Transgenically altered lignin biosynthesis affects photosynthesis and water relations of field-grown *Populus trichocarpa*

Running Head: PHYSIOLOGY OF FIELD-GROWN *POPULUS TRICHOCARPA*

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Abbreviations

R_d : daytime respiration rate
 $K_{L,p}$: leaf-specific whole-plant hydraulic conductance
 V_{cmax} : maximum velocity of Rubisco for carboxylation
 g_m : mesophyll conductance
 Ψ_{leaf} : midday leaf water potential
 A_n : net CO₂ assimilation rate
 J : photosynthetic electron transport rate
 g_s : stomatal conductance
 E : transpiration rate
 TPU : triose phosphate use
 k_s : xylem-specific hydraulic conductivity

Abstract

Concerns over energy security and environmental sustainability have stimulated interest in development of high-yield, low-lignin trees for bioenergy. Black cottonwood (*Populus trichocarpa*) has been targeted as a potential bioenergy species due to its high productivity, but it is unclear how transgenically altered lignin biosynthesis will affect plant function. We investigated the physiology of two transgenic *P. trichocarpa* genotypes grown in short rotation woody cropping systems at two sites in southeastern USA: (1) mesic mountain site and (2) warmer, drier Piedmont site. Our results suggest that lignin is fundamental for tree growth and survival in field environments. Lignin deficiency can decrease biochemical photosynthetic processes and interfere with the temperature-response of photosynthesis. Significantly, hydraulic conductivity of transgenic genotypes was 15–25% that of wildtype trees, resulting in decreased leaf-specific whole-plant hydraulic conductance. In the Piedmont, decreased hydraulic efficiency drastically reduced productivity of low-lignin genotypes by 50–70% relative to wildtype. Transgenic trees at the mountain site recovered stem lignin concentrations to levels observed in wildtype trees, but still had severely impaired hydraulic traits, highlighting the major consequences of genetic transformation on whole-plant function. Surprisingly, substantial loss of hydraulic conductivity had only minor effects on productivity at the mesic site and resulted in an alternative advantage for bioenergy systems – lower water consumption. In the hottest month (July), higher intrinsic water use efficiency resulted in total water savings of roughly 1 kg d⁻¹ per transgenic tree without sacrificing productivity. Decreased hydraulic conductivity could therefore be a promising trait for selection of water-efficient genotypes in *Populus*.

Introduction

Biofuel production from cellulosic perennial crops, such as grasses and poplars, reduces greenhouse gas emissions relative to gasoline [1] and provides numerous advantages over non-cellulosic crops such as corn [2]. Poplars (i.e. *Populus* species) are among the fastest growing trees in temperate regions and thus are ideal candidates for short rotation woody cropping systems (SRWC) for bioethanol production in the United States [3, 4]. The high productivity of poplars requires a large water supply [5], so they are restricted to regions with adequate water availability. The genus *Populus* contains species that are highly susceptible to drought stress due to low stomatal sensitivity to water potential [6] and high vulnerability to xylem cavitation [7-10]. Higher temperatures in the future are expected to increase the probability of drought and temperature-induced drought stress [11], so selecting genotypes that minimize water use and maximize carbon assimilation and growth will be of critical importance [12]. Explicit quantification of stand-level water use efficiency (WUE), and its improvement, will be important components of engineering climatically robust and sustainable bioenergy production systems [12].

Before commercial-scale production of cellulosic biofuels can occur, however, the cost of converting cellulose to ethanol or other biofuels must be decreased to a level competitive with gasoline and corn-starch ethanol (i.e. first generation biofuels). Reduction of lignin in cellulosic plant biomass, in particular, holds promise to increase ethanol yield while decreasing processing inputs [13]. Lignin in plant secondary cell walls hinders the enzymatic degradation of cell wall polysaccharides to simple sugars destined for fermentation to ethanol [13]. Another strategy for increasing the biochemical efficiency of cellulose conversion to biofuels is to increase the ratio of syringyl (S) to guaiacyl (G) monolignol units in angiosperm lignin [14]. Syringyl-rich lignin is

substantially easier to separate from cellulose than guaiacyl-rich lignin during pulping for paper production [15], which should theoretically decrease pretreatment costs during biofuel production. Genetic transformations of genes that encode for enzymes along the lignin biosynthetic pathway have been developed that successfully decrease lignin quantity and increase the S/G ratio of stem tissue in *Populus* [16-19]. Although transgenic plants grown in controlled conditions have dramatic decreases in stem lignin content [18, 20], evidence is mounting that transgenic alteration of lignin biosynthesis may not remain stable when grown under natural field conditions over long time periods [21-23]. There are 23 genes that likely encode monolignol biosynthesis enzymes during wood formation in *P. trichocarpa* (Torr. & Gray), and there is likely functional redundancy in many of these enzymes [24]. In the 4-coumarate:coenzyme A ligase (*4CL*) gene family, three genes are expressed in wood-forming tissue of *P. trichocarpa* and were suggested for biochemical roles in lignin synthesis [24]. Increased lignin biosynthesis during wood formation can be induced by biotic or abiotic environmental stressors, including low temperature [25], pathogen attack [26], and drought [27]. Lignin composition has also been observed to change in response to natural abiotic factors such as wind [28]. Better understanding of the role of lignin in whole-plant functional responses to environmental variation is needed in order to improve purpose-grown trees.

Lignins are functionally important in mechanical support [29, 30], wood strength [31], water conductance [32], carbon storage, and disease resistance [26]. Lignified secondary cell walls provide significant compressive strength against stresses up to 40 MPa, relative to non-lignified walls [29]. Low-lignin *Populus* trees have been found to have decreased hydraulic conductivity and a greater susceptibility to cavitation [33, 34] as a result of collapsed cells and blockage of vessels by tyloses and phenolics [35]. Such reductions in water transport capacity

are likely to have negative effects on the productivity of transgenic trees, especially *Populus* because of its large water requirement [5]. It is unknown how universal these physiological responses are among *Populus* genotypes and different genetic transformations, especially for plants grown under field conditions for multiple years.

The main objective of this study was to quantify physiological water relations of three genotypes of *P. trichocarpa* (wildtype and two low-lignin transgenics) under natural environmental conditions in the southeastern USA. A previous study by Stout et al. [22] established that biomass production of these two low-lignin transgenic genotypes (PT-1, PT-3) was high, but the low-lignin and high S/G phenotypes were not fully maintained after three years of growth in North Carolina (Table 1). Therefore, we compared physiological responses at two different study sites: (1) a warmer ‘Piedmont’ site more prone to drought stress where the two transgenics maintained lower wood lignin concentrations (but not higher S/G ratio) than the wildtype, and (2) a mesic ‘mountain’ site where low-lignin phenotypes and high S/G ratio were not stable (i.e. not significantly different from wildtype after three years) but total yields were competitive with other proposed perennial cellulosic biofuel crops [22]. We measured seasonal changes in growth and hydraulic traits (leaf water potential, gas exchange, leaf-specific whole-plant hydraulic conductance ($K_{L,p}$), intrinsic WUE) of each genotype and compared responses of transgenic lines to the wildtype control at each site. We also determined if photosynthesis, foliar nitrogen concentration, and wood density differed among genotypes. We hypothesized that transgenic trees will have decreased rates of biophysical and biochemical photosynthetic processes (e.g. maximum carboxylation velocity of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco), V_{cmax} ; electron transport rate, J) and decreased hydraulic efficiency ($K_{L,p}$) relative to the wildtype control.

Materials and Methods

Study species

Populus trichocarpa (black cottonwood) is the largest hardwood tree in western North America and can grow to over 60 m in height [36]. It grows primarily on moist sites west of the Rocky Mountains and is most productive in bottomlands of major streams and rivers [36]. The female *P. trichocarpa* (genotype Nisqually-1) was found near the Nisqually River in central Washington, USA, and was the first tree to have its complete genome sequenced [37]. Stout et al. [22] performed an environmental screening of twelve transgenic *P. trichocarpa* lines, and in the current study, we performed a detailed analysis of the physiology and water relations of the two most promising genotypes. These two transgenic lines (PT-1, PT-3) were produced by antisense suppression of the gene encoding *4CL* in the Nisqually-1 genotype (V. Chiang, North Carolina State University, Raleigh, NC, USA; see [38]). The transformations used the *4CL* promoter from *P. tremuloides* and were mediated by *Agrobacterium tumefaciens*. The transgenic trees were produced from tissue culture under greenhouse conditions during fall 2008. After several months of growth, the pre-transplants were transferred to small pots and placed in a covered greenhouse in January 2009 for cold acclimation before planting in April 2009. Under greenhouse conditions, the two low-lignin lines had mean lignin mass fractions of 13.9% and 17.4%, while wildtype contained 22% lignin ([22], Table 1). The S/G ratios of the low-lignin lines were approximately 40% greater than the control at the time of planting (Table 1). After three years of growth in North Carolina, wood lignin concentration of all genotypes increased by 1.4–9.2% and S/G ratios decreased by 0.5–2.2, with the largest changes occurring in the transgenic lines (Table 1). As a result, lignin concentration or S/G ratios did not vary between transgenic and wildtype

trees at the mountain site in the study year 2012. At the Piedmont site in 2012, the PT-1 and PT-3 transgenic lines had lower wood lignin concentrations than wildtype by 3.4% and 4.8%, respectively.

Experimental study sites

Trees were planted in the mountain, Piedmont, and coastal regions of North Carolina, USA, although only mountain and Piedmont sites were measured in this study as physiological responses at the coastal site were intermediate. The mountain site was located in Fletcher, Henderson County, NC (35° 25.700' N, 82° 33.467' W, 647 m a.s.l.), and the Piedmont site was located in Oxford, Granville County, NC (36° 18.167' N, 78° 36.733' W, 146 m a.s.l.). At the cooler and wetter mountain site, mean annual temperature is 12.9 °C and mean annual precipitation is 1312 mm. Mean annual temperature at the warmer and drier Piedmont site is 14.4 °C and mean annual precipitation is 1158 mm. Mean growing season temperature (May–September) during 2012 was lower at the mountain site than the Piedmont site (21.2 vs. 23.5 °C, respectively; Fig. S1). Growing season precipitation (May–September) was lower at the mountain site than the Piedmont site in 2012 (456 vs. 808 mm, respectively), which is contrary to the 30-year average precipitation patterns for these regions (mountain: 584 mm, Piedmont: 526 mm from 1971–2000). Despite having lower rainfall, measurements of predawn leaf water potential indicated that plant-available soil water was greater at the mountain site during the 2012 growing season (Table 2), mostly due to soil differences between sites. Soil at the mountain site is a well-drained loam (Hayesville loam), while soil at the Piedmont site is a moderately well-drained sandy-loam (Helena sandy loam) with a compacted plow pan soil layer that restricts

root growth and water movement. Soil nitrogen mass fractions were higher at the mountain than the Piedmont site (0.22% vs. 0.05%, respectively; [39]).

Trees were planted in the field at a spacing of 0.6 m within rows and 1.2 m between rows, which would correspond to a planting density of approximately 13,500 ha⁻¹. Each row consisted of a single transgenic line, with two rows of wildtype trees at each end of the plot. At the mountain site, 78 trees were planted over an area of approximately 45 m², whereas at the Piedmont site, 149 trees were planted over an area of approximately 100 m². Due to the small size of the plots, we considered within-plot variability of environmental conditions to be negligible. All trees were coppiced in January 2010 at 5 cm above the base of the tree and in January 2011 and 2012 at 10 cm above the base of the tree. Further details of growth conditions can be found in Stout et al. [22].

Tree growth measurements

Seasonal changes in stem height and leaf area were estimated for each tree ($n=5$ trees per genotype) in May, July, and September, 2012. Height (m) was measured for all stems and length (m) of all sylleptic and proleptic branches, while leaf number and leaf area were measured for a subset of stems and branches of varying height or length for each plant. Total shoot length (m) per tree was calculated by summing all stem heights and branch lengths. Total leaf area (A_L , m²) per tree was estimated as:

$$A_L = \sum(n \cdot n_{L,H} \cdot a_{L,H})$$

where n is the number of stems of a given height/length, $n_{L,H}$ is the mean leaf number for that stem height/length, and $a_{L,H}$ is the mean leaf area for that stem height/length. Allometric equations relating stem height to leaf number and leaf area were used to determine $n_{L,H}$ and $a_{L,H}$,

respectively, within each genotype at each sampling date. Leaf area was measured using a portable laser leaf area meter (CI-202, CID Bio-Science Inc., Camas, WA, USA), and specific leaf area ($\text{m}^2 \text{kg}^{-1}$, $n=3$ leaves per genotype) was determined after oven-drying leaves at 60 °C to constant mass.

Gas exchange, photosynthetic processes, and foliar nitrogen content

Gas exchange measurements were made under local environmental conditions using a portable infrared gas analyzer equipped with a red–blue light source (LI-6400, Li-Cor, Lincoln, NE, USA) on sunny days in May, July, and September 2012 from 11:00 to 15:30 h. All measurements were made under saturating PAR ($1500 \mu\text{mol m}^{-2} \text{s}^{-1}$) and ambient CO_2 concentration ($400 \text{ cm}^3 \text{m}^{-3}$). Each sampling day, the cuvette block temperature was held constant at ambient temperature and the relative humidity was held within 10% of ambient conditions. Fully-developed leaves ($n=15\text{--}31$ per genotype per month) were measured at a range of stem heights (top, middle, base) on each plant to examine how gas exchange varied along the stem. Photosynthesis measurements were recorded for a period of 2–3 minutes after conditions inside the cuvette stabilized (usually 1–2 minutes) and then averaged to determine net CO_2 assimilation (A_n), stomatal conductance (g_s), transpiration (E), and intrinsic WUE ($A_n:g_s$) for each leaf.

To determine if the genetic transformation affected biophysical and biochemical photosynthetic processes of *P. trichocarpa* leaves, the response of A_n to varying concentrations of intercellular CO_2 (C_i) was measured between 11:00 and 15:30 h in July, 2012. These A_n/C_i curves were measured on five leaves per genotype at each site. Photosynthesis was measured using the LI-6400 at ambient temperature and relative humidity, under saturating PAR (1500

$\mu\text{mol m}^{-2} \text{ s}^{-1}$). The external CO_2 (C_a) was lowered stepwise from 400–50 $\text{cm}^3 \text{ m}^{-3}$ and then increased from 400–2000 $\text{cm}^3 \text{ m}^{-3}$ with a total of 12 points per A_n/C_i curve. Rates of photosynthesis were recorded after two minutes at each C_a . Values of A_n were corrected for CO_2 leakage by subtracting ‘apparent’ photosynthesis quantified with six photosynthetically inactive leaves from the wildtype control, following [40]. For comparisons among genotypes, the parameters V_{cmax} (maximum carboxylation velocity of Rubisco), J (photosynthetic electron transport rate), TPU (triose phosphate use), R_d (daytime respiration), and g_m (mesophyll conductance) were estimated at 25 °C for each curve using the procedure described in [41].

To determine foliar C:N ratios, fully-developed sun leaves were collected from the top of the stem on the south side of the tree ($n=5-7$ trees per genotype) in October 2011 at both sites. Leaves were oven-dried at 70 °C, and three leaves from each plant were combined and ground to a fine powder in liquid nitrogen. Leaf tissue samples (1.8–2.2 mg) were analyzed for carbon and nitrogen mass fractions (%) with an elemental analyzer (Carlo Erba, Model 1110, Milan, Italy) at the Stable Isotope Mass Spectrometry Laboratory (Kansas State University, Manhattan, KS, USA).

Leaf-specific whole plant hydraulic conductance

Based on an Ohm’s-law analogy of water transport, we estimated leaf-specific whole-plant hydraulic conductance ($K_{L,p}$, $\text{mmol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$, $n=5$ trees per genotype) at both sites on three dates throughout summer 2012 (May, July, September) using the method described in [42]:

$$K_{L,p} = \frac{(D_L/P_{\text{atm}}) * g_w}{(\Psi_{\text{soil}} - \Psi_{\text{leaf}})}$$

where D_L (kPa) is vapor pressure deficit at the leaf surface, P_{atm} (kPa) is atmospheric pressure for each study site, g_w ($\text{mmol m}^{-2} \text{ s}^{-1}$) is leaf conductance to water vapor, Ψ_{soil} (MPa) is soil water

potential, and Ψ_{leaf} (MPa) is midday leaf water potential. This expression assumes a steady state in which transpiration is equal to the rate of water flow through the plant. Measurements of air temperature and relative humidity (CS215-L sensor; Campbell Scientific Inc., Logan, UT, USA) were measured every 2 minutes during the midday sampling periods (11:30–14:30 h), recorded by automated dataloggers (CR1000; Campbell Scientific Inc.), and combined with leaf temperature measurements to calculate D_L for each plant. We used predawn leaf water potential (Ψ_{predawn}) as a proxy for Ψ_{soil} [43]; we collected one or two fully-developed leaves per tree per sampling date from the top of the stem between 5:00 and 6:30 h. For Ψ_{leaf} , two to three fully-developed leaves per tree were collected from the top of the stem between 11:30 and 14:30 h. Predawn and midday leaf water potentials were measured with a pressure chamber (Model 1000, PMS Instruments, Corvallis, OR, USA) and averaged to determine Ψ_{predawn} and Ψ_{leaf} of each plant.

Leaf conductance (g_w) was calculated following Nobel [44] and incorporated both stomatal (g_s) and boundary layer (g_b) conductance:

$$g_w = \left(\frac{1}{g_s} + \frac{1}{g_b} \right)^{-1}$$

Midday g_s was measured as described above on the same two leaves sampled for Ψ_{leaf} , immediately before excision. To estimate g_b , we used the relationship described in [44]:

$$g_b = \frac{D_w}{\delta_b} \left(\frac{P_{\text{atm}}}{R \cdot T} \right)$$

where D_w ($\text{m}^2 \text{s}^{-1}$) is the diffusion coefficient of water vapor in air, δ_b (mm) is boundary layer thickness, R ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) is the ideal gas constant, and T (K) is air temperature. To estimate δ_b , we used the relationship: $\delta_b = 4.0 \cdot (w/v)^{1/2}$, where w (m) is the mean leaf width and v (m s^{-1}) is the ambient wind speed. We measured wind speed with a cup anemometer

(Inspeed, Sudbury, MA, USA) every 5 seconds and recorded mean wind speed over 2-minute periods on a datalogger (CR1000, Campbell Scientific Inc.). We determined the average leaf width for each plant using a portable laser leaf area meter (CI-202, CID Bio-Science Inc.).

Hydraulic conductivity and wood density

Xylem-specific hydraulic conductivity (k_s) of stems ($n=5$ per genotype per site) was measured in September 2012 at both sites. Unbranched stems less than 1 year in age and at least 1 m in length were excised from the plant and immediately recut under water. The stems were transported to the laboratory with the cut end submerged in water and the uncut end covered in an opaque plastic bag. In the laboratory, branches were again cut underwater to remove at least 25 cm from each end, leaving stem segments of 15–25 cm in length with xylem diameter of 3–6 mm. The branch was connected to a hydraulic apparatus filled with deionized water at an applied pressure of 13 kPa. Volumetric flow rates were measured 3–4 times over a 10-min period to ensure that flow had stabilized; measurements were averaged for each stem. All measurements were completed within 1 day of stem collection. Xylem-specific hydraulic conductivity (k_s , $\text{g m}^{-1} \text{s}^{-1} \text{kPa}^{-1}$) was calculated according to Darcy's law:

$$k_s = \frac{Q \cdot l}{A_s \cdot P}$$

where Q (g s^{-1}) is the volume flow rate, l (m) is the length of the segment, A_s (m^2) is the conductive sapwood area, and P (kPa) is the pressure applied to the segment. Both ends of the stem segment were perfused with methylene blue solution before determining A_s . Small discolored patches of non-conducting xylem were observed within stems of both transgenic lines, but not in wildtype trees (Fig. S2). To avoid overestimating conductive A_s of transgenic

trees, we scanned stained cross-sections of wood and estimated the percentage of non-conducting xylem using ImageJ v1.49v [45]; this percentage was then subtracted from total A_s .

To measure wood density (kg m^{-3}), the stems collected for hydraulic conductivity were cut to about 6.0 cm length and stripped of bark. Each segment was split and the pith was removed before determining fresh volume using the water displacement method. The sample was then dried to a constant mass at 70 °C and weighed.

Statistical analyses

When determining physiological differences among genotypes, statistical comparisons were analyzed within the same site due to pronounced site differences in climate, soil characteristics, and plant phenotypes [22]. We performed one-way, univariate ANOVAs with genotype as the main effect and total lignin content, S:G ratio, A_L , foliar N and C content, wood density, k_s , V_{cmax} , J , TPU , R_d , g_m , A_n , g_s , E , WUE, Ψ_{leaf} , and $K_{L,p}$ as dependent variables. If differences among means were found ($p \leq 0.05$), we used the Tukey HSD to test for significant differences among genotype means. Seasonal differences in A_L , shoot height, A_n , g_s , E , WUE, Ψ_{leaf} , and $K_{L,p}$ were analyzed for each site using a repeated measures analysis with month as the within-subjects factor and genotype as the between-subjects factor using SPSS version 21 (IBM Corporation, Armonk, NY, USA). To test for differences among months, we used pairwise significance tests adjusted for multiple comparisons with the Bonferroni correction.

We analyzed for genotype–environment interactions in hydraulic and photosynthetic traits to determine if there was intraspecific variation in phenotypic plasticity. Specifically, differences in A_L , shoot height, foliar C:N, wood density, k_s , V_{cmax} , J , TPU , R_d , g_m , A_n , g_s , E , WUE, Ψ_{leaf} , and $K_{L,p}$ were analyzed using multivariate, full-factorial analyses of variance

(ANOVAs) with genotype and environment as the main effects. Data from the three sampling dates (when relevant) were combined for analyses to ensure observed differences were consistent over time. All data were tested for normality with the Shapiro and Wilk's test and A_L , shoot height, $K_{L,p}$, k_s , and g_m were ln-transformed to achieve normality. Analyses were performed using JMP 9.0 (SAS Institute, Cary, NC, USA). Means were considered significantly different at $p \leq 0.05$.

Results

Growth and physiology differences at the Piedmont site

At the warmer Piedmont site, total shoot length and leaf area was 2–4 times higher in the wildtype control than the two transgenic lines (Fig. 1a,c). Rates of A_n declined throughout the growing season at this drought-prone site (Fig. 2a). In May, there was no difference in A_n among the three genotypes, but A_n was 5–6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ higher in wildtype trees by July, and remained higher in September (Fig. 2a). The low-lignin line PT-1 also had significantly lower J , TPU , and g_m when compared to the wildtype control (Table 2). No significant differences in foliar nitrogen or carbon content were observed between the transgenic and wildtype trees (Table 3).

There were also differences in hydraulic traits among genotypes at the Piedmont site. Hydraulic conductivity in the transgenic lines PT-1 and PT-3 was 15 and 25% of wildtype trees in September, respectively (Table 3). Stomatal conductance of transgenic lines was only about half that of wildtype trees in July (Fig. 2c). We observed that g_s of leaves at the top of stems was lower than in leaves at the base of the stem in both low-lignin genotypes, while no decreases in g_s were found with increasing stem height in wildtype trees (Fig. 3). Transpiration of transgenic lines was 4–6 $\text{mol m}^{-2} \text{hr}^{-1}$ less than wildtype trees in the middle to late growing season (Fig. 4a).

By September, midday Ψ_{leaf} and $K_{L,p}$ were significantly lower in the transgenic line PT-1 than in wildtype trees (Fig. 4c,e). Hydraulic efficiency (i.e. $K_{L,p}$) of the transgenic line PT-1 was 43% of the wildtype control (Fig. 4e).

Growth and physiology differences at the mountain site

At the mesic mountain site, there were only minor differences in growth among *P. trichocarpa* genotypes (Fig. 1b,d). There was some canopy dieback (i.e. leaf and branch mortality) in the transgenic line PT-3 near the end of the growing season ($t_4=11.76$, $p<0.001$), while no significant dieback was observed in wildtype trees ($t_4=2.093$, $p=0.104$; Fig. 1b,d). We found no differences in A_n (Fig. 2b) or biophysical and biochemical photosynthetic parameters among genotypes (Table 2), but leaves of wildtype trees had higher specific leaf area (11.9 vs. 10.4 m² kg⁻¹; $F_{2,42}=5.389$, $p=0.008$) and nitrogen mass fraction than the transgenic lines (Table 3). Interestingly, intrinsic WUE of transgenic lines was significantly higher than the wildtype control in the hottest month, July (0.056 vs. 0.051 $\mu\text{mol mmol}^{-1}$, respectively; $F_{2,42}=3.627$, $p=0.036$). The increase in intrinsic WUE was caused by a trend for lower g_s in transgenic trees relative to wildtype trees ($F_{2,42}=2.876$, $p=0.068$; Fig. 2d), rather than any significant change in A_n ($F_{2,42}=0.787$, $p=0.462$; Fig. 2b).

There were numerous effects of the genetic transformation on hydraulic traits at the mountain site that matched observations at the Piedmont site. Hydraulic conductivity of both transgenic lines was about 25% that of wildtype trees in September (Table 3). The two transgenic genotypes had significantly lower g_s (Fig. 2d), E (Fig. 4b), midday Ψ_{leaf} (Fig. 4d), and $K_{L,p}$ (Fig. 4f) compared to the wildtype control. Hydraulic efficiency (i.e. $K_{L,p}$) of the transgenic lines PT-1 and PT-3 was 78 and 71% of wildtype controls, respectively (Fig. 4f). No significant differences

in wood density or foliar carbon content were observed between the transgenic and wildtype trees (Table 3).

Genotype–environment interaction

We found significant genotype–environment interactions among *P. trichocarpa* genotypes for growth (total shoot length, A_L), photosynthetic traits (A_n , J , TPU , foliar C:N), and one hydraulic trait (E , Table 4). At the mesic mountain site, total shoot length of PT-3 trees was significantly lower than wildtype trees during the 2012 growing season ($F_{2,44}=3.517$, $p=0.039$; Fig. 5a), while PT-1 trees (rather than PT-3 trees) had the lowest total shoot length at the drier Piedmont site ($F_{2,44}=49.512$, $p<0.001$; Fig. 5a). The same pattern was observed among genotypes for leaf area and A_n (data not shown). Photosynthetic electron transport rate of wildtype trees was higher at the drier Piedmont site than the mesic mountain site (Table S1), but the transgenic trees had lower J at the Piedmont than the mountain site (Fig. 5b). A similar pattern was observed for TPU (data not shown). Foliar C:N was lowest in wildtype trees at the mountain site (Table S1), but equivalent foliar C:N was observed in transgenic trees at both sites (data not shown). All genotypes had lower E at the Piedmont site relative to the mountain site, but the decrease between sites was largest for the transgenic trees (24–28% vs. 9% in wildtype; Fig. 5c).

Discussion

This study examined the effect of genetic transformation by insertion of the antisense *4CL* gene on leaf-level and whole-tree physiology of field-grown *P. trichocarpa* trees. Most previous studies investigating the function of low-lignin plants under field conditions have quantified changes solely in productivity and tissue chemistry [19, 21–23]. This study is the first to report

decreases in biochemical photosynthetic processes (i.e. electron transport rate, triose phosphate use) and mesophyll conductance of field-grown, low-lignin transgenic trees (Table 2). We also document that genetic transformation can decrease whole-plant hydraulic efficiency (i.e. $K_{L,p}$) of *P. trichocarpa* while increasing intrinsic WUE (Fig. 2, 4). Our findings support our hypotheses and agree with previous studies that have documented decreased hydraulic conductivity in low-lignin transgenic *Populus* [33-35].

Many of the observed changes in hydraulic traits of the transgenic trees were observed at both study sites (Table 4), despite large differences in wood chemistry (Table 1). The low-lignin phenotype was not maintained over time at the mountain site (Table 1), which is surprising as other field trials have achieved stable lignin reductions in *Populus* after 4–5 years [23, 46]. Despite recovering stem lignin concentrations to the level observed in the wildtype control (approximately 23%, Table 1), the transgenic lines PT-1 and PT-3 still had severely impaired hydraulic conductivity and efficiency (Table 3, Fig. 4). It is unclear what mechanism is responsible for the collapse or blockage of xylem vessels (see discussion below). Our results highlight the fact that genetic transformation can have major consequences, direct and indirect, on whole-plant function.

Effects of genetic transformation on photosynthesis

Modulation of lignin biosynthesis clearly and directly impacts downstream processes, such as wood synthesis [31], but our study demonstrates that upstream processes (i.e. photosynthesis) can also be indirectly impacted through complex physiological source-sink feedback mechanisms. The genetic transformation decreased photosynthetic processes of low-lignin PT-1 trees, resulting in lower electron transport rates, triose phosphate usage, mesophyll conductance

(Table 2, Fig. 2a), and ultimately, lower productivity (Fig. 1a,c). Photosynthesis was also significantly decreased in low-lignin PT-3 trees (Fig. 2a), although we did not find any significant change in rates of individual photosynthetic processes (Table 2), perhaps due to low sample sizes ($n=5$ leaves per genotype). Lignin biosynthesis provides a strong sink for removal of carbohydrates from tree leaves, but accumulation of high levels of starch and soluble sugars can occur in leaves of low-lignin *Populus* genotypes [33]. When carbohydrates accumulate in leaves, photosynthesis declines as a result of decreasing regeneration of ribulose-1,5-bisphosphate, RuBP [47]. Although we did not measure carbohydrate concentrations in leaves, our observations are consistent with photosynthetic limitation caused by a loss of sink strength. It is also possible that stimulation of genes for lignin biosynthesis in PT-1 and PT-3 resulted in suppression of genes for photosynthesis, which has been observed in elongation zones of poplar stems [48].

Photosynthesis was not affected by the genetic transformation at the mesic mountain site, where stem lignin concentrations were higher, despite significant reductions in leaf nitrogen content of transgenic trees relative to wildtype trees (Table 2, Fig. 2b). Plants with lower leaf nitrogen content typically have lower photosynthetic capacities [49-51], although changes in specific leaf area can compensate for lower leaf nitrogen on a mass basis [52]. Indeed, transgenic trees had lower specific leaf area than wildtype trees, allowing for equivalent rates of A_n (Fig. 2b) and similar levels of productivity (Fig. 1b,d).

Our results also suggest that lignin biosynthesis can indirectly affect the temperature response of photosynthesis. Biochemical photosynthetic processes of wildtype trees were higher at the Piedmont site than the mountain site (Table S1, Fig. 5b), as expected due to higher temperatures at the Piedmont site (Fig. S1). Photosynthetic processes of the transgenic lines were

lower at the warmer Piedmont site than the mountain site (Fig. 5b), however. Evidently, the indirect effects of genetic modification on whole-plant function can outweigh the well-documented expectation for the temperature response of photosynthesis (i.e. warmer temperatures increase rates of photosynthesis [53, 54]). This confirms the importance of analyzing genotype–environment interactions to improve our understanding of the influence of phenotypic plasticity on tree productivity [55].

Effects of genetic transformation on water relations

Poplars are among the most vulnerable tree species to xylem cavitation, with air-seeding embolism beginning at xylem tensions of about -1.0 to -1.5 MPa [7, 8, 10, 56]. Resistance to xylem cavitation has been shown to be even lower in transgenic poplar lines than in wildtype trees [33, 34], although this characteristic does not always result in decreased hydraulic conductivity of low-lignin transgenic trees [57]. The genetic transformation in this study decreased hydraulic conductivity (Table 3) and efficiency of water transport in trees at both field sites (Fig. 4e,f), even in trees with stem lignin content equivalent to levels observed in the wildtype control. Decreased hydraulic conductivity in other transgenic low-lignin poplars occurred as a result of collapsed xylem cells [33, 34] and blockage of vessels by tyloses and phenolics [35]. Two-year-old stems of the PT-1 transgenic line contained more embolized vessels than the wildtype control [39]. We also observed patches of non-conductive xylem in transgenic stems (Fig. S2) that contained embolized vessels, collapsed vessels, or vessels blocked by tyloses and phenolic deposits. There are several plausible mechanisms why low-lignin stems are vulnerable to embolism and collapse, including (1) larger or more abundant voids exist in vessel cell walls, (2) the edges of pit membranes are more easily stretched and

ruptured, or (3) the entire wall and pit chamber complex more readily collapses under pressure [31, 34, 58]. It is less clear why transgenic stems with high lignin (i.e. approximately 23% mass fraction) contained patches of non-conductive xylem. Downregulation of *4CL* may have disrupted the orientation and order of cellulose fibers in stem tissues, leading to vessel collapse [59].

Lignin content of about 23% is likely adequate for normal function of *Populus* at sites with low environmental stress (Table 1), but stem lignin content of 23% did not prevent stunted growth of the genotype PT-1 at the warmer, drier Piedmont site (Fig. 1a,c). Wildtype trees at this site increased stem lignin content to 26% (Table 1), suggesting that lignin plays an important role in drought tolerance. Previous studies have also found that drought can trigger lignin biosynthesis in plant roots [60, 61] and woody stems [27]. Loss of vascular integrity was highest at the tops of stems (Fig. 3), where stomatal closure in response to low leaf water potential (Fig. 4c,d) decreased carbon gain. Water stress at the Piedmont site limited productivity of *P. trichocarpa* (Fig. 1a) and indicates that cropping systems of low-lignin trees may be restricted to mesic locations.

Productivity of transgenic trees at the mesic mountain site was nearly equal to that of wildtype trees (Fig. 1b,d), despite loss of about 75% in stem conductivity relative to wildtype trees (Table 3). Maintaining water supply to the canopy is crucial for plant growth [62], and clearly, hydraulic continuity of the soil–leaf pathway was adequately preserved in transgenic *P. trichocarpa* genotypes, despite patches of non-conductive xylem in stems (Fig. S2). The trees may not have experienced much water deficit, as soil water availability was relatively high on the May, July and September sampling dates (Ψ_{predawn} did not drop below -0.5 MPa). Moderate amounts of xylem cavitation can increase water conservation by reducing transpiration [63-65].

Decreased conductivity of vascular tissues in the transgenic lines significantly increased intrinsic WUE during the hottest sampling date (July, Fig. 2f), with only minor sacrifices to total productivity (Fig. 1b,d). Other studies confirm that highly productive *Populus* trees can have relatively high WUE [66]. Estimated water savings is nearly 1 kg d⁻¹ per transgenic tree, assuming a reduction in transpiration of 1 mol m⁻² h⁻¹ and a leaf area of 4.5 m². Even a small increase in WUE could be advantageous and help to reduce water stress, which is important, since climate change is expected to increase the occurrence of drought and temperature-induced drought stress in coming decades [11, 12].

Conclusions

Populus trichocarpa ranks as one of the most productive tree crop species being considered for bioenergy production, due to its high rates of photosynthesis and growth. Woody cropping systems using *Populus* and similar genera in the southeastern US may perform better in the mountainous regions, which are cooler and wetter than Piedmont and coastal regions, and therefore more similar to climate conditions in native *P. trichocarpa* habitats. Biomass production at the mountain site was sustained without any fertilization or irrigation, with comparable yields to other low-input bioenergy production systems. Lignin is important for maintaining high productivity in field environments, and lignin deficiency can decrease biochemical photosynthetic processes and interfere with the temperature-response of photosynthesis. This demonstrates that functional consequences of genetic modification (e.g. downregulation of *4CL*) are far-reaching, as untargeted biological processes (e.g. photosynthesis) can also be indirectly impacted through complex physiological mechanisms. This study also

identified that decreased hydraulic conductivity is a promising trait for selection of water-efficient and highly-productive *Populus* genotypes.

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Figure 1. Seasonal changes in (a-b) total shoot length, including all stems and branches, and (c-d) total leaf area at two field sites in North Carolina in 2012. The wildtype control is indicated by a gray triangle, while the two transgenic lines are indicated by circles. Months with significant differences among the genotypes are indicated by a star (*). Values are means of 5 trees, and error bars indicate SE. Note the different scales used for each site.

Figure 2. Seasonal changes in gas exchange parameters of fully-developed leaves ($n=15-31$ per genotype per month), including (a-b) net assimilation rate (A_n), (c-d) stomatal conductance (g_s), and (e-f) intrinsic water use efficiency (WUE) at two field sites in North Carolina in 2012. The wildtype control is indicated by a gray triangle, while the two transgenic lines are indicated by circles. Months with significant differences among the genotypes are indicated by a star (*). Values are means of 5 trees, and error bars indicate SE. Note the different scales used for each site.

Figure 3. Variation in stomatal conductance (g_s) along the height of the main stem at the (a) Piedmont site and (b) mountain site in North Carolina in May 2012. Leaves ($n=5-34$ per height) were sampled at the base of the stem, mid-stem, and the top of the stem. Means not connected by the same letter within each set of bars are significantly different (Tukey HSD, $p<0.05$). Error bars indicate SE.

Figure 4. Seasonal changes in (a-b) transpiration (E), (c-d) midday leaf water potential (Ψ_{leaf}), and (d-e) leaf-specific whole-plant hydraulic conductance ($K_{L,p}$) at two field sites in North Carolina in 2012. Leaves at the top of stems were measured for Ψ_{leaf} ($n=10$ stems per genotype). The wildtype control is indicated by a gray triangle, while the two transgenic lines are indicated by circles. Months with significant differences among the genotypes are indicated by a star (*). Values are means of 5 trees, and error bars indicate SE.

703 **Figure 5.** Genotype–environment interaction for (a) total shoot length, including all stems and
704 branches, (b) photosynthetic electron transport rate, and (c) transpiration at two field sites in
705 North Carolina in 2012. The wildtype control is indicated by a gray triangle, while the two
706 transgenic lines are indicated by circles. Data points are seasonal mean measurements (when
707 relevant; $n=5-77$) on individual trees.

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