford, Granville County, NC. Mean annual precipitation at the Piedmont site ranges from 94 to 152 cm, mean annual temperature 15.0–18.8 °C, frost free period 200–240 days, and elevation 146 m. The soil is Helena sandy loam, a moderately well-drained sandy-loam. The Coastal Plain site was located in Wallace, Duplin County, NC. Mean annual precipitation at the Coastal Plain site ranges from 94 to 140 cm, mean annual temperature 15–21 °C, frost free period 210–265 days, and elevation 116 m. The soil is Noboco loamy fine sand.

The transgenic trees were produced from tissue-culture under greenhouse conditions during fall of 2008 and transferred to a covered but not heated greenhouse in January 2009 to acclimate the trees to cold in preparation for planting out. Trees were kept in small pots in the cold greenhouse until planting in April 2009. A minor aphid infestation before

Table 1 – Promoters, genes, and terminators used to create constructs of transgenic *Populus trichocarpa*. (Data provided by Dr. Vincent Chiang, North Carolina State University).

Construct	Promoter	Gene(s)	Terminator	Hypothesized phenotype	No. of lines
As4CL	35s from Cauliflower mosaic caulimovirus	antisense 4CL from *P. trichocarpa*	Nopaline synthase from *Agrobacterium tumefaciens*	• Decreased lignin • Increased cellulose • Increased growth	6
CH	4CL promoter from *P. trichocarpa*	• antisense 4CL from *P. trichocarpa* • sense CAld5H from *Liquidambar styraciflua*	Nopaline synthase from *Agrobacterium tumefaciens*	• Decreased lignin • Increased cellulose • Increased growth • Increased S/G	2
PT	4CL promoter from *P. tremuloides*	antisense 4CL from *P. tremuloides*	Nopaline synthase from *Agrobacterium tumefaciens*	• Decreased lignin • Increased cellulose • Increased growth	4

planting out was controlled by spraying foliage with volk oil. Trees were planted in the field in rows at a spacing of 0.6 m within rows, and 1.2 m between rows to achieve a final planting density of approximately 13,513 trees ha^{-1}. Each row consisted of a single construct line, with two rows of wildtype at each end of each plot. At the Mountain site, 78 trees were planted over an area of approximately 45 m^2. At the Piedmont and Coastal Plain sites, 149 trees were planted over an area of approximately 100 m^2. Due to the small size of the plots, we considered that within-plot variability of environmental conditions would be negligible.

Throughout the first growing season, all three sites were watered by drip tape irrigation to aid in establishment. The drip tape was removed after the establishment year so we could adequately test how the trees grew without water inputs under the given environmental conditions of each region. Throughout the experiment, the site was hand weeded and mowed with a push mower to decrease weed competition. If defoliators pests occurred, they were controlled with Conserve SC (Spinosad, Dow Agrosciences) according to the label.

2.3. Growth measurements and tree biomass

Height, diameter, and number of stems were measured monthly or bi-monthly between March and October in 2009, 2010, and 2011. The heights of the two tallest stems were measured to the nearest 0.5 cm with a meter stick or height pole. Stem diameter was measured on the same stems to the nearest 0.1 mm with digital calipers at 10 cm from ground level. In January 2011, the number of sylleptic branches for the 2010 growing season was counted on every tree. In the analysis, lines As4CL-1-5 were excluded from sylleptic branch comparisons because of the difficultly of distinguishing sylleptic branches from proleptic branches or stems because of the extreme bush-like phenotype that occurred in these construct lines (Fig. 1). In January 2010, all trees were coppiced at 5 cm above the base of the tree, and the biomass divided into stems and branches. Wood was air-dried until constant mass and then weighed. Subsamples from each tree were oven-dried at 65 °C to determine water content of air-dried samples, and a correction factor for water weight was subtracted from the mass of the air-dried samples to determine oven-dried mass. In January 2011, trees were coppiced at 10 cm above the base of the tree, and the biomass divided into stems and branches. Wood was weighed fresh in the field, and subsamples were oven-dried at 65 °C to determine water content of fresh samples. A correction factor for water weight was subtracted from the mass of the fresh samples to determine oven-dried mass.

2.4. Wood lignin and S/G

After the 2011 harvest, four bark-free wood samples per construct line from individual trees (e.g. four trees per line) and site were oven-dried and milled to pass a fine mesh using a Wiley mill. Klason calibrated estimates of insoluble lignin and S/G ratio were determined by molecular beam mass spectroscopy (MBMS) at the National Renewable Energy Laboratory (NREL) in Golden, CO., U.S [35].

Fig. 1 – Example of consistency of growth form within construct lines as well as the stunted growth of the As4CL low-lignin construct lines. Picture is from the Mountain site, October 2010 after one year of growth in the field. Construct lines from left to right; wildtype, As4CL-1, As4CL-2, As4CL-3, PT-1.

2.5. Statistical analysis

Due to the large variance of initial total lignin content and S/G ratio between the construct lines within the same construct, we chose to use construct line as the experimental unit, with individual trees within each construct line as replicates. We also chose to limit our statistical comparisons to differences in growth between construct lines within the same site. We performed one-way, univariate ANOVAs with construct line as the main effect and total lignin, S/G ratio, biomass coppice one, height coppice one, sylleptic branches, biomass coppice two, height coppice two, and number of stems after coppice as dependent variables. If differences between means were found, we used the Dunnett Procedure to test for differences between treatments means and the wildtype mean. Means were considered significantly different at $P \leq 0.05$.

Least squares regression was used to examine relationships between total lignin concentration and S/G ratio, total lignin concentration and biomass, and S/G ratio and biomass. Because of strong multicollinearity between total lignin content and S/G ratio, we used simple linear regression to estimate the effect of total lignin on biomass and S/G ratio on biomass separately. All statistical analyses were carried out using SAS JMP 8 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Survival, growth, tree architecture, and biomass

Within construct line variability for survival, growth, and tree architecture was low. Each construct line quickly conformed into a recognizable phenotype that was maintained in all specimens, adopting either a normal tree architecture with growth concentrated in one or two stems, or a stunted growth

architecture, with growth spread between multiple, short stems (Fig. 1). Patterns of survival and growth among the construct lines were generally maintained across sites and across years. Growth and tree architecture differences between construct lines become more pronounced as the field trial progressed, with low-performing construct lines becoming even less successful or dying out over time or after the first coppice, and high-performing construct lines continuing to thrive after the first coppice.

The Mountain site was markedly the most productive site for wildtype and transgenic construct lines (Fig. 2). After three years in the field, 98.7% of the trees survived at the Mountain site (Table 2), 77.2% survived at the Coastal Plain site (Table 3), and 61.8% survived at the Piedmont site (Table 4). In the first coppice, average biomass at the Mountain site was twice as great as the Coastal Plain site and eight times greater than at the Piedmont site. In the second coppice, average biomass at the Mountain site was five times greater than at the Coastal Plain site, and 12 times greater than at the Piedmont site. The number of stems that grew following the first coppice appeared correlated with biomass for the second coppice at the Piedmont and Coastal Plain sites, with fewer stems correlating to lower biomass, but the correlation did not persist at the Mountain site.

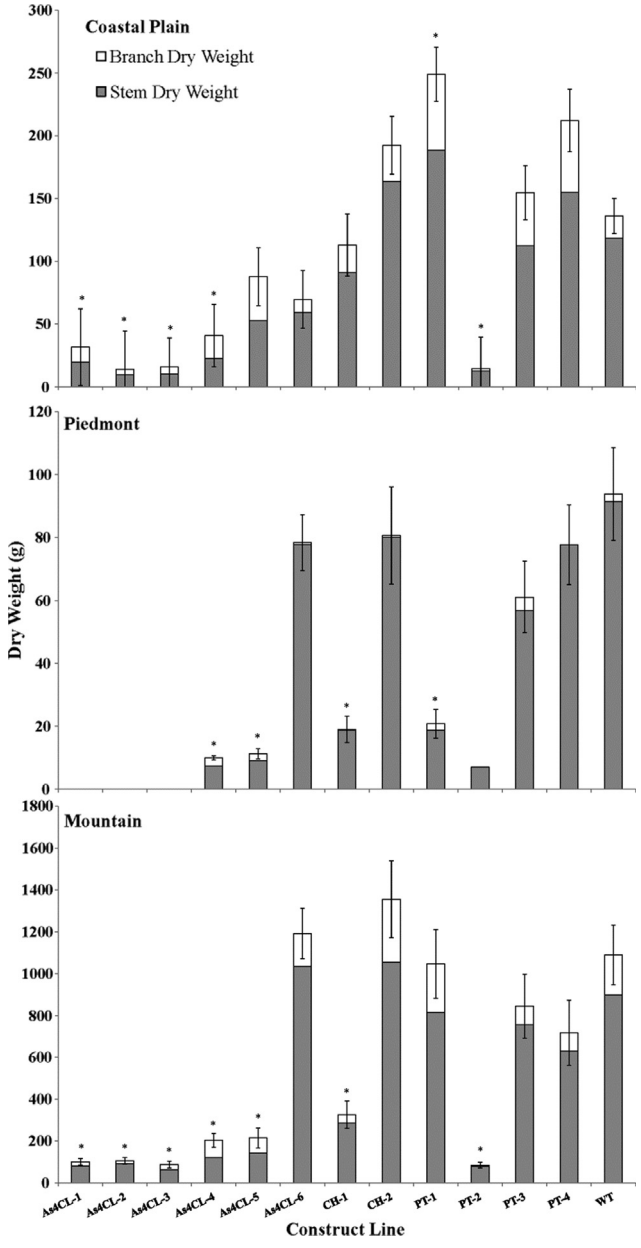

Fig. 2 – Biomass at the time of the second coppice. Construct lines significantly different from wildtype are denoted with an asterisk (*). Note different *X*-axis scales due to the widely divergent productivity between sites.

At all the sites, spring and early summer were the most productive times of the growing season. At the Coastal Plain and Piedmont sites, height and diameter growth on all construct lines generally stopped by the end of June. However, at the Mountain site, height and diameter growth stopped only for the low-performing lines, As4CL-1 through 5, CH-1, and PT-2, while the high-performing lines, As4CL-6, CH-2, PT-1, PT-3, PT-4, and wildtype, continued to grow until September. At all sites, construct lines As4CL-1 through 5 exhibited a loss of apical dominance, stunted growth, repeated dieback of the leader stem, copious epicormic branches, and increased sensitivity to pathogens (data not shown).

Overall, higher total lignin and S/G ratio were positively correlated with total aboveground biomass (Fig. 3) and with each other (Fig. 4). Total lignin concentration and S/G ratio were strongly positively correlated at all sites (Mountain $r^2 = 0.85$, $p < 0.0001$, $n = 52$; Piedmont. $r^2 = 0.82$, $p < 0.0001$, $n = 35$; Coastal Plain $r^2 = 0.88$, $p < 0.0001$, $n = 45$). However, all prediction equations using total lignin concentration or S/G ratio alone as explanatory variables for total aboveground biomass explained less than 42% of the variation (data not shown). Survival, tree architecture, and biomass were very consistent within construct lines. Generally, lines that were originally engineered with the lowest total lignin (less than 16.2%) had the worst survival and growth (As4CL-1 through 5, CH-1, PT-2). However, one construct line, PT-1, with original total lignin of 13.9% was the most productive line at the Coastal Plain site and had productivity similar to wildtype at the Mountain site.

Further, in the second coppice, several construct lines had total lignin percentages significantly less than wildtype without significant loss in biomass. At the Coastal Plain site this included four construct lines, As4CL-5 (lignin 20.6%, S/G 1.8), CH-1 (lignin 20.7%, S/G 1.2), PT-1 (lignin 21.9%, S/G 1.6), and PT-3 (lignin 20.5%, S/G 1.4),compared to wildtype (lignin 25.5%, S/G 1.9). At the Piedmont site this included two construct lines, As4CL-6 (lignin 23.3%, S/G 1.5), and PT-3(lignin 21.6%, S/G 1.4), compared to wildtype (lignin 26.4%, S/G 1.7). However, at the Mountain site, all construct lines with significantly lower total lignin and S/G ratio had significantly lower aboveground biomass than wildtype.

Construct line PT-1 at the Coastal Plain site was the only construct line to have significantly greater mean whole tree biomass than wildtype, with about 2.5 times wildtype biomass in the first coppice, and 1.8 times wildtype biomass in the second coppice (coppice one: $p < 0.0001$; coppice two: $p = 0.0004$). PT-1 at the Coastal Plain site also had significantly lower ($p = 0.0470$) total lignin percentage than wildtype after

Table 2 – Means, SE, and Dunnett *p*-values for lignin and S/G ratio after two years of growth in the field, biomass coppice one, height coppice one, sylleptic branches, biomass coppice two, height coppice two, number of stems, and percent survival at the Mountain site. Bold typeface denotes significant difference from wildtype.

	WT	As4CL-1	As4CL-2	As4CL-3	As4CL-4	As4CL-5	As4CL-6	CH-1	CH-2	PT-1	PT-2	PT-3	PT-4
Lignin (%)[a]	**23.4**	**18.9**	**19.7**	**17.8**	**19.7**	**20**	23.7	**18.9**	22.8	23.1	23.8	23.4	23.3
SE	**0.3**	**0.3**	**0.5**	**0.4**	**0.8**	**0.5**	0.3	**0.5**	0.2	0.5	0.6	0.2	0.7
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	0.9997	**<0.0001**	0.9727	1	0.995	1	1
S/G ratio[a]	**2**	**0.7**	**0.8**	**0.8**	**1**	**1.1**	2	**1.1**	2.1	2.1	2	2.1	2.1
SE	**0.04**	**0.03**	**0.1**	**0.03**	**0.1**	**0.2**	0.04	**0.1**	0.03	0.1	0.1	0.04	0.1
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	1	**<0.0001**	0.9942	0.9903	1	0.8329	0.9903
Biomass coppice 1 (g)	**692.1**	183.1	182.7	154.8	251	371.6	968.8	234.1	707.1	637.7	**81.3**	903.5	352.6
SE	**82.3**	168.5	168.5	168.5	188.4	168.5	188.3	168.5	168.5	168.5	**168.5**	168.5	188.4
Dunnett		0.0936	0.0932	0.0637	0.3253	0.636	0.8799	0.1778	1	1	**0.0215**	0.9606	0.683
Ht. coppice 1 (cm)	**299.4**	**75.6**	**75.2**	**70**	**100.3**	**88.2**	367.9	**140.6**	314.6	279.3	**150.6**	318.5	298.9
SE	**12**	**23.9**	**23.9**	**23.9**	**26.8**	**23.9**	26.8	**23.9**	23.9	23.9	**23.9**	23.9	26.8
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.2192	**<0.0001**	0.999	0.9986	**<0.0001**	0.9991	1
Sylleptic branches[b]	**11.5**						9.8	2.2	10.6	**21.8**	2.2	19	6
SE	**1.6**						3.6	3.2	3.3	**3.2**	3.2	3.2	3.6
Dunnett							0.9992	0.0864	1	**0.0438**	0.0864	0.2516	0.6975
Biomass coppice 2 (g)	**1088.9**	**100.3**	**105.4**	**87.3**	**202.8**	**214.4**	1191.8	**325.5**	1355.5	1046.3	**84.3**	844.2	717.5
SE	**88.5**	**177.1**	**177.1**	**177.1**	**198**	**177.1**	198	**177.1**	177.1	177.1	**177.1**	177.1	198
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**0.0015**	**0.0005**	1	**0.0032**	0.8767	1	**<0.0001**	0.9257	0.6316
Ht. coppice 2 (cm)	**325.2**	**94.6**	**105**	**80.2**	**112**	**120.8**	364	**178.8**	365.8	336.6	**175**	346.6	348.3
SE	**6.2**	**12.4**	**12.4**	**12.4**	**13.8**	**12.4**	13.9	**12.4**	12.4	12.4	**12.4**	12.4	13.9
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.135	**<0.0001**	0.0525	0.9967	**<0.0001**	0.7583	0.776
No. stems coppice 2	**20**	20.8	14.4	17.6	12	10	19.8	16.6	24.2	21.4	**8.4**	23	19
SE	**1.6**	3.3	3.3	3.3	3.9	3.3	3.7	3.3	3.3	3.3	**3.3**	3.3	3.7
Dunnett		1	0.7721	0.9996	0.4267	0.0902	1	0.9911	0.9559	1	**0.0279**	0.9969	1
% survival[a]	**100**	100	100	100	80	100	100	100	100	100	100	100	100

[a] At time of the second coppice.
[b] At time of the first coppice.

Table 3 – Means, SE, and Dunnett *p*-values for lignin and S/G ratio after two years of growth in the field, biomass coppice one, height coppice one, sylleptic branches, biomass coppice two, height coppice two, number of stems, and percent survival at the Piedmont site. Bold typeface denotes significant difference from wildtype.

	WT	As4CL-1	As4CL-2	As4CL-3	As4CL-4	As4CL-5	As4CL-6	CH-1	CH-2	PT-1	PT-2	PT-3	PT-4
Lignin (%)[a]	**26.4**				**19.1**	**17.7**	**23.3**	**19.8**	26	**23**		**21.6**	24.8
SE	**0.4**				**1.2**	**0.7**	**0.7**	**0.1**	0.3	**0.8**		**1**	0.2
Dunnett					**<0.0001**	**<0.0001**	**0.0175**	**<0.0001**	0.9989	**0.0076**		**0.0001**	0.4286
S/G ratio[a]	**1.7**				**1.1**	**0.7**	1.5	**1**	2	1.7		1.4	1.9
SE	**0.1**				**0.2**	**0.02**	0.1	**0.02**	0.02	0.1		0.2	0.1
Dunnett					**0.0006**	**<0.0001**	0.6885	**0.0002**	0.324	1		0.1828	0.6717
Biomass coppice 1 (g)	**81**	33.8	24.6	**19.1**	21.5	48	83	60	76.9	77.3	**13.6**	68.2	66.5
SE	**8.1**	27	19.1	**15.6**	15.6	13.5	12.1	13.5	12.7	12.7	**14.4**	12.7	13.5
Dunnett		0.6437	0.0814	**0.0076**	0.0119	0.3369	1	0.875	1	1	**0.0012**	0.9953	0.9902
Ht. coppice 1 (cm)	**136.1**	**29.5**	**29.2**	**20.1**	**64.7**	**60.1**	**162.5**	**88**	150	104.6	**84.6**	109.3	147.5
SE	**5.7**	**20.2**	**16.5**	**11.6**	**9**	**10.8**	**10.1**	**11.6**	10.1	10.1	**10.8**	10.1	10.1
Dunnett		**0.001**	**<0.0001**	**0.001**	**<0.0001**	**<0.0001**	0.2361	**0.0041**	0.9368	0.0829	**0.007**	0.2188	0.9848
Sylleptic branches[b]	**3.8**						0.5	1	0.5	7.9	0.1	5.5	0.8
SE	**0.8**						1.4	1.6	1.4	1.5	1.5	1.4	1.4
Dunnett							0.3025	0.6373	0.3025	0.1507	0.2464	0.9273	0.396
Biomass coppice 2 (g)	**93.8**				**9.9**	**11.1**	78.3	**19**	80.6	**20.8**	7	61.1	77.6
SE	**9.2**				**18.3**	**13.9**	13	**13**	13	**13**	36.7	13	13
Dunnett					**0.001**	**<0.0001**	0.9543	**0.0001**	0.9826	**0.0002**	0.1775	0.2868	0.9416
Ht. coppice 2 (cm)	**137.5**				**44.3**	**50.7**	135.8	**74.8**	131.1	**94.4**	76	119.9	143.6
SE	**7.3**				**14.6**	**11**	10.3	**10.3**	10.3	**10.3**	29.2	10.3	10.3
Dunnett					**<0.0001**	**<0.0001**	1	**<0.0001**	0.9996	**0.0094**	0.2954	0.7458	0.9997
No. stems coppice 2	**15.4**				**6.5**	**8.1**	13.3	**6.3**	12.9	**4.4**	1	12.4	10.8
SE	**1.3**				**2.6**	**2**	1.8	**1.8**	1.8	**1.8**	5.2	1.8	1.8
Dunnett					**0.0238**	**0.0225**	0.9675	**0.001**	0.8924	**<0.0001**	0.0669	0.763	0.2677
% survival[a]	**57.1**	0	0	0	50	87.5	100	100	100	100	14.3	100	100

[a] At time of the second coppice.
[b] At time of the first coppice.

Table 4 – Means, SE, and Dunnett *p*-values for lignin and S/G ratio after two years of growth in the field, biomass coppice one, height coppice one, sylleptic branches, biomass coppice two, height coppice two, number of stems, and percent survival at the Coastal Plain site. Bold typeface denotes significant difference from wildtype.

	WT	As4CL-1	As4CL-2	As4CL-3	As4CL-4	As4CL-5	As4CL-6	CH-1	CH-2	PT-1	PT-2	PT-3	PT-4
Lignin (%)[a]	**25.5**		**16.1**	**17.7**	**19.6**	**20.6**	25.6	**20.7**	26.1	**21.9**	26.8	**20.5**	25.4
SE	**0.5**		**0.6**	**1**	**1.1**	**1.9**	0.4	**0.5**	0.3	**1**	0.4	**0.9**	0.3
Dunnett			**<0.0001**	**<0.0001**	**0.0003**	**0.003**	1	**0.0226**	0.9998	**0.047**	0.9307	**0.0023**	1
S/G ratio[a]	**1.9**		**0.9**	**1**	**1.2**	**1.2**	1.8	**1.2**	2	1.6	1.8	1.4	1.8
SE	**0.1**		**0.1**	**0.04**	**0.1**	**0.3**	0.1	**0.1**	0.1	0.1	0.04	0.1	0.04
Dunnett			**<0.0001**	**0.0007**	**0.0028**	**0.0021**	0.9981	**0.0131**	0.9194	0.5962	1	0.0683	1
Biomass coppice 1 (g)	**237.2**	77.1	92.4	96.3	131	343.6	167	251.6	169	**633.8**	**43.8**	298.9	265
SE	**32.6**	71.1	63.6	53.7	53.7	53.7	58.1	58.1	53.7	**50.3**	**58.1**	50.3	58.1
Dunnett		0.3652	0.3785	0.2505	0.6289	0.6265	0.971	1	0.9645	**<0.0001**	**0.0507**	0.9758	1
Ht. coppice 1 (cm)	**136.6**	**39.8**	**37**	**43.3**	**55.6**	**78.9**	126.7	**102.4**	131	160.5	**87.3**	123.5	148.3
SE	**4.6**	**10**	**9**	**7.6**	**7.6**	**7.6**	8.2	**8.2**	7.6	7.1	**8.2**	7.1	8.2
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.9702	**0.0055**	0.9997	0.0639	**<0.0001**	0.7332	0.9137
Sylleptic branches[b]	**5.1**						1	5	9.3	**47.3**	3.7	13.1	6.7
SE	**2.1**						3.7	3.7	3.4	**3.2**	3.7	3.2	3.7
Dunnett							0.9267	1	0.8843	**<0.0001**	0.9999	0.219	0.9997
Biomass coppice 2 (g)	**136.1**	**31.6**	**13.8**	**15.8**	**40.8**	87.7	69.6	112.9	192.5	**249.1**	**14.6**	154.6	212.2
SE	**14**	**30.5**	**30.5**	**23.1**	**24.9**	23.1	23.1	24.9	23.1	**21.6**	**24.9**	21.6	24.9
Dunnett		**0.0281**	**0.0054**	**0.0003**	**0.0144**	0.5476	0.1534	0.9965	0.3398	**0.0004**	**0.0007**	0.9989	0.0962
Ht. coppice 2 (cm)	**148.8**	**62.3**	**49.3**	**54.1**	**64.5**	**93.1**	142.9	128.6	161.3	158.3	**73.2**	152	172.3
SE	**5.2**	**11.2**	**13**	**5.2**	**9.2**	**8.5**	8.5	9.2	8.5	8	**9.2**	8	9.2
Dunnett		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	0.9998	0.4662	0.911	0.9815	**<0.0001**	1	0.2594
No. stems coppice 2	**15.8**	**3.5**	7.6	**6.4**	**2.7**	**6.6**	9.7	13.5	18.6	10.8	**4.8**	6.5	18.2
SE	**1.3**	**2.8**	2.5	**2.1**	**2.3**	**2.1**	2.1	2.3	2.1	2	**2.3**	2	2.3
Dunnett		**0.0019**	0.0535	**0.0039**	**<0.0001**	**0.0047**	0.164	0.993	0.9644	0.3156	**0.001**	0.0023	0.9934
% survival[a]	**67.9**	50	50	87.5	75	87.5	87.5	75	87.5	100	85.7	100	75

[a] At time of the second coppice.
[b] At time of the first coppice.

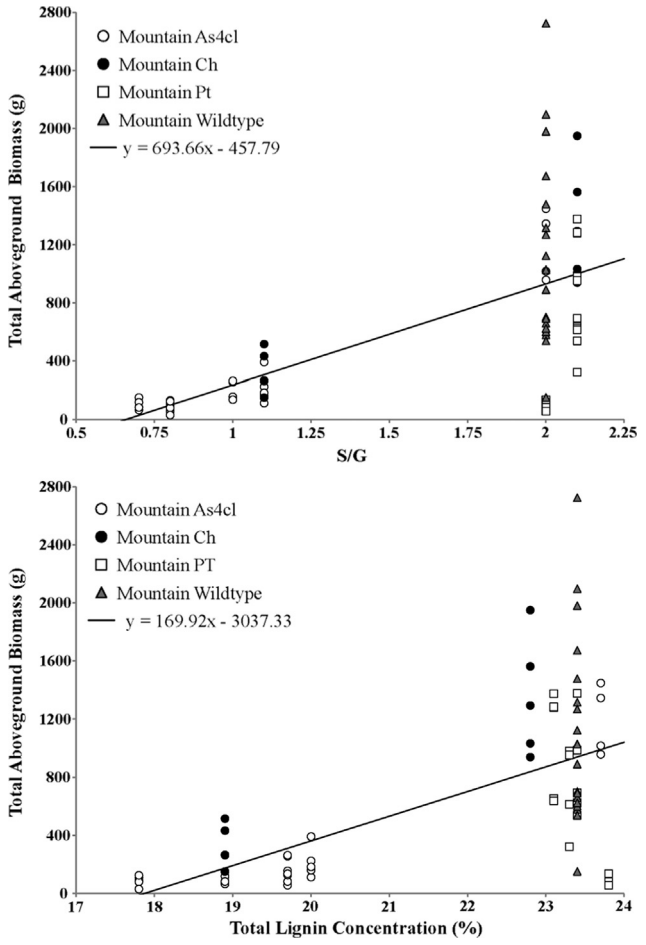

Fig. 3 – One year aboveground biomass growth after coppice one at the Mountain site by total lignin concentration ($r^2 = 0.36$, $p < 0.0001$, $n = 77$) and S/G (Adj. $r^2 = 0.42$, $p < 0.0001$, $n = 77$).

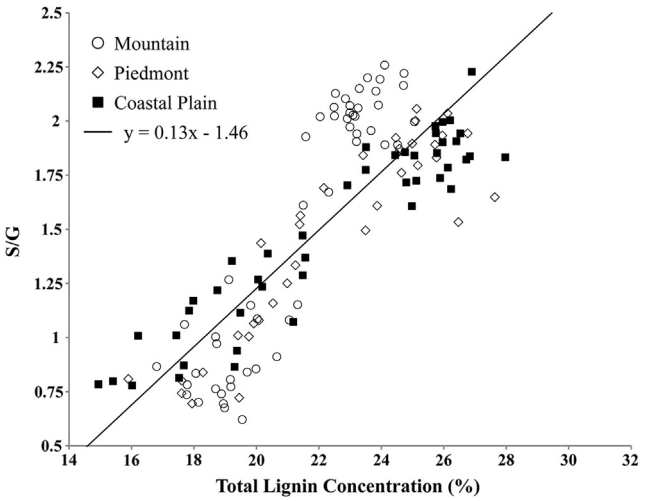

Fig. 4 – S/G ratio by total lignin concentration for Mountain site ($r^2 = 0.85$, $p < 0.0001$, $n = 52$), the Piedmont site ($r^2 = 0.82$, $p < 0.0001$, $n = 35$) and Coastal Plain site ($r^2 = 0.88$, $p < 0.0001$, $n = 45$) sites after three years in field environment.

Table 5 – Initial greenhouse lignin concentration and S/G ratio, field values and percent change of *Populus trichocarpa* transgenically modified for decreased wood lignin after 3 years growth in the field.

		WT	As4CL-1	As4CL-2	As4CL-3	As4CL-4	As4CL-5	As4CL-6	CH-1	CH-2	PT-1	PT-2	PT-3	PT-4	Average
Total lignin year 3	Initial lignin	22.00	11.8	12.1	13.4	15	15.7	21	15.7	16.7	13.9	16.2	17.4	20.5	16.3
	Coastal Plain	25.50		16.1	17.7	19.6	20.6	25.6	20.7	26.1	21.9	26.8	20.5	25.4	**22.2**
	Piedmont	26.4				19.1	17.7	23.3	19.8	26	23		21.6	24.8	**22.4**
	Mountain	23.4	18.9	19.7	17.8	19.7	20	23.7	18.9	22.8	23.1	23.8	23.4	23.3	**21.4**
	Average	**25.1**	**18.9**	**17.9**	**17.8**	**19.5**	**19.4**	**24.2**	**19.8**	**25.0**	**22.7**	**25.3**	**21.8**	**24.5**	
Δ Lignin (%)	Coastal Plain	15.9		33.1	32.1	30.7	31.2	21.9	31.8	56.3	57.6	65.4	17.8	23.9	**36.6**
	Piedmont	20.0				27.3	12.7	11.0	26.1	55.7	65.5		24.1	21.0	**37.8**
	Mountain	6.4	60.2	62.8	32.8	31.3	27.4	12.9	20.4	36.5	66.2	46.9	34.5	13.7	**31.7**
	Average	**14.1**	**60.2**	**47.9**	**32.5**	**29.8**	**23.8**	**15.2**	**26.1**	**49.5**	**63.1**	**56.2**	**25.5**	**19.5**	
	Initial S/G	2.50	2.3	3.4	3	3.2	3.7	2.3	1.8	2.9	3.4	2	3.6	2.7	**2.8**
S/G Ratio Year 3	Coastal Plain	1.9		0.9	1	1.2	1.2	1.8	1.2	2	1.6	1.8	1.4	1.8	**1.5**
	Piedmont	1.7				1.1	0.7	1.5	1	2	1.7		1.4	1.9	**1.4**
	Mountain	2	0.7	0.8	0.8	1	1.1	2	1.1	2.1	2.1	2	2.1	2.1	**1.5**
	Average	**1.9**	**0.7**	**0.9**	**0.9**	**1.1**	**1.0**	**1.8**	**1.1**	**2.0**	**1.8**	**1.9**	**1.6**	**1.9**	
Δ S/G Ratio (%)	Coastal Plain	−24.0		−73.5	−66.7	−62.5	−67.6	−21.7	−33.3	−31.0	−52.9	−10.0	−61.1	−33.3	**−47.6**
	Piedmont	−32.0				−65.6	−81.1	−34.8	−44.4	−31.0	−50.0	−100.0	−61.1	−29.6	**−49.0**
	Mountain	−20.0	−69.6	−76.5	−73.3	−68.8	−70.3	−13.0	−38.9	−27.6	−38.2	0.0	−41.7	−22.2	**−45.9**
	Average	**−25.3**	**−69.6**	**−75.0**	**−70.0**	**−65.6**	**−73.0**	**−23.2**	**−38.9**	**−29.9**	**−47.1**	**−36.7**	**−54.6**	**−28.4**	

three years in the field, with wildtype at 25.5% (SE 0.5) and PT-1 with 21.9% (SE 1.0). S/G ratio did not differ ($p = 0.5962$) between wildtype (1.9 (SE 0.1)) and PT-1 (1.6 (SE 0.1)) at the Coastal Plain site. PT-1 was also the only construct line to have significantly greater sylleptic branches than wildtype, with three times as many sylleptic branches than wildtype at the Mountain site, and seven times as many as wildtype at the Coastal Plain site.

The mean biomass accumulation of wildtype trees only at the first coppice (two year's growth) assuming 13,513 trees ha^{-1} (the approximate study planting density) was 9.4 dry Mg ha^{-1} at the Mountain site, 3.2 dry Mg ha^{-1} at the Coastal Plain site, and 1.1 dry Mg ha^{-1} at the Piedmont site. The mean biomass accumulation of wildtype trees only at the second coppice (one year's growth) assuming 13,513 trees ha^{-1} was 14.7 dry Mg ha^{-1} at the Mountain site, 1.8 dry Mg ha^{-1} at the Coastal Plain site, and 1.3 dry Mg ha^{-1} at the Piedmont site.

3.2. *Lignin change from greenhouse levels*

After three years in the field environment, total wood lignin concentration increased in all construct lines at all sites relative to the original greenhouse values (Table 5). The original total lignin percentages of the transformed trees ranged from 11.8% to 20.5%, with wildtype at 22.0%. At the time of the second coppice, the range was from 16.1% to 26.8%. Total lignin concentration increased on average more than 30.0% at all sites. Construct lines at the Mountain site had the lowest increase in lignin, approximately 31.7% increase, followed by the Coastal Plain site with a 36.6% increase, and then the Piedmont site with a 37.8% increase. Overall, construct lines with lower initial total lignin percentages had larger increases in lignin than construct lines with initially higher total lignin percentages. Construct line PT-1 had the largest average increase in total lignin, with an average increase of 63.1% across the three sites, followed by As4CL-1 with an average increase of 60.2% and PT-2 with an average increase of 56.2%. Wildtype had the lowest percent increase in total lignin, with an average increase of 14.1% across the three sites, followed by As4CL-6 with an increase of 15.2% and PT-4 with an increase of 19.5%.

Conversely, S/G ratio decreased universally. The original greenhouse S/G ratio of the transformed trees ranged from 1.8 to 3.7, with wildtype at 2.5. At the time of the second coppice, the field-grown range was 0.7–2.1. S/G ratio decreased on average more than 40.0% across the three sites. Much like total lignin, S/G ratio decreased the least at the Mountain site, with an average decrease of 45.9%, followed by the Coastal Plain site with a decrease of 47.6%, and the Piedmont site with a decrease of 49.0%. Construct lines As4CL-1 through 5 seemed to have the greatest decrease in S/G ratio, with average decreases greater than 65%. Despite the sense Cald5H transformation, S/G ratio decreased 30% in the CH construct lines.

4. Discussion

A clear relationship between total lignin content and growth or S/G ratio and growth was not evident in this study. While overall, increases in total lignin and increases in S/G ratio were associated with greater biomass production, the effects of decreased lignin content were not consistent between construct lines. Instead, construct line appeared to play an important role in survival and productivity beyond the effect of total lignin content and/or S/G ratio alone. For instance, construct lines within the As4CL construct with initial total lignin content ranging from 29% to 46% of the control had poor survival and uniform stunted growth across all sites. Yet construct line PT-1, with an initial reduction in lignin of 37% of the control, was more productive than wildtype at the Coastal Plain site and just as productive as wildtype at the Mountain site. At these sites, PT-1 had a large number of sylleptic branches, which can indicate a highly productive clone in *Populus* [36]. Sylleptic branches develop from lateral axes in the same season the lateral axes are formed [37]. Furthermore, several other construct lines had total lignin contents significantly less than wildtype without a significant loss in biomass (As4CL-6 and PT-3 at the Piedmont site, and As4CL-5, CH-1, and PT-3 at the Coastal Plain site).

In addition to wood lignin affecting growth in the field environment, the field environment affected lignin in our study. Lignin biosynthesis is induced by biotic and abiotic stresses, including pathogen attacks, drought, and mechanical stresses [38,39]. The average lignin increase was least at the Mountain site, the most productive site and the site that is most similar to natural *P. trichocarpa* habitat. Lignin increase in the field was highest at the Piedmont site, the least productive site. The capacity to upregulate lignin biosynthesis despite transgenic modification for decreased lignin biosynthesis in the event of environmental stress may have helped some of the low-lignin lines to survive. Construct line PT-1 had 100% survival, despite an initial greenhouse lignin concentration of 13.9%. At the time of the second harvest, this construct line had an average lignin concentration of 22.7%.

While environmental stress may have led to an increase in total lignin, the relative amount of lignin monomers may have also been affected by environmental stress. S/G ratio decreased more than 40% at all sites, with the As4CL construct lines with stunted growth displaying the largest decrease in S/G ratio. Lignin biosynthesis is a metabolically expensive process, and efficiency of lignin biosynthesis is important to plant carbon and metabolic energy budgets [40]. The glucose requirements for biosynthesis of the three monolignol subunits are thought to vary, with H lignin requiring the least amount of glucose, 2.473 g glucose per gram of lignin synthesis, followed by G lignin, 2.547 g glucose per gram of lignin, and lastly S lignin, 2.600 g glucose per gram of lignin [40]. Given the increased energy requirement of synthesizing S lignin over G or H lignin, S/G ratio may have decreased as a result of a need for increased efficiency of lignin biosynthesis in response to stressful field conditions. This effect may have been exacerbated in plants that were under the most stress because of large reductions in total lignin.

These results are similar but slightly more promising than a field study in Oregon, USA, where transgenic *P. tremula* × *P. alba* were transformed with *Agrobacterium tumefaciens* carrying an antisense aspen PT4CL1 gene [29]. Consistent with our study, *Voelker* et al. (2010) [29] found that there was no clear relationship between biomass production and total lignin content for transgenic events with small reductions in lignin. They did find, however, that decreases in lignin of 10% or more of the control were associated with reduced productivity,

stunted growth and a shrubby appearance, and the presence of wood of a reddish-brown color occupying 24%–60% of cross-sectional area. The brown wood coloration was associated with altered wood chemistry and morphology, most significantly collapsed vessels and the deposition of phenolic "extractives" that occluded vessels [41]. In our study, we attempted to quantify production of reddish-brown wood through digital analysis, but found essentially no detectable change in wood color. Unlike our study, changes in total lignin content or S/G ratio from greenhouse to field were not specifically addressed by Voelker et al., [29].

Wang et al. (2011) [32] found that a 6–10% reduction in total lignin did not cause altered growth rates over a five year field trial of two lines of antisense 4CL *Populus tomentosa* in Beijing, China. However, Wang et al. (2011) [32] did find that insoluble lignin content increased throughout development, from 10 to 15% in one year old clonal siblings to 19% after five years.

Lastly, although not specifically addressed in this study, Voelkler et al. (2010) [29] and Wang et al. (2011) [32] found that total glucose and xylose release by enzymatic hydrolysis of pretreated wood did not increase despite the reduction of lignin content by antisense 4CL. In addition, Wang et al. (2011) [32] found a strong negative correlation between soluble lignin content (higher S content) and the amount of glucose released by enzymatic hydrolysis.

5. Conclusion

The ability to decrease lignin and increase S/G ratio in trees for improvement of ethanol feedstocks, while retaining or enhancing productivity, could have a profound impact on the economics of cellulosic biofuels. The U.S. Department of Agriculture estimates that in order to be economically viable, perennial biofuels feedstocks will require sustained agronomic yields in excess of 24.71 ton ha^{-1} $year^{-1}$ [3]. The results from this study indicate that wildtype or low-lignin transgenic *P. trichocarpa* may not be well suited for SRWC in the Coastal Plain or Piedmont of North Carolina and that some lines of antisense 4CL *Populus* develop unfavorable phenotypes when exposed to field conditions. However, this study demonstrates that wildtype *P. trichocarpa* can be grown in the Mountain region of North Carolina (and surrounding states) at yields that can be competitive as a biofuel feedstock, although large-scale field trials are necessary to estimate biomass production rates comparable that would better simulate operational bioenergy SRWC plantations. In addition, several transgenic low-lignin lines (As4CL-6, CH-2, PT-1, PT-3, and PT-4) remain promising possibilities for further studies. More research focused on promising construct lines in appropriate environmental conditions and with larger samples sizes is needed to clarify if a significant reduction in total lignin content can be achieved on a plantation scale, and whether that reduction will translate into the increased efficiency of enzymatic hydrolysis.

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