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Transgenic poplars with reduced lignin show impaired xylem conductivity, growth efficiency and survival

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ABSTRACT

We studied xylem anatomy and hydraulic architecture in 14 transgenic insertion events and a control line of hybrid poplar (*Populus* spp.) that varied in lignin content. Transgenic events had different levels of down-regulation of two genes encoding 4-coumarate:coenzyme A ligase (4CL). Two-year-old trees were characterized after growing either as free-standing trees in the field or as supported by stakes in a greenhouse. In free-standing trees, a 20 to 40% reduction in lignin content was associated with increased xylem vulnerability to embolism, shoot dieback and mortality. In staked trees, the decreased biomechanical demands on the xylem was associated with increases in the leaf area to sapwood area ratio and wood specific conductivity (k_s), and with decreased leaf-specific conductivity (k_l). These shifts in hydraulic architecture suggest that the bending stresses perceived during growth can affect traits important for xylem water transport. Severe 4CL-downregulation resulted in the patchy formation of discoloured, brown wood with irregular vessels in which water transport was strongly impeded. These severely 4CL-downregulated trees had significantly lower growth efficiency (biomass/leaf area). These results underscore the necessity of adequate lignification for mechanical support of the stem, water transport, tree growth and survival.

Key-words: biomass; cavitation; embolism; hydraulic architecture; leaf area; moisture content; P_{50} ; sapwood area; shoot dieback; xylem

INTRODUCTION

Many studies have characterized the biochemical composition of wood in transgenic trees with altered lignification, including those with 4-coumarate:coenzyme A ligase (4CL) down-regulation (Baucher *et al.* 1996; Hu *et al.* 1999; Lapiere *et al.* 1999; Meyermans *et al.* 2000; Huntley *et al.* 2003; Li *et al.* 2003; Leple *et al.* 2007; Wagner *et al.* 2009; Voelker *et al.* 2010). 4CL acts early in the phenylpropanoid pathway. In angiosperm dicots, this step is governed by a gene family that

encodes enzymes important for the synthesis of lignin and an array of other secondary metabolites (Harding *et al.* 2002; Hamberger & Hahlbrock 2004; Costa *et al.* 2005; Tsai *et al.* 2006). It has been hypothesized that transgenic modifications that reduce lignin content should result in greater carbon availability for primary metabolism and growth (Hu *et al.* 1999). Proportional increases in cellulose content associated with reduced lignification have been found, but a stunted growth form and irregular or collapsed vasculature have often accompanied these changes in cell wall properties (Anterola & Lewis 2002; Coleman *et al.* 2008; Davin *et al.* 2008; Wagner *et al.* 2009; Kitin *et al.* 2010; Voelker *et al.* 2010). The underlying physiological alterations of these phenotypes, however, have only started to be unravelled. In a study of suppression of coumaroyl 3'-hydroxylase in poplar, Coleman *et al.* (2008) found that reduced sink strength, photosynthesis and xylem resistance to embolism had a role in the stunted growth of these transgenic poplars. Another study found that wood anatomy was not changed by 4CL downregulation in aspen (*Populus tremuloides* Michx.) (Horvath *et al.* 2010a). In previous work we provided visual evidence that tyloses and the deposition of complex phenolics could obstruct normal stem water transport in wood of 4CL-downregulated poplars (Kitin *et al.* 2010). Apart from these few studies, the physiological consequences of reduced lignification have received little attention.

The increasing demand for wood fibre may require the expansion of tree plantations to increasingly marginal sites (Tuskan 1998). Because poplar (*Populus* spp.) clones used for biomass production tend to be sensitive to water supply (Shock *et al.* 2002), much work has focused on increasing their water use efficiency and drought tolerance while retaining adequate productivity under water-limited conditions (Tschaplinski *et al.* 2006). These efforts are relevant for the development of transgenic trees with altered lignin because transpiration in poplars is closely regulated by stomata to avoid excessive xylem tension and a consequent loss of hydraulic function due to embolism (Sparks & Black 1999). If compromised lignification results in xylem with elevated susceptibility to embolism at less negative xylem pressure than the plant's threshold pressure for stomatal closure, plants will incur a greater risk of catastrophic hydraulic failure (Sperry & Tyree 1988), causing shoot

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dieback (Cochard, Ridolfi & Dreyer 1996; Sparks & Black 1999; Rood *et al.* 2000), reduced carbon gain and an increased likelihood of mortality (McDowell *et al.* 2008).

Support for the hypothesis that there is an innate tradeoff between wood strength or stiffness and xylem hydraulic function have mostly been based on natural experiments consisting of surveys across species and environmental gradients (Gartner 1991a,b; Mencuccini, Grace & Fioravanti 1997; Wagner, Ewers & Davis 1998; Spicer & Gartner 2002; Gartner, Roy & Huc 2003; Woodrum, Ewers & Telewski 2003; Christensen-Daalsgaard *et al.* 2007; Jacobsen *et al.* 2007a,b; Pratt *et al.* 2007). These studies have largely concluded that suites of antagonistic traits result in inevitable tradeoffs between stem mechanical support versus water supply to the leaves. However, experimental tests of specific hypotheses of the nature of antagonistic trait linkages are few. Direct alterations of woody cell walls at the molecular level provide a means for more rigorous and precise experimental tests than are usually afforded by natural surveys. For example, the absence of experimental manipulations has made it very difficult to attribute differences in the resistance to embolism strictly to the strength and stiffness of wood because of co-variation in the size and morphology of tracheary elements (Hacke *et al.* 2001), their pit morphology (Domec *et al.* 2008) and pit membrane porosity (Domec & Gartner 2002). Meanwhile, the few experimental studies on the suspected tradeoff between wood mechanical support and xylem hydraulic sufficiency (Gartner 1991a,b; Spicer & Gartner 2002; Gartner *et al.* 2003; Kern *et al.* 2005) have yielded mixed results that have been largely explained by changes in cell identity (i.e. the proportions of cell types). Even more rarely addressed is how differences in lignin content may affect wood anatomical or hydraulic properties (Koehler, Ewers & Telewski 2006; Horvath *et al.* 2010a).

To understand the potential tradeoffs between the hydraulic function and structural integrity of the xylem, we undertook a detailed evaluation of transgenic trees that had modified cell walls as a result of lignin reduction. We earlier reported that xylem-specific down-regulation of the *P4CL1* gene (Li *et al.* 2003) caused a significant lignin reduction in about one third of the events studied (Voelker *et al.* 2010). Transgenic events with the greatest *4CL* down-regulation tended to grow slowly and their xylem was characterized by patches of reddish or brown-coloured wood associated with altered cell wall structure and deposits of brown-coloured phenolic extractives (Voelker *et al.* 2010). In late August and early September of each of the two years before the trees were harvested, we noted that some transgenic events experienced shoot dieback and increased mortality. Subsequent microscopy of excised branches revealed that reduced water transport was associated with phenolic extractive deposition and the formation of tyloses in vessels of low-lignin, brown-coloured wood (Kitin *et al.* 2010). The aim of the current study was to determine whether variation in lignin content and the occurrence of brown wood interact with differences in mechanical stresses during growth to alter hydraulic architecture and physiological function in *4CL* transgenic poplars. To test the effects of differing mechanical stresses

we characterized free-standing trees grown outdoors in a field trial and a sub-set of trees grown staked in a greenhouse to keep their developing xylem from sensing significant bending stresses. To shed light on potential changes in these plants, for both the free-standing and staked groups, we characterized variation in dieback, mortality, growth efficiency (aboveground biomass produced per unit leaf area), wood moisture contents, wood anatomy, xylem water transport and xylem vulnerability to embolism. We report that lignin-reduced transgenic trees had substantially impaired growth efficiency and xylem function that was more strongly manifest under field conditions.

METHODS

Plant material

Hybrid white poplars (*P. tremula* × *P. alba*, INRA-France 717-1B4) were used for all transformations, as described by Filichkin *et al.* (2006). *Agrobacterium* strain C58 containing the antisense *P. tremuloides 4CL1* construct (Li *et al.* 2003) was used to propagate each of 14 independent, regenerated and PCR-confirmed gene insertion events. Genomic DNA isolation and PCR conditions are described elsewhere (Voelker *et al.* 2010). Free-standing and staked non-transgenic poplars that had also been propagated *in vitro* at the same time as the transgenic trees served as controls.

Plant preparation and growth conditions

Growing conditions are described elsewhere (Voelker *et al.* 2010, Voelker *et al.* in press). Briefly, the field trial was planted in November 2005 with dormant plants near Corvallis, Oregon. Trees were well-watered throughout the field trial. The planting consisted of 10 to 15 ramets from each transgenic event and a control line spaced at 3 m intervals. In February 2007, two individuals from each transgenic event (except event 90 because it had the fewest trees at the time of planting, $n = 10$) and four individuals from the control line were transferred to an unheated greenhouse and re-planted in pots with 66 L of sandy loam soil per tree. Each tree was watered and fertilized regularly. We inserted a wooden stake into each pot, and then guyed the stake with steel wires from the stake's top to the bases of the pots. At weekly intervals after the first flush, the main stem was fixed from heights of about 0.5 and up to 5 m from the pot soil-level between the stake and the steel guy wire using numerous plastic zip-ties from heights of about 0.5 to 5 m above the soil level.

Lignin content

Lignin content of the control line was estimated to be 223 mg g⁻¹. Using molecular beam mass spectroscopy methods while total monomer release by thioacidolysis was used to characterize material from the same control trees and each of the transgenic lines (Voelker *et al.* 2010). Total thioacidolysis yields were used to calculate the lignin

contents relative to the control value assuming a 70% yield (Boudet, Lapiere & Grima-Pettenati 1995), giving estimates ranging from 132–221 mg g⁻¹ for the transgenic lines (Voelker *et al.* in press).

Aboveground biomass, survival and shoot dieback

Tree heights and basal diameters were measured in late 2005 (when the field trial was established) and at the end of the 2006 and 2007 growing seasons. Total aboveground biomass for a sub-sample of unsupported trees grown in the field trial were established for the control line ($n = 6$ trees) and each transgenic event ($n = 4$ trees per event) (Voelker *et al.* 2010). For the field trial, allometric estimates of biomass were developed for the control line and each transgenic event using height and diameter measurements from the same trees. Aboveground biomass was also measured on all staked trees. During late August 2007 the basal stem diameters were estimated and all leaves were counted on the trees eventually harvested for biomass measurements. During the same leaf counts we excised each 5th leaf from small trees or each 10th leaf on larger trees. Leaves were scanned on a leaf area meter (LI-3100C, Li-Cor Biosciences, Lincoln, NE, USA), and then these data were scaled to total leaf area per tree. For each free-standing tree we used the allometric relationship of leaf area to stem diameter established in August 2007 to estimate the total leaf area (A_l) associated with the final stem diameter measured at the end of the growing season.

Tree mortality was recorded just after leaf-flush in May 2006 and 2007 and again in November 2007 just before harvesting for biomass measurements. In early September 2006, we first observed dieback in distal branches and this often continued on different branches within the same tree until leaf fall occurred after the first frost. In November 2006, the occurrence of dieback was recorded for each tree. Similar levels of dieback, often recurring in the same trees, occurred in September of 2007 and 2008 but no data were collected.

Estimation of brown wood

Among transgenic events, wood colour varied from yellow-green (like control wood), to consistently light pink throughout, to having distinct reddish-brown or brown patches (Voelker *et al.* 2010). The cross-sectional area in brown wood was determined for each event by overlaying a grid of dots onto three cross-sections per tree at the stem base, and at 20 and 40 cm above ground. We recorded the number of dots over brown wood relative to the total number of dots over the cross-section to estimate relative frequency of brown wood.

Wood histochemistry and *in vivo* xylem dye ascent

Wood histochemistry was visualized using light microscopy of thin transverse sections of wood. To differentiate regions

with or without phenolics such as lignin present in the wood we used a dual staining method with astra-blue (binds to cellulosic material free of lignin) and safranin (binds to phenolics such as lignin) (Jourez, Riboux & Leclercq 2001). Xylem dye trace experiments were conducted in September 2008 on branches of control plants and transgenic event 712, which had the greatest levels of brown wood within the main stems (Voelker *et al.* 2010). At dawn, apparently healthy branches were excised under water and transported to the laboratory. Once there, the branches were re-cut under water with a razor blade and then placed in a beaker of 0.2% acid fuchsin solution that had been filtered to 0.22 μ m. To minimize the diffusion of dye outside of water-conducting vessels, branches were frozen in liquid Nitrogen as soon as the acid fuchsin was observed in the most distal petiole (Sano, Okamura & Utsumi 2005; Umebayashi *et al.* 2007). Cryo-fixed branch segments were planed in the frozen state at -10 to -30 °C on a sliding cryo-microtome and observed at -30 °C with an epi-fluorescence microscope (Nikon E400, Tokyo, Japan) equipped with a custom cryo-stage. Images were recorded with a digital CCD camera (Q Imaging, Micropublisher 5.0 RTV).

Quantification of xylem hydraulic and anatomical traits

At the end of 2007, we measured xylem specific conductivity (k_s , kg m⁻¹ s⁻¹ MPa⁻¹) of two segments from near the base of the main stem for four trees from each transgenic event and six control trees in the field trial, and all trees in the greenhouse. A measure of xylem transport efficiency, k_s , can be estimated according to Darcy's law as

$$k_s = \frac{Ql}{A\Delta P}$$

where Q is the volume flow rate (kg s⁻¹), l is the length of the segment (m), A is the cross-sectional sapwood area of the segment (m²) and ΔP is the pressure difference across the segment (MPa). In stems less than 1 cm in diameter, the entire segment was used, cut to a length of 12 cm (axial direction). From larger stems, two segments were chiselled from the outermost sapwood to dimensions of approximately 1 × 1 cm in cross-section and at least 12 cm in length. A razor blade was used for shaping of the samples to follow the wood grain. All segments were submerged in pH 2 HCl solution filtered to 0.2 μ m and placed under vacuum overnight to remove embolisms. Before each k_s measurement, segment ends were cut under water with a razor blade, and segment dimensions re-measured with digital callipers. Segments chiselled from stems were tightly wrapped in parafilm (Pechiney Plastic Packaging, Inc.) to reduce leakage through radial or tangential flowpaths.

Next, maximum k_s was measured by perfusing fresh, filtered HCl solution through embolism-free segments at a pressure head of approximately 0.006 MPa. The solution was near 20 °C for all measurements. The solution entered and exited the samples through tightly-fitting latex tubes.

The volume flow rate was estimated from the mean time required for the solution to pass at least four successive marks on a 1 or 0.1 mL pipette attached with latex tubing to the distal end of the sample.

To estimate the sufficiency of water supply by stem xylem to the leaves we estimated leaf area to sapwood area ratios (A_l/A_s) and leaf specific conductivity (k_l). For A_l/A_s , leaf area was measured as described above. Digital callipers were used to estimate the pith diameter and the diameter inside bark and outside bark of each basal stem segment harvested for biomass measurements. These measurements were used to estimate the cross-sectional sapwood area assuming circularity of the pith and xylem. To estimate sapwood areas of trees not harvested for biomass, a linear regression relationship was used to estimate the diameter inside bark from the diameter outside bark and then the average pith area was subtracted from the estimated circular cross-sectional area. The calculation of k_l was carried out by multiplying k_s by the stem sapwood area of the tree (A_s , m^2) and then dividing by the total leaf area (A_l , m^2).

We made thin transverse sections by hand with a razor blade from each of the k_s samples for anatomical analyses. Images were obtained using a light microscope with 20× objective lens, leading to a total magnification of 200×. Digital images photographed with light microscopy were used to estimate vessel lumen areas (later converted to diameters assuming circularity) and vessel frequencies with ImageJ software (<http://rsbweb.nih.gov/ij/>). A series of six digital images distributed radially across the 2007 growth ring of each k_s sample were used for vessel measurements. To equitably represent the contribution of vessels located along a radial transect, one image was located in the first one-third of the ring, two in the centre one-third of the ring and three in the outer one-third of the ring. Using the average vessel lumen diameters and vessel frequencies we calculated theoretical k_s based on the Hagen-Poiseuille equation (Zimmerman 1983).

Xylem vulnerability to embolism was determined using the air injection method (Cochard, Cruziat & Tyree 1992). A two-ended pressure collar was used to inject N_2 gas at pressures of 0.6, 1.2, 1.8, 2.4 and 3.4 MPa for each segment on which k_s was measured. After each round of pressurization, stem segments were wrapped in a wet paper towel and allowed to equilibrate before k_s was measured again. After each k_s measurement samples were re-cut under water with a fresh razor blade and their length recorded. Xylem vulnerability curves were generated by plotting the percent loss of conductivity from the initial, maximum k_s values as a function of the pressure applied. A total of 1056 k_s measurements were used (approximately 72 per transgenic event and 120 for the control line) to estimate the pressure causing a 50% loss of conductivity (P_{50}). For each sample a second order polynomial regression was fit to the pressure data plotted against percent loss in conductivity data (y-axis). Means for each event/line were calculated from the P_{50} value estimated for each sample. The 12–15 cm length of the segments tested did not exceed the maximum vessel size for *Populus* stems (Zimmerman & Jeje 1981; Hacke &

Sauter 1996). However, any overestimates of k_s or P_{50} values resulting from a few vessels exceeding the sample length would have applied equally across the samples tested and should not have affected our comparisons of the control line to the 4CL-downregulated transgenic events.

To estimate wood moisture contents, trees harvested for biomass measurements were stripped of all leaves early in the morning, cut into sections and placed in plastic bags for transport to the laboratory. We recorded fresh and then oven-dry mass of lower bole stem sections from each tree. On a subsample of transgenic events and the control line, we selected stems spanning a range of diameters. From these samples we carefully removed the bark of fresh stem segments and measured bark and xylem component masses separately. The relationship between whole stem and wood-only moisture contents as a function of diameter was used to estimate wood moisture contents without removal of the bark from the remaining whole-stem samples. No differences were found in the size-related scaling of wood to whole cross-section moisture content among the control line and transgenic events so the pooled overall relationship was used for each tree.

We estimated the volumetric proportion of the trunk xylem that contains cell wall, gas and liquid. These three components must sum to 100% (Gartner, Moore & Gardiner 2004). Wood density was measured on the same stem samples used to determine moisture contents described above. Wood density was determined by dividing the oven dry mass of the samples by the volume (water displacement method) of the samples when green. The proportion of cell wall was estimated by dividing the wood density of each sample by 1.53 $g\ cm^{-3}$, the assumed density of cell wall material. The volume of liquid within each sample was estimated by dividing the wood density by the moisture content and dividing by 100. The volumetric proportion of the wood that was gas was found by subtracting the proportions of cell wall and liquid from 100%.

We chose not report values for xylem vulnerability to embolism or moisture contents for staked trees (Table 1) because an inadvertent shutdown of the greenhouse irrigation system near the end of the 2007 growing season caused severe drought stress (leaves wilted) for most trees. Daily watering for two weeks afterward until harvesting for biomass did not result in the wood moisture contents recovering (i.e. little re-filling occurred) and the much less negative P_{50} values we measured indicated the xylem had been severely weakened by the acute drought stress.

Statistical analyses

Least-squares regression methods were used to assess relationships between tree form, size and wood mechanical properties. Variation in trait values was compared among the control line and transgenic events, treatments (i.e. free-standing versus staked trees) and their interaction using analysis of variance (PROC GLM, SAS version 9.2, SAS Institute Inc., Cary, NC, USA). Further analyses of the free-standing trees only compared means among events/lines

Table 1. Functional hydraulic traits in relation to lignin content for field-grown and staked trees. Values in parentheses are one standard deviation. Lignin contents were determined only on free-standing trees. Units for k_s and theoretical k_s are $\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$. For k_l the same units were multiplied by 1000. Leaf area to sapwood area is A_l/A_s . Bold values were significantly different than field-grown controls ($P < 0.05$, Tukey HSD). No post-hoc comparisons were conducted on staked trees because only two trees per event were grown staked. The dotted lines separate brown wood versus normal transgenic events

	Line or event	Lignin content (%)	k_s	Theoretical k_s	Vessel diameter (μm)	Vessel frequency (mm^{-2})	k_l	A_l/A_s	Wood moisture content (%)	P_{50} (MPa)
Field	Control	100.0	3.0 (0.6)	10.7 (4.5)	34.1 (12.5)	157 (87)	1.7 (0.5)	2006 (537)	100 (5)	−2.7 (0.7)
	224	98.9	2.6 (0.6)	8.9 (5.3)	34.2 (11.7)	131 (78)	1.9 (1.3)	1748 (1028)	105 (2)	−2.5 (0.5)
	90	95.4	2.8 (0.0)	8.7 (4.1)	30.9 (12.1)	174 (95)	1.2 (0.3)	2175 (567)	100 (2)	−2.1 (0.1)
	17	94.9	3.4 (1.2)	9.0 (3.9)	34.4 (11.3)	150 (41)	2.2 (1.0)	1939 (989)	111 (6)	−2.7 (0.9)
	115	88.8	1.7 (0.7)	7.6 (2.8)	32.8 (9.8)	172 (56)	1.1 (0.3)	1927 (820)	111 (11)	−2.1 (0.3)
	209	93.9	3.7 (0.4)	9.7 (5.6)	38.4 (13.6)	101 (46)	2.0 (0.8)	2023 (127)	115 (7)	−2.9 (0.4)
	210	91.9	2.9 (0.5)	8.8 (3.4)	37.6 (11.3)	112 (55)	1.9 (0.6)	1880 (508)	108 (7)	−2.6 (0.2)
	204	91.2	4.4 (0.8)	11.9 (5.8)	36.8 (11.5)	153 (46)	2.2 (0.6)	1925 (573)	112 (5)	−2.5 (0.2)
	640	91.7	2.7 (0.8)	9.5 (4.8)	35.7 (13.5)	123 (53)	1.1 (0.5)	2294 (750)	108 (8)	−2.2 (0.1)
	225	88	3.9 (1.2)	9.5 (5.0)	36.0 (11.1)	134 (78)	1.9 (0.6)	1910 (617)	106 (6)	−2.2 (0.2)
	713	91.4	1.2 (1.1)	7.2 (3.1)	37.7 (9.0)	107 (25)	1.2 (0.7)	1597 (857)	114 (11)	−2.5 (0.2)
	150	88	0.3 (0.4)	7.7 (2.9)	31.7 (10.3)	189 (48)	0.5 (1.0)	1903 (1011)	121 (8)	−1.5 (0.5)
	350	70	0.4 (0.2)	5.1 (1.1)	31.8 (5.7)	168 (29)	0.6 (0.0)	1467 (1302)	127 (14)	−1.6 (0.2)
	671	76.9	0.9 (0.6)	7.0 (4.7)	35.4 (7.7)	142 (45)	0.7 (0.7)	1727 (313)	122 (15)	−1.9 (0.1)
	712	58.5	0.5 (0.4)	8.1 (4.6)	36.0 (10.2)	123 (46)	0.9 (0.6)	1518 (584)	119 (15)	−2.6 (0.7)
Staked	Control	100.0	4.2 (0.9)	12.7 (4.7)	39.3 (10.7)	148 (45)	1.1 (0.4)	4171 (215)		
	224	98.9	4.7 (0.2)	9.9 (4.9)	36.5 (9.4)	161 (57)	1.7 (0.7)	3083 (174)		
	90	95.4								
	17	94.9	6.8 (1.4)	12.9 (5.0)	38.3 (12.6)	147 (32)	1.5 (0.3)	5063 (871)		
	115	88.8	4.0 (2.0)	9.7 (2.8)	39.8 (7.6)	128 (21)	0.9 (0.6)	4867 (175)		
	209	93.9	4.1 (0.8)	10.7 (7.0)	34.5 (11.6)	175 (69)	1.1 (0.4)	4166 (30)		
	210	91.9	3.7 (1.2)	12.8 (4.7)	43.0 (9.0)	123 (24)	1.0 (0.4)	3974 (410)		
	204	91.2	6.1 (0.1)	12.6 (5.4)	40.4 (9.9)	140 (44)	1.2 (0.4)	5896 (1841)		
	640	91.7	4.4 (0.3)	10.2 (5.2)	38.1 (11.2)	130 (44)	1.2 (0.4)	4186 (88)		
	225	88	4.2 (0.7)	13.6 (4.9)	41.6 (9.3)	137 (44)	1.4 (0.2)	3289 (549)		
	713	91.4	4.4 (0.4)	9.6 (4.5)	37.3 (8.0)	156 (28)	1.0 (0.3)	5103 (228)		
	150	88	0.9 (0.2)	7.7 (4.0)	34.2 (9.8)	150 (49)	0.2 (0.1)	5948 (1230)		
	350	70	1.9 (0.1)	6.5 (2.6)	34.4 (8.3)	137 (35)	1.3 (1.0)	2382 (1226)		
	671	76.9	0.1 (0.1)	6.7 (3.5)	36.4 (8.1)	122 (27)	0.1 (0.0)	3991 (537)		
	712	58.5	2.5 (2.5)	8.6 (6.7)	35.7 (10.2)	134 (26)	0.9 (0.1)	5474 (128)		

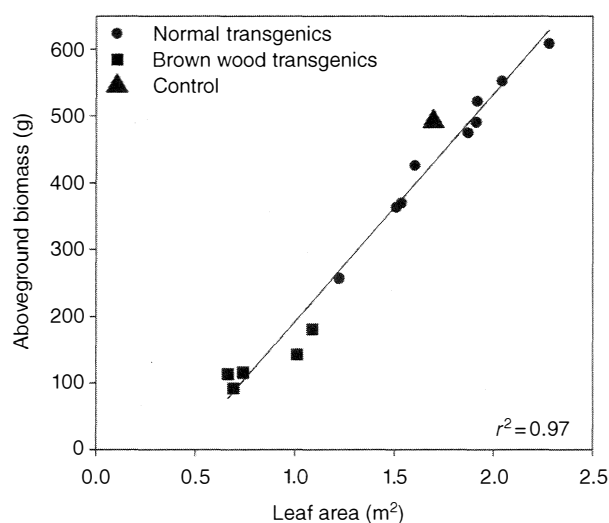


Figure 1. The relationship of aboveground biomass to leaf area. Each point is the mean from one event or the control line for free-standing trees only.

with Tukey HSD tests to control for Type 1 experiment-wise error. We employed standardized major axis regression to compare the scaling of the log-log relationship between vessel frequency and vessel diameter among the control line, normal transgenics and brown wood transgenics using the SMATR program (Warton *et al.* 2006).

RESULTS

Biomass, survival and dieback

Biomass accumulation of free-standing trees was closely related to leaf area for controls and transgenics ($P < 0.0001$; Fig. 1). The control line did not achieve the greatest biomass, but did have the greatest growth efficiency (biomass per leaf area, Fig. 2). Compared to the controls, growth efficiency of 'normal transgenics' was about 10% less, whereas growth efficiency of the 'brown wood transgenics' was about half of the control value. There appeared to be a threshold level of lignin reduction beyond which growth efficiency was sharply reduced that corresponded to the formation of extensive patches of brown wood.

There was no shoot dieback observed for the control trees. The frequency of shoot dieback increased from about 10% of the trees of the normal transgenics to near 90% of trees with the highest levels of brown wood occurrence (Fig. 3). Similarly, mortality of free-standing trees was 1.5% in controls, about 6% in normal transgenics and about 25% of trees in brown wood events (Fig. 3). None of the trees grown in the greenhouse died. Branch dieback progressed from initial dark lesions on leaf tips (Fig. 4a) to desiccation of the entire distal portion of affected branches within 1–3 d (Fig. 4b).

Wood anatomy and plant hydraulics

Microscopic observations revealed differences in anatomy, histochemistry and water transport of brown wood versus

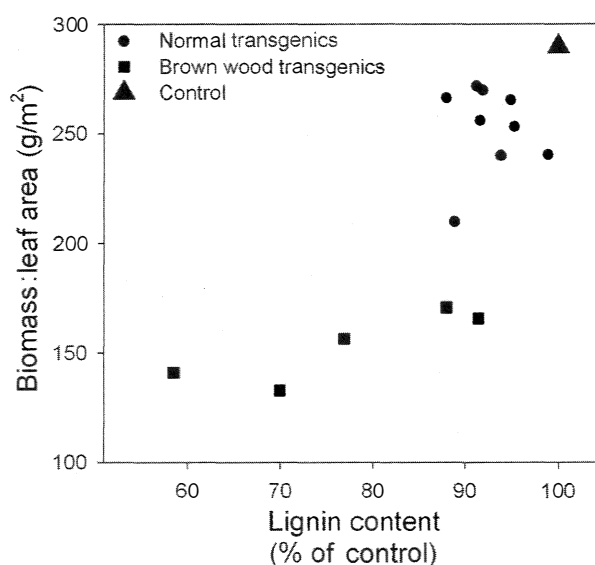


Figure 2. The ratio of aboveground biomass to leaf area plotted against lignin content. Each point is the mean from one event or the control line for free-standing trees only.

control wood (Fig. 4c–f). Dye trace experiments through excised branches showed acid fuchsin stained most vessels of the control trees (Fig. 4c), whereas in xylem from brown wood event 712, the dye was largely restricted to vessels nearer the pith where the wood was normal in colour (Fig. 4e).

Control wood harvested at the end of the growing season had normal appearing xylem stained consistently red by the safranin (Fig. 4d). In contrast, brown wood xylem harvested at the same time often had patches of mature cell walls that

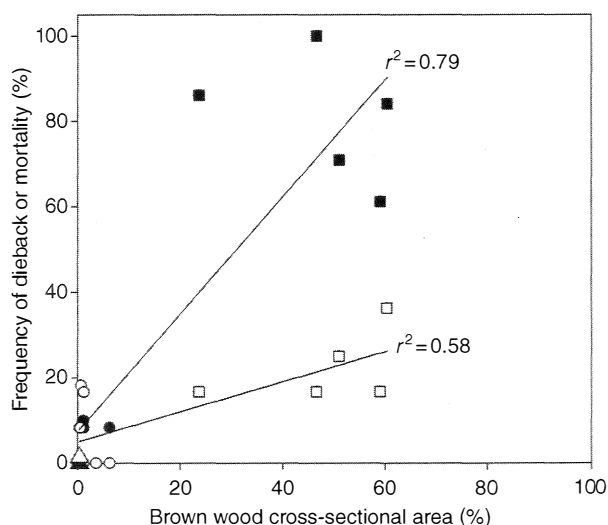


Figure 3. Frequency of shoot dieback (closed symbols) and mortality (open symbols) versus the average brown wood proportion at stem bases. Each point is the mean from one event or the control line for free-standing and staked trees combined.

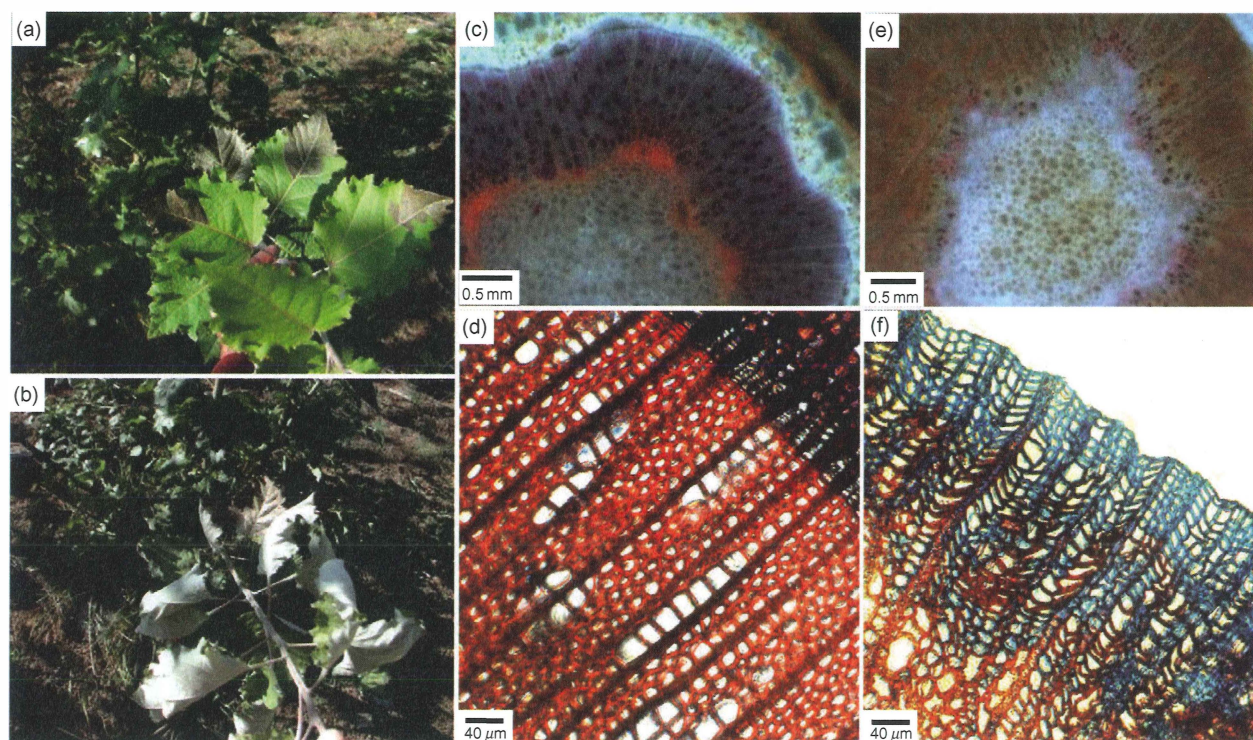


Figure 4. Shoot dieback in well-watered brown wood transgenic event 712; morning of 10 September 2008, necrotic areas on tips of basal leaves (a) and the same individual on the morning of 12 September 2008, basal leaves on shoot completely wilted and distal leaves (not shown) completely desiccated (b). Cryo-Fluorescence microscopy image from a dye trace experiment showing most vessels were dye-filled in the control line (c), whereas dye was only faintly observed within a few vessels next to the pith in the brown wood of event 712 (e). Light microscopy of thin wood cross-sections stained with safranin (bright red is lignified tissue) and astra-blue (bright blue is non-lignified tissue) show control wood with a normal appearance (the darker region is the intact cambial zone and bark) (d) as well as irregularly shaped and collapsed xylem from brown wood (cambial zone and bark sloughed off during preparation) (f).

contained phenolics that stained orange, purple or blue that apparently indicated altered or reduced lignin contents (Fig. 4f).

Vulnerability to embolism was inversely proportional to lignin content ($r^2 = 0.58$). A 30% reduction in lignin content relative to controls was associated with an increase in P_{50} of about 1 MPa ($P = 0.0025$, Fig. 5a). For this relationship we did not include the outlier, event 712, in which k_s values and estimations of P_{50} had to be conducted on small branches because at the time of harvest this event had a shrubby form and did not have branch-free trunk sections of at least 10 cm in length required for the air-injection technique.

Moisture content of stem wood in free-standing trees in the field trial increased with declining lignin content ($P = 0.0057$, Fig. 5b; Table 1). Wood specific conductivity (k_s) had a near threefold decrease between the control values and the brown wood transgenics, with the normal transgenics varying widely around the k_s value for the control line ($P = 0.05$, Fig. 5c; Table 1). Theoretical k_s values, based on measurements of vessel diameters and frequencies, were substantially higher than measured k_s (Fig. 5c–d). In contrast to the threefold range in k_s (Fig. 5c) the relationship between theoretical k_s and lignin contents showed

a gradual but significant ($P = 0.03$, Fig. 5d) trend, with theoretical k_s of brown wood events about one third less than the control line (Table 1).

Among transgenic events, there were no significant differences from the control line in mean vessel diameters (Table 1). Five transgenic events had significantly different vessel frequencies, but there was no relationship between these trait means and their corresponding lignin contents (Table 1). As expected, comparisons of the slopes of vessel frequency versus vessel diameter using SMATR software (Warton *et al.* 2006) indicated no differences occurred among the three groups ($P < 0.001$, Fig. 6). However, post-hoc comparisons using WALD tests found the controls and normal transgenics to have significant shifts in elevation as compared to the brown wood transgenics ($P < 0.008$), indicating these groups were closer to the theoretical packing limit for xylem conduits. Controls did not differ from the normal transgenics in their proximity to the packing limit ($P = 0.168$). These data are consistent with the weak trend between theoretical k_s and lignin content being driven by the brown wood transgenics (Fig. 5d).

Theoretical k_s was strongly correlated with measured k_s ($P < 0.0001$, Fig. 7a). However, this relationship does not necessarily indicate that xylem anatomy was primarily

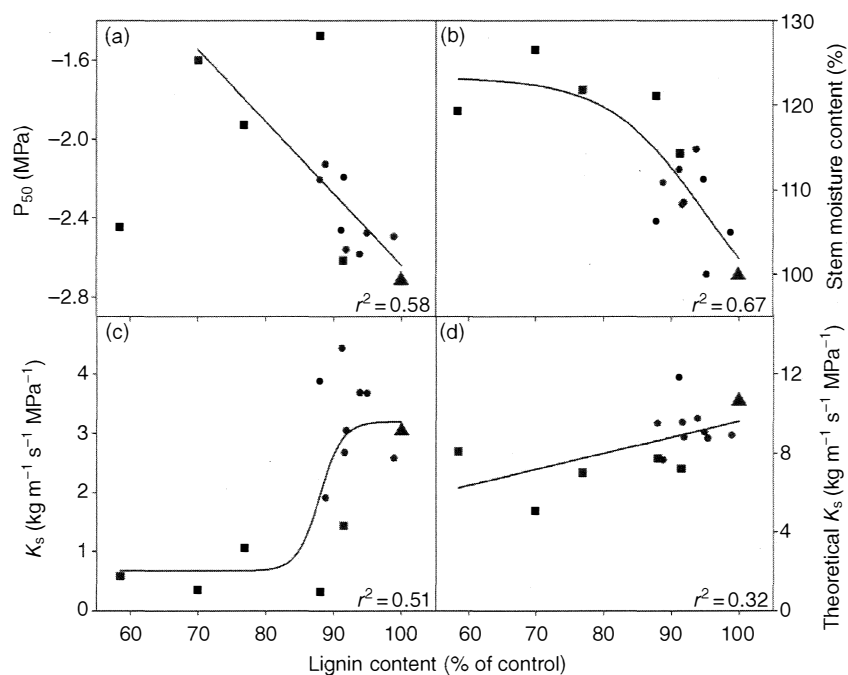


Figure 5. Plotted against lignin content are hydraulics related traits including the negative xylem pressure required to reduce wood specific conductivity (k_s) by 50% (a), stem moisture content (b) maximum k_s (c) and theoretical k_s estimated from wood anatomical measurements (d). Each point is the mean from one event or the control line for free-standing trees only. Symbols as in Fig. 1.

responsible for the lower k_s associated with brown wood occurrence. The percentage of theoretical k_s (observed k_s /theoretical k_s) in brown wood lines fell well below the mean percentage of theoretical k_s for diverse angiosperms (i.e. 44%, Wheeler *et al.* 2005; Hacke *et al.* 2006). Staked poplars spanned the entire range of the data whereas

free-standing trees were limited to generally lower values (Fig. 7). When plotted against the amount of brown wood in the samples on which k_s was measured for each event (not the overall mean across all trees), the strong reductions in conductive efficiency caused by brown wood, as well as the effect of staking were distinct ($P < 0.0001$ for both regressions, Fig. 8). Staked trees had much higher A_l/A_s than free-standing trees regardless of whether the sapwood areas included brown wood or not (Fig. 9a,b).

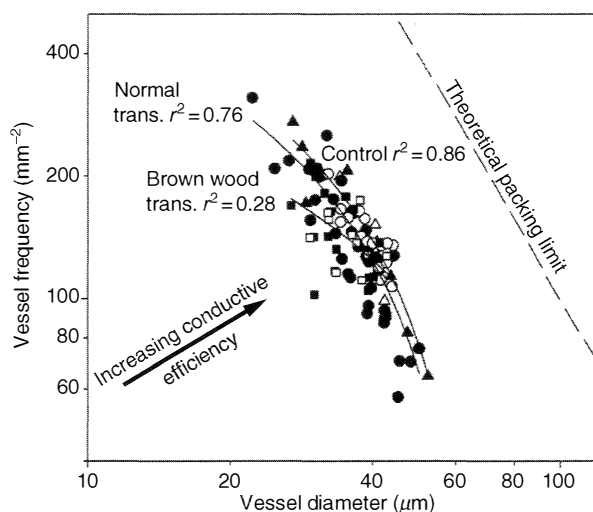


Figure 6. Trends in vessel density and vessel diameter across individual tree stems, log-scale. The theoretical packing limit assumes the entire wood area is made up of vessel lumens for a given mean diameter. Closed symbols are free-standing trees, open symbols are staked trees. Controls = triangle, normal transgenics = circles and brown wood events = squares. Linear regression lines combined field-grown and staked trees within a group.

Comparisons of free-standing and staked poplars

Both free-standing and staked trees had strong correlations between k_s and theoretical k_s (Fig. 7) but self-supported trees had lower absolute values of k_s for a given amount of brown wood (Fig. 8). This difference was associated with significant treatment effects for both k_s and theoretical k_s (Table 2). The parallel regression lines (Fig. 8) indicate the effect of staking was greater for brown wood transgenics, and this was supported by a significant treatment $\times$ line interaction for k_s . Staked trees had k_s values that were about 2.1-fold greater than free-standing trees and their A_l/A_s was about 2.4-fold greater. This imbalance between leaf area and stem water transport capacity resulted in k_l being significantly less for staked versus free-standing trees (Tables 1 & 2). However, it should be noted that the A_l/A_s used to calculate values of k_l (Table 1) were 'unadjusted' for brown wood (Fig. 9a). As such the effective k_l values of brown wood events would have been lower than those reported. Both aboveground biomass and leaf area of free-standing trees tended to be slightly greater on average than for staked trees from the same events (*data not shown*) such

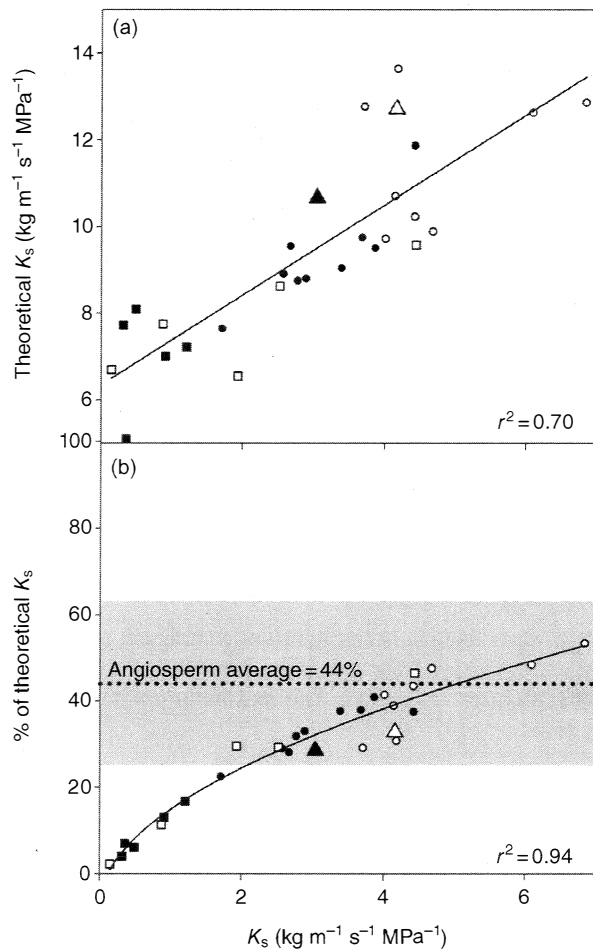


Figure 7. Relationship of measured k_s to theoretical k_s (a) and to percentage of theoretical k_s (b). Each point represents the mean value for either the staked trees (open symbols) or free-standing (closed symbols) trees within a line. Mean of 44% and one standard deviation of 19% conductive efficiency of angiosperms is shown as a dotted line and shaded region (after Hacke *et al.* 2006). Closed symbols are free-standing trees, open symbols are staked trees. Controls = triangle, normal transgenics = circles and brown wood events = squares.

that biomass produced (and associated stem water storage capacity) per unit leaf area was not significantly different between staked and free-standing trees (Table 2).

DISCUSSION

Lower lignin decreases xylem resistance to embolism

Reduced lignification in transgenic 4CL poplars increased their xylem vulnerability to embolism. This finding is consistent with those of other studies reporting relationships between xylem vulnerability to embolism, wood mechanical properties and the corresponding dimensions of xylem conduits across diverse species (Wagner *et al.* 1998; Hacke *et al.*

2001; Jacobsen *et al.* 2005; Jacobsen *et al.* 2007a,b Pratt *et al.* 2007). Although these extensive studies speak to the generality of a hypothesized correlation between wood strength and xylem resistance to embolism, this type of cross-species comparison of xylem safety can be confounded by co-variation in cell types, sizes and wall thickness. By contrast, our intensive work on a single species with altered wood chemistry helps clarify that xylem resistance to embolism relies on adequate cell wall stiffness and strength being provided by normal or near-normal lignin contents.

In a previous study, low-lignin transgenic poplars had greater vulnerability to embolism (i.e. less negative P_{50} values) than the wild type (Coleman *et al.* 2008). In that study only the most affected transgenic event was compared to the wild type, making it unclear whether increased vulnerability to embolism resulted from reduced lignin in the wood as a whole, a parallel reduction in guaiacyl lignin that tends to be localized in vessels (Saka & Goring 1985; Nakashima *et al.* 2008), or the irregular 'collapsed' geometry of many of the vessels. In the poplars we studied collapsed vessels would have contributed very little to k_s and to the P_{50} values we report because these cells rarely carried dye (Fig. 4). In contrast to Coleman *et al.* (2008), the low lignin poplar wood studied here showed a greater reduction in syringyl than guaiacyl moieties and small increases in p -hydroxyphenyl units incorporated into the lignin (Voelker *et al.* 2010). As would be expected from differences in lignin moieties among cell types (Saka & Goring 1985; Nakashima *et al.* 2008), these changes were accompanied by greater reductions of lignin in fibre cell walls compared to vessel cell walls (Voelker *et al.* 2010). Another study of transgenic poplars suggested that up-regulation of

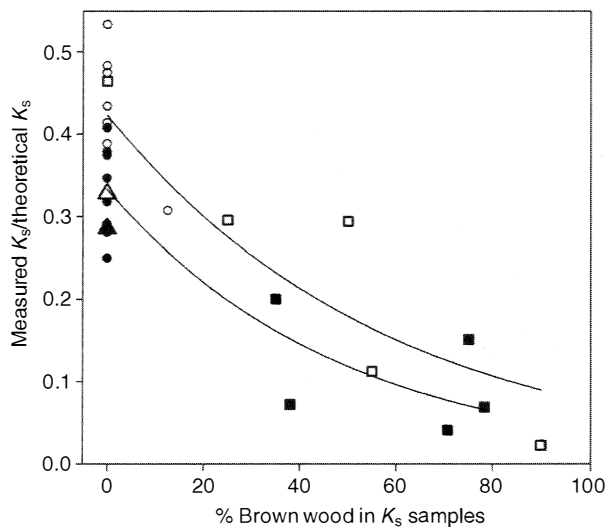


Figure 8. Brown wood incidence plotted against conductive efficiency for free-standing and staked poplars. Brown wood formation in each point is the mean value within those stem segments measured for k_s . Closed symbols are free-standing trees, open symbols are staked trees. Controls = triangle, normal transgenics = circles and brown wood events = squares.

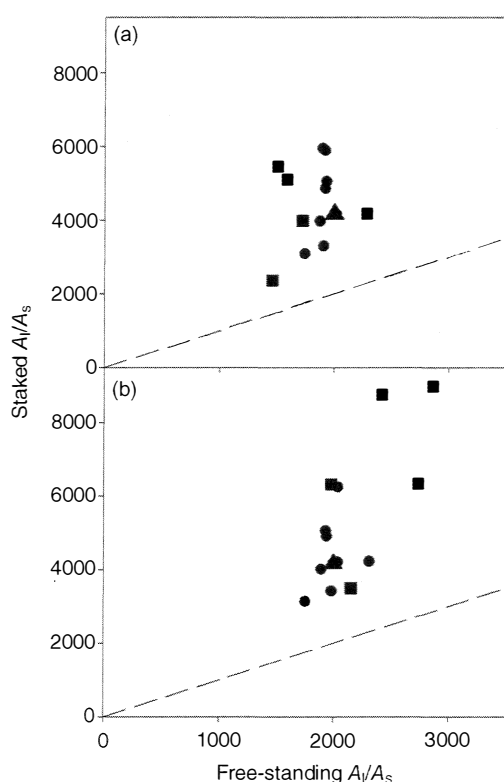


Figure 9. Leaf area to sapwood area ratios for free-standing and staked poplars. Actual values measured (a) and for sapwood values adjusted by average % brown wood in main stems of each event such that only normal appearing sapwood is included (b). Dashed lines show the 1:1 relationship. Controls = triangle, normal transgenics = circles and brown wood events = squares.

ferulate-5 hydroxylase increased wood stiffness (e.g. Huntley *et al.* 2003; Koehler *et al.* 2006), but that this did not decrease P_{50} values (L. Koehler, pers. comm.). This evidence is consistent with the hypothesis that native lignin contents and compositions are evolutionarily 'optimized' for the greatest stiffness and associated resistance to embolism. Moreover, even if transgenic modifications for reduced lignin can be localized within fibre secondary cell walls, the resistance to embolism of vessels may still be compromised by decreased tissue-level stiffness (Jacobsen *et al.* 2005).

There are a few plausible mechanisms by which reduced lignin could cause wood to be more vulnerable to air-seeded embolisms. Altered lignification may have resulted in larger or more abundant voids in vessel cell walls. Under this scenario, micro-fractures might propagate more readily under the hoop stresses that bend vessel walls inward when the xylem stream is under tension (or when pressurized externally, as in our measurements). Thus, air could conceivably pass through compromised cell walls more easily, resulting in embolism of water columns under *in vivo* hydrostatic tensions. The second scenario envisages the edges of pit membranes, made of randomly oriented cellulose microfibrils and embedded with pectic substances, being stretched and or ruptured so as to cause increased

porosity. Extensive membrane stretching (that increases membrane porosity) or even membrane rupture might occur if next to the pit chamber the lignin-pectin matrix of the middle lamella does not have a sufficient interface with the hemicelluloses which are in turn hydrogen bonded to the cellulose microfibrils in the pit membranes. Membrane stretching could also promote embolism if reduced lignification caused the entire wall and pit chamber complex to be more readily bent inward towards the lesser of the pressures between adjacent gas-filled versus water-filled vessels (see Jacobsen *et al.* 2005). This last mechanism for how tissue-level properties can affect membrane stretching seems particularly likely to have contributed to the greater xylem vulnerability to embolism we report because of the established reductions of wood stiffness of low-lignin 4CL poplar (Voelker *et al.* in press; Horvath 2010; Horvath *et al.* 2010b).

Wood moisture content is affected by reduced lignification

Stem water storage is an important component of diurnal water supply for trees (Meinzer *et al.* 2003; Phillips *et al.* 2003; Meinzer *et al.* 2006). The maximum potential water

Table 2. Selected ANOVA results for traits compared within and between field-grown unstaked trees and staked greenhouse-grown trees. Significant treatment effects indicate where staking altered various traits related to plant form or physiological functioning as compared to field-grown trees

Trait	Source	D.F.	F	P
Brown wood	Overall	28	19.01	<0.01
	Treatment	1	1.34	0.25
	Line	14	35.60	<0.01
	Trt. × Line	13	2.51	<0.01
k_s	Overall	28	14.04	<0.01
	Treatment	1	67.38	<0.01
	Line	14	20.41	<0.01
	Trt. × Line	13	3.09	<0.01
A_l/A_s	Overall	28	7.27	<0.01
	Treatment	1	162.89	<0.01
	Line	14	1.18	0.31
	Trt. × Line	13	1.87	0.05
k_1	Overall	28	3.83	<0.01
	Treatment	1	14.80	<0.01
	Line	14	5.65	0.01
	Trt. × Line	13	1.03	0.43
Biomass per unit leaf area	Overall	28	2.20	<0.01
	Treatment	1	2.95	0.09
	Line	14	2.83	<0.01
	Trt. × Line	13	1.46	0.13
Theoretical k_s	Overall	28	1.89	0.01
	Treatment	1	4.85	0.03
	Line	14	2.97	<0.01
	Trt. × Line	13	0.50	0.92
Total biomass	Overall	28	1.77	0.01
	Treatment	1	4.30	0.04
	Line	14	2.95	<0.01
	Trt. × Line	13	0.30	0.99

storage will vary inversely with the proportional volumes of cell wall material, gas in embolized cells and other intercellular spaces (Gartner *et al.* 2004). We expected greater moisture contents in low-lignin wood because of a decrease in the volumetric proportion of cell wall and/or a relative increase in hydrophilic (polysaccharide) cell wall components. This hypothesis was supported (Fig. 5b), but the moisture contents were greater than expected for a given reduction in lignin.

Reductions to lignification of normal cell walls hypothetically could decrease average basic cell wall density (i.e. increased void space within cell walls with reduced lignin). Although this has not been directly measured to our knowledge, Raman imaging microscopy has shown increased water content within cell walls of low-lignin 4CL transgenic aspen as compared to the wild type (Horvath 2010). The wood density, and thus the volume of solid material, did not change appreciably among the transgenic events we studied, but there was a threefold increase in tension wood for the lowest lignin events compared to the control line (Voelker *et al.* in press). Assuming a 24% increase in cellulose content of tension wood as compared to normal wood (Meier 1985), the pore space between cellulose fibrils (if this exists to the same extent that would be present in a normal cell wall without lignin) can explain about 3% of the 23% variation in moisture content. In addition, about a 3% increase in moisture content would result from greater amounts of water bound to cell walls with a proportional shift from phenolic to polysaccharide cell wall polymers (the amount of bound water is usually about 30% of the wood mass). Therefore, only about 6% of the 23% increase in moisture content was explainable due to changes in cell wall composition. The remaining 17% is likely attributable to the hydraulic isolation of water in vessel lumens that were found to contain abundant tyloses (Kitin *et al.* 2010).

Mechanical environment (staking) impacts tree hydraulic architecture

Trees supported by stakes had an altered hydraulic architecture compared to free-standing trees. For the control line and normal transgenic events, trees supported by stakes had about 55% greater k_s on average. Increased k_s in staked plants was found by Gartner (1991a) and decreased k_s with manual stem bending was observed by Kern *et al.* (2005). Staking also increased k_s values for four of five brown wood events, but the magnitude of the increases were much less, corresponding to the significant treatment $\times$ line interactions for both of these traits (Tables 1 & 2). Theoretical k_s of staked trees was also significantly greater, but this difference only amounted to a shift of about 20% on average (Tables 1 & 2). In comparing these traits brown wood had an overriding effect on conductive efficiency that was similar in effect among free-standing versus staked growing environments despite the average conductive efficiency being lower for free-standing trees (Fig. 8). Indeed, the significant treatment $\times$ line interaction for brown wood occurrence and a lack of an interactive effect for theoretical k_s

(Table 2) indicates that rather than changes in wood anatomy, it was brown wood occurrence as modified by growth environment that most affected plant water transport. The increased values of measured k_s /theoretical k_s for staked trees should reflect variation in the hydraulic resistance due to characteristics of vessel design other than size, such as the frequency or porosity of inter-vessel pit fields (Wheeler *et al.* 2005; Hacke *et al.* 2006). Because we would not expect the porosity of inter-vessel pit characteristics to have changed between growth environments we speculate that the reduced bending and faster shoot extension of staked trees was associated with longer vessel lengths and fewer inter-vessel pits for water to cross for a given length of stem xylem.

Lower mechanical demands for support in staked trees corresponded to reduced diameter growth and thus a greater A_l/A_s (Tables 1 & 2; Fig. 9). Although k_s was greater in staked trees, it was not proportional to their increased A_l/A_s , causing the relative ability of the xylem to supply the leaves with water (k_l) to be significantly lower in staked trees. This contrasts with the findings of Gartner (1991a) where k_l values did not differ between staked versus free-standing plants. Thus the staked poplars, with lower biomechanical demands on their xylem, would have had greater xylem tensions for a given rate of stomatal conductance.

Brown wood causes reduced xylem hydraulic sufficiency

Both measured and theoretical k_s were strongly reduced in brown wood. Differences in measured k_s should not be directly attributable to differences in lignin content because lignification occurs after the cellulosic framework determining cell size and the locations of pit fields are largely determined (Esau 1967; Panshin & de Zeeuw 1980; Donaldson 2001). The lower k_s of brown wood events compared to normal wood (Fig. 8; Table 1) can largely be explained by the increased incidence of phenolic extractives or tyloses occurrence in vessels (Kitin *et al.* 2010). However, collapsed xylem, as well as shifts in the size and frequency of uncollapsed vessels, also contributed to reductions in k_s . To account for these differences in wood anatomy, we determined the maximum potential conductivity of a collection of vessels, assuming they are circular conduits (Zimmerman 1983). Measured k_s should always be lower than theoretical k_s because the latter does not take into account the resistance due to inter-vessel pit fields and vessel perforation plates. Although theoretical k_s is not realistic as an absolute value, it allows comparison of the expected trend in k_s among events without the influence of phenolic deposits, tyloses or other occlusions. Lower theoretical k_s values of brown wood events indicate that some portion of the observed reductions in k_s owed to reductions in vessel sizes and frequencies (Figs 5d & 6). For individual trees the ranges of vessel sizes and frequencies overlapped for the controls, normal and brown wood transgenics (Fig. 6). However, standardized major axis regression found there to be a significant reduction in vessel frequency for a given vessel diameter for the

brown wood transgenics in comparison to the other two groups, indicative of this plant vasculature being further away from the theoretical packing limit and having a substantial decrease in conductive efficiency (Sperry, Meinzer & McCulloh 2008; McCulloh *et al.* 2010; Zanne *et al.* 2010). Reduced wood stiffness in low-lignin trees (Voelker *et al.* in press) likely was an indirect cause of the reduction in vessel frequencies in the brown wood transgenics. Decreased wood stiffness would have caused greater bending stresses in free-standing trees which in turn caused up to a threefold increase in tension wood (Voelker *et al.* in press). In most angiosperms, bending stresses associated with the formation of tension wood increases the frequency of fibres at the expense of vessel size and frequency (Gartner 1991a; Yoshizawa *et al.* 2000; Gartner *et al.* 2003; Kern *et al.* 2005). Surprisingly, we detected no difference in tension wood occurrence between staked and free-standing poplars (Voelker *et al.* in press), which explains why trees from both treatments had comparable ranges of vessel sizes and frequencies (Fig. 6; Table 1).

Measured k_s correlated strongly with theoretical k_s for free-standing and staked trees, suggesting that variation in wood porosity and resulting hydraulic function was closely associated with altered lignification (Fig. 7a). By comparing measured to theoretical k_s values of the same wood samples, Hacke *et al.* (2006) found that on average measured k_s was only 44% of theoretical k_s across a diverse set of hardwood species. The values for the poplars studied here were within the expected range for angiosperms measured by Hacke *et al.* (2006). Brown wood events tended to have ratios of measured k_s to theoretical k_s that approached 0%, demonstrating that vessels in these trees had extreme reductions in their hydraulic function (Fig. 7b).

Shoot dieback is associated with altered hydraulic architecture in low-lignin transgenics

Brown wood transgenics had up to nine times more frequent dieback and four times more frequent mortality than did controls (Fig. 3). Hydraulic failure appears to have caused the shoot dieback at the end of each summer because desiccation of distal leaves occurred so abruptly after leaf necroses appeared that no yellowing or apparent remobilization of cell contents was observed (Fig. 4a–b). The introduction of dye into the xylem, with subsequent localization using a cryo-fixing procedure provided visual evidence of reductions in water transport in brown wood (Fig. 4c,e; Kitin *et al.* 2010).

The above results, in combination with knowledge of sapwood area and leaf area, can be used to draw inferences about whole plant water status and risk of catastrophic xylem embolism. Trees were kept well-watered throughout the field trial, diminishing the likelihood that dieback was caused exclusively by xylem with increased vulnerability to embolism. Rather, the deposition of phenolic extractives and formation of tyloses within vessels caused severe reductions in k_s and effectively decreased the sapwood area in brown wood transgenics (Kitin *et al.* 2010). In brown wood

events the functional sapwood area was reduced in relation to leaf area and transpirational demand by about 20% in the field and 55% for staked trees. Xylem tension gradients in brown wood events would have increased in direct proportion to the increase of A_l/A_s in the absence of reductions in stomatal conductance to compensate for their reduced k_l . Therefore, we tentatively conclude that both increased vulnerability to embolism in low-lignin xylem and reductions in k_l that increased xylem tensions were likely to have caused catastrophic, or runaway xylem embolism (*sensu* Sperry & Tyree 1988) that, in turn, resulted in the rapid branch dieback observed.

In conclusion, major disruptions of poplar physiological function were induced by *4CL*-downregulation. In addition to modified wood chemistry, in previous work we demonstrated major changes to wood structure and strength (Kitin *et al.* 2010; Voelker *et al.* 2010, in press). In the current work, changes in lignin biosynthesis and deposition of phenolic extractives led to greater xylem drought susceptibility, xylem dysfunction, plant dieback and mortality. Our work suggests that crop plants designed with lower lignin content are very likely at risk for reduced carbon gain and higher mortality, especially if grown in high stress environments.

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