

# Reduced wood stiffness and strength, and altered stem form, in young antisense *4CL* transgenic poplars with reduced lignin contents

Steven L. Voelker<sup>1</sup>, Barbara Lachenbruch<sup>1</sup>, Frederick C. Meinzer<sup>2</sup> and Steven H. Strauss<sup>3</sup>

<sup>1</sup>Department of Wood Science & Engineering, Oregon State University, Corvallis, OR 97330, USA; <sup>2</sup>USDA Forest Service, Pacific Northwest Research Station, 3200 Jefferson Way, Corvallis, OR 97330, USA; <sup>3</sup>Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97330, USA

## Summary

Author for correspondence:

Steven L. Voelker

Tel: +1 541 829 9094

Email: [steve.voelker@oregonstate.edu](mailto:steve.voelker@oregonstate.edu)

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**Key words:** buckling safety factor, lignin, stem form, tension wood, transgenic poplar, wood stiffness, wood strength.

- Reduced lignin content in perennial crops has been sought as a means to improve biomass processability for paper and biofuels production, but it is unclear how this could affect wood properties and tree form.
- Here, we studied a nontransgenic control and 14 transgenic events containing an antisense 4-coumarate:coenzyme A ligase (*4CL*) to discern the consequences of lignin reduction in poplar (*Populus* sp.). During the second year of growth, trees were grown either free-standing in a field trial or affixed to stakes in a glasshouse.
- Reductions in lignin of up to 40% gave comparable losses in wood strength and stiffness. This occurred despite the fact that low-lignin trees had a similar wood density and up to three-fold more tension wood. In free-standing and staked trees, the control line had twice the height for a given diameter as did low-lignin trees. Staked trees had twice the height for a given diameter as free-standing trees in the field, but did not differ in wood stiffness.
- Variation in tree morphogenesis appears to be governed by lignin × environment interactions mediated by stresses exerted on developing cells. Therefore our results underline the importance of field studies for assessing the performance of transgenic trees with modified wood properties.

## Introduction

Despite wide recognition that lignin is crucial for water conduction and plant self-support (Raven, 1977; Niklas, 1992; Boyce *et al.*, 2004), it is unclear how variation in lignin content affects biomechanics at the cell, tissue and whole-plant level. The strength, stiffness and other basic mechanical properties of lignin, cellulose and hemicellulose have been estimated (Mark, 1967; Cousins, 1976, 1978; Niklas, 1992), but there is only a rudimentary understanding of how polymers interact within cell walls and between cells (Salmén & Burgert, 2009). For long-lived woody plants, tissue behavior under bending loads, such as those imposed by wind or ice, are important for determining structural stability and survival (King, 1986; Gartner, 1995; Niklas, 1994; Peltola *et al.*, 1999). When a stem is subjected to bending, it simultaneously experiences tensile stresses on the side being pushed and compressive stresses on the opposite side. Cellulose provides most of the

strength in tension, whereas lignin appears to play an important role in compression and shear resistance (Niklas, 1992; Gindl & Teischinger, 2002; Horvath, 2010).

Research on the effect of reduced lignin for wood stiffness and strength has found conflicting results depending on the methods used and range of lignin contents investigated. Lignin has been found to be important for the strength of wetted wood, above the fiber saturation point, but, in dry tissues, there is little difference (Klauditz *et al.*, 1947; Klauditz, 1952; Köhler & Spatz, 2002). One interpretation of these data is that the foremost mechanical role of lignin is to protect the structural polysaccharides (cellulose and hemicelluloses) from interactions with water. However, these experiments may have exaggerated the role of lignin in cell wall mechanics because the chemical removal of lignin probably affects other cell wall components (Burgert *et al.*, 2005a,b; Jungnikl *et al.*, 2008). Isolated plant fibers differing in lignin content have also been tested, but co-variation with the orientation of cellulose microfibrils within the S2

cell wall layer probably obscures the effect of lignin content (Salmén, 2004; Burgert, 2006). Moreover, the axial properties of fibers are indirectly related to their transverse properties and thus to the behavior of wood under bending stresses (Bergander & Salmén, 2000). Nano-indentation has been used to test average wood moduli (i.e. not influenced by microfibril angle or material anisotropy) of specific cell wall layers, indicating that lignin contents of conifer tracheids above *c.* 200 mg g<sup>-1</sup> result in progressive decreases in stiffness (Gindl *et al.*, 2004).

Lignin appears to be important for resisting compressive stresses in the axial direction. The compressive strength was positively correlated with lignin content, but there was only about a 10% increase in compressive strength for a near-doubling in lignin content from *c.* 180 to 350 mg g<sup>-1</sup> (Gindl & Teischinger, 2002). Lignin contents are thought to be more important for developing xylem, where stiffness is *c.* 75% of the value for a mature cell wall (Gindl *et al.*, 2002). It is uncertain how this information may apply to cell walls that are fully developed but have low lignin contents. Lignin-deficient wood often includes areas of cell deformation or collapse (Donaldson, 2002; Coleman *et al.*, 2008; Wagner *et al.*, 2009; Voelker *et al.*, 2010), suggesting that, after apoptosis and the removal of turgor pressure, vessel cell walls become deformed under compressive stresses induced by xylem tension. Although low lignin contents probably promote cell collapse, the proximate cause is poorly understood. In tension wood fibers, the G-layer is a highly cellulosic innermost wall layer with cellulose microfibrils of this layer oriented close to the cells' axial direction. In addition to maturation stresses induced by the cellulose lattice spacing in the S2 cell wall layer (Clair *et al.*, 2010), a strong swelling action of the G-layer creates additional tensile stresses that help angiosperms re-orient their stems (Goswami *et al.*, 2008). Hence, considerable lateral and/or axial compressive stresses may be induced by tension wood on the surrounding cells (Clair *et al.*, 2006). This could explain the observation that, in low-lignin poplar, tension wood cells were not deformed even though a tangential band of surrounding fibers and vessels lacking G-layers was collapsed (Voelker *et al.*, 2010).

The stresses exerted on developing cells and the resulting strains perceived are determined by the stiffness of the surrounding cells and the shape and orientation of the plant or organ under consideration (Coutand *et al.*, 2009; Hamant & Traas, 2009; Moulia & Fournier, 2010). Hence, any decrease in the rigidity of the wood will increase the likelihood of catastrophic stem failure if the tree stem geometry is not altered to provide a lower center of gravity and reduced bending moment (Cannell & Morgan, 1989; Niklas, 1994; Peltola *et al.*, 1999). The 'uniform stress hypothesis' posits that cambial growth along the stem (and, by extension, cellular identity in proportions of vessels, normal and tension wood fibers) is regulated so as to distribute stresses equitably along the outer margin of the stem

(Wilson & Archer, 1979; Archer, 1987; Morgan & Cannell, 1994; Gartner, 1995; Telewski, 1995). Similarly, normal 'growth stresses' are used in many biological systems to achieve an upright form (Moulia & Fournier, 2010). By extending the uniform stress hypothesis to include changes in wood mechanical properties, it can be predicted that trees of the same species with reduced wood stiffness will have greater allocation of growth towards the base of the tree. However, the high planting densities used to maximize forest biomass productivity promote slender tree growth forms (i.e. little taper) (Jaffe, 1973; King, 1986; Holbrook & Putz, 1989; Telewski, 1995, 2006; Ennos, 1997). Thus, in the high-density stands common for plantation and bioenergy plantings, low-lignin trees – if compromised in strength as proposed above – could be at an especially high risk of mechanical failure.

The mechanical properties of transgenic low-lignin trees have been characterized in detail by only a few research groups (Koehler & Telewski, 2006; Koehler *et al.*, 2006; Horvath, 2009, 2010; Horvath *et al.*, 2010). With the exception of increased tensile strength in low-lignin flax (Wróbel-Kwiatkoska *et al.*, 2007), studies of other transgenic low-lignin plant species have found a reduction in the material properties (Hepworth & Vincent, 1999; Merali *et al.*, 2007; Patten *et al.*, 2007). *4CL* down-regulated trees have been reported to show strong reductions in lignin content (Hu *et al.*, 1999; Li *et al.*, 2003; Jia *et al.*, 2004; Wagner *et al.*, 2009; Voelker *et al.*, 2010), but there has been little study of the effects on tree biomechanical stability. This knowledge will be critical for weighing the tradeoffs between the potential positive impact of lignin reductions on the end uses of wood fiber vs the negative impact of severely reduced lignin on structural integrity of the xylem (Anterola & Lewis, 2002; Davin *et al.*, 2008).

To study how lignin reduction affects wood properties and stem architecture under differing biomechanical demands on the xylem, we compared free-standing trees in the field vs staked, glasshouse-grown trees. We report here that transgenic *4CL* poplar with reduced lignin shows lower stiffness (modulus of elasticity, MOE) and strength (modulus of rupture, MOR). To avoid mechanical failure, transgenic poplars with low lignin content and reduced wood stiffness altered their stem form by increasing the radial rates of cell division at the tree base relative to apical cell division rates, resulting in greater stem taper. Low-lignin trees also had an increased proportion of tension wood.

## Materials and Methods

### Plant genotypes and transformation

Hybrid white poplar (*Populus tremula* × *P. alba*, INRA-France 717-1B4) was used for all transformations, as

described by Filichkin *et al.* (2006). *Agrobacterium strain* C58 containing the antisense *P. tremuloides 4CL1* construct was provided by Dr Vincent L. Chiang at North Carolina State University, NC, USA (Li *et al.*, 2003). To ensure transformation events were independent, transgene presence was confirmed by PCR and a single regenerating shoot per individual explant, termed a transgenic 'event,' was propagated. Genomic DNA isolation and PCR conditions have been described elsewhere (Voelker *et al.*, 2010).

### Plant preparation and growth conditions

PCR-positive events were propagated *in vitro* (Filichkin *et al.*, 2006). Seven-to-eight-week-old plantlets were transferred to soil in small pots in a glasshouse and grown for 2 months under a 16 h : 8 h photoperiod with supplemental lighting (April–May 2005), followed by transfer to tubular pots (6.7 × 24.8 cm) for another 2 months (June–July 2005). A total of 14 transgenic events (i.e. independent gene insertions) with 10–17 ramets and 108 nontransgenic controls were produced. Plants were relocated to an outdoor shadehouse for 3 months of acclimatization (August–October 2005). Transgenic controls were not employed because the transformation protocol used suggests very little somaclonal variation (Strauss *et al.*, 2004).

The field trial, planted in November 2005 with dormant plants, was conducted just outside Corvallis, OR, USA (44.65°N, 123.3°W, 140 m elevation). All trees were kept well watered during the duration of the field trial. The planting was arranged as a randomized complete block with 10–15 ramets from each transgenic event and a control line planted at a square spacing with 3 m between trees. In February 2007, before the second year of growth, two individuals from each transgenic event (except event 90 because it had the fewest trees,  $n = 10$ ) and four individuals from the control line were randomly selected to be transferred to an unheated glasshouse. These trees were carefully dug from the initial field site and replanted in large pots (*c.* 66 l of soil volume per tree) full of a local sandy loam soil. Each tree was watered and fertilized regularly. The main stem was affixed to a 1.5 × 3 cm wooden stake from heights of *c.* 0.5 and up to 5 m from the pot soil level at weekly intervals. The stakes themselves were guyed to the bases of the pots with steel wires to prevent their movement. Trees were fixed between the stake and the steel guy wire using numerous plastic zip-ties that prevented any significant sway below the growing tip. Trees were moved within the planting arrangement once per month to minimize any effects of spatial variation in growth conditions. The roof and sides of the glasshouse opened automatically when indoor temperatures exceeded 29°C and closed when temperatures dropped below 20°C.

### Lignin content and brown wood transgenics

In a parallel study, molecular beam mass spectroscopy (MBMS, Laboratory of Gerald Tuskan at the Department of Energy, Bioenergy Science Center, Oak Ridge, TN, USA) was used to estimate that the lignin content of the control line was 223 mg g<sup>-1</sup> and that each of the transgenic events had reduced lignin contents in comparison with the control line (Voelker *et al.*, 2010). In the same study, the total monomer release by thioacidolysis was used to characterize the trees (Laboratory of Norman Lewis at Washington State University, Pullman, WA, USA). Thioacidolysis yields do not give an absolute value of lignin content, but are probably more accurate than MBMS estimates for the transgenic events that formed brown wood. Brown wood events were characterized by increases in phenolic extractives that probably interfered with the accuracy of the MBMS methods. Therefore, we calculated the lignin contents relative to the control value using the total thioacidolysis yields and assuming a 70% yield for hardwoods (Boudet *et al.*, 1995). These values are provided in Supporting Information Fig. S1, Table S1.

This parallel study also found that severe *4CL* down-regulation resulted in wood with decreased lignin contents and a patchy reddish-brown color in *c.* 20–60% of the stem cross-sectional area (Voelker *et al.*, 2010). Hereafter, we refer to these as 'brown wood transgenics', whereas the other transgenic events had 6% or less brown wood and are referred to as 'normal transgenics'.

### Tree form

Tree height and basal diameter were measured in November 2005 (planting), 2006 and 2007. At the end of the 2007 growing season, six control trees and three or four trees that spanned the range in tree size for each transgenic event were harvested from the field planting to determine the proportions of oven-dried biomass in the main stems, branches and leaves. The same measurements were made for all staked trees that were transferred back to the glasshouse and staked the previous winter.

### Wood properties and tree biomechanics

To calculate wood stiffness (Young's modulus or MOE) and wood strength (MOR), we subjected stem sections with bark intact to standard three-point static bending tests. All stem sections were from 'main stems' that were near vertically aligned. Because the samples varied in size, the larger samples may have included 2 yr of growth, whereas smaller samples generally included just 1 yr of growth. For all stem samples, we used a Sintech Model 1/G (MTS Systems Corp., Eden Prairie, MN, USA), fitted with a Sensotec 230 kg load cell (Model 41/571-07; Honeywell

International Inc., Morristown, NJ, USA). Sample dimensions and taper were calculated from the diameter inside the bark at each end of a sample, its total length and the span tested. The span tested for each sample was 16–20 times the sample's mid-point diameter. For these dimensions, shear force stresses should be negligible compared with bending stresses. Moduli were corrected for taper following Maki & Kuenzi (1965).

All samples were tested at 12% wood moisture content. Samples were first air-dried and left with the bark on, which slowed the process, except at the sample ends. During air-drying, checks (radial cracks) formed at the outer ends (2–3 cm) of larger diameter samples (control line and transgenic events), but otherwise checking was rarely observed. Data from the bending tests employed largely reflect wood properties at the center of the sample, and so checks located at the ends of samples were outside the span tested and should not influence our results.

Because the range of diameters tested differed within each line or event (see the Results section), we calculated MOE and MOR values normalized to the regression-predicted value for that diameter of the control line. Therefore, these normalized values provide estimates of the variation in MOE and MOR that should be unbiased with respect to variation in the stem diameter distributions tested for the different events. For most samples, the wood tested contained a single year of growth. Two growth rings were present in a few of the larger samples for each line or event. Although radial changes in wood properties are well known, the difference between two consecutive years is generally very small and should have little influence on the wood properties of the few samples with two growth rings.

To compare biomechanical function for trees that differed in both material properties and form, we estimated the safety factor for buckling as the critical buckling height ( $H_{crit}$ ; Greenhill, 1881) divided by the actual height of individual trees. This model oversimplifies tree form, but has been shown to approximate well the height at which a tree of a given material and geometry becomes unstable and buckles under its own self-loading (Holbrook & Putz, 1989). Greenhill's formula is as follows:

$$H_{crit} = 1.26 \times (E/W)^{1/3} \times (D)^{2/3}$$

[ $E$ , apparent MOE (Pa);  $W$ , green wood density (i.e. density of wood and water,  $\text{kg m}^{-3}$ );  $D$ , basal diameter of the tree (m)].

For the data presented here,  $D$  was measured just above the basal flare associated with the root collar, 5–10 cm above the soil surface. It was assumed that the values for  $E$  corresponded to those MOE values measured for each line or event at 12% moisture content, but reduced by 28%, the overall average loss in stiffness of green wood vs that tested at

12% moisture content (Green *et al.*, 1999). Moisture contents (fresh wood mass/oven-dried wood mass) were measured on two basal stem sections (50–200 mm in length depending on stem diameter) from free-standing and staked trees. Wood densities (oven-dried wood mass/green wood volume) were measured on trunk and branch samples that ranged from 1.5 to 60 mm in diameter and 50 to 100 mm in length. Branch samples were generally < 5 mm in diameter and trunk samples were generally > 5 mm in diameter. The volume of green wood was measured by water displacement and the mass was measured for the same sample pieces after being oven-dried. Wood was largely from the second year of growth, although a small amount of wood from the first year of growth may have been present in larger diameter stem samples. The same two basal sections measured for moisture contents were used to calculate green wood density (green wood mass/green wood volume).

### Tension wood

Tension wood was estimated using two methods: staining/light microscopy and visual estimation. The staining/light microscopy method (described below) is well established, but time intensive, and so only a subsample of trees was used. Tension wood can also be identified by its lighter color and smooth and shiny appearance compared with surrounding normal wood fibers (Badia *et al.*, 2006; Barbacci *et al.*, 2008; Fig. S2). The visual estimation method (described below) employed this macroscopic characteristic, and was more rapidly conducted than staining/light microscopy. Our comparison of results confirmed that estimates from the two methods provided substantial agreement (Fig. S2).

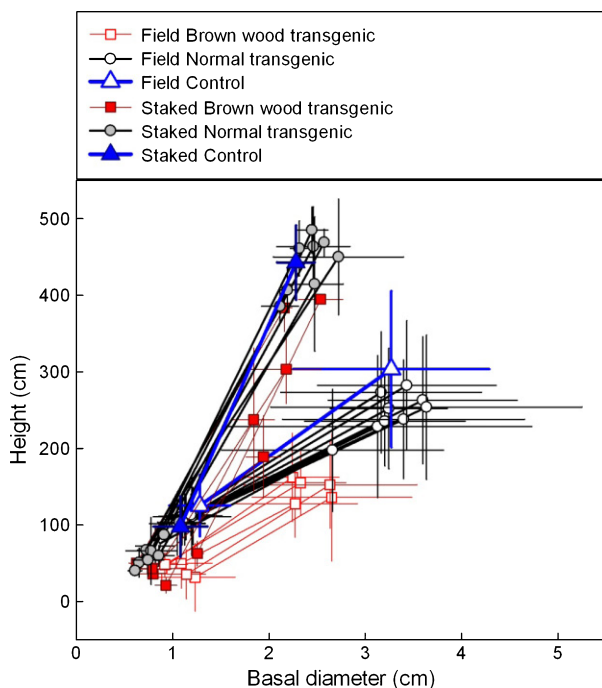
From near the base of trees harvested for biomass measurements, a razor blade was used to make thin, transverse hand-sections located along a radial pith to bark path across the stem. These transverse sections were stained with safranin and astra-blue following Jourez *et al.* (2001) to distinguish the gelatinous G-layers of tension wood fibers (Fig. S2). Using a bright-field light microscope (Nikon E400, Tokyo, Japan), three radial scans were conducted across hand-sections to visually estimate the relative radial position (to the nearest 5%) at which patches of astra-blue-stained tension wood fibers began and ended. The resulting distance and frequency distributions were used to estimate the percentage of the xylem cross-sectional area formed as tension wood fibers, assuming stems were circular in cross-section. Visual estimations of tension wood were made from the cut-ends of trees not harvested for biomass measurements. Tension wood was quantified by overlaying a grid of dots on a transparent plastic sheet over three cross-sections from each tree at three heights (stem base, 20 and 40 cm) and recording the relative frequency of tension wood relative to the entire cross-sectional wood area.

## Statistical analyses

Least-squares regression methods were used to assess the relationships between tree form, size and wood mechanical properties. To compare trait values among the controls and transgenic events, we conducted analysis of variance (ANOVA) tests. Traits were first compared with a global ANOVA (PROC GLM, SAS version 9.2; SAS Institute Inc., Cary, NC, USA) to determine whether significant variation existed among events, treatments (i.e. staked vs free-standing) and their interaction. Further analyses compared means among transgenic events and the control with Tukey honestly significant difference (HSD) tests to control for Type 1 experiment-wise error. ANOVA results for MOE and MOR used values that were normalized to the regression-predicted control value for a given diameter.

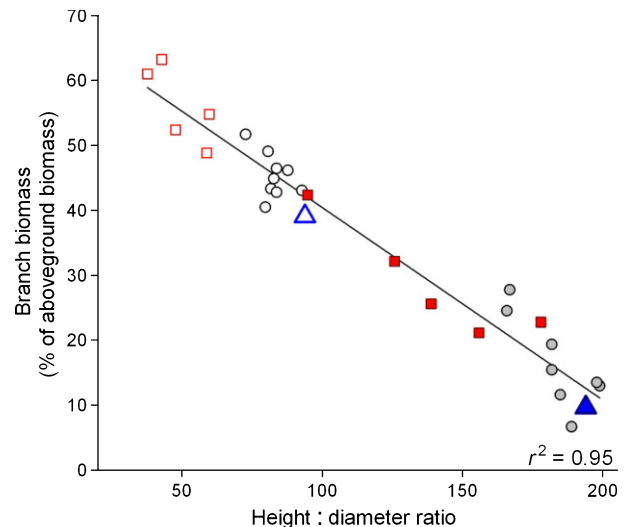
## Results

Compared with the free-standing trees grown in the field trial, staked trees were consistently taller and thinner, and had less branching and larger leaves (Figs 1–3; Table S2). The differences in growth trajectories between staked and free-standing trees appeared to be greatest for brown wood transgenics (Fig. 1). Indeed, for a given stem diameter, increases in height growth of staked transgenics resulted in height : diameter ratios having a strong treatment  $\times$  line

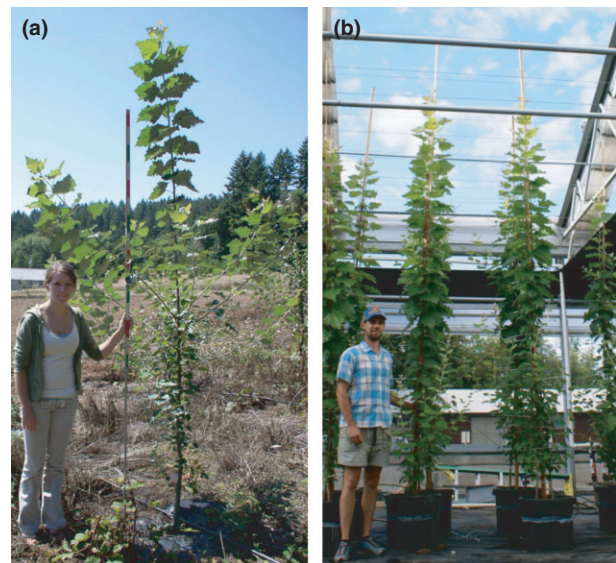


**Fig. 1** Height vs diameter of free-standing field-grown and staked glasshouse-grown hybrid poplar trees. Each pair of symbols and connecting line represent the mean growth trajectory from 2006 to 2007 for each event and the control line. Error bars are 1 standard deviation.

interaction (Tables 1, S2). In comparison with staked trees, the free-standing controls and transgenics had at least a two-fold increase in proportional branch biomass that was strongly correlated with the height : diameter ratio (Fig. 2; Table S2). Within each growth environment, there was a



**Fig. 2** Proportion of aboveground biomass in branches vs height : diameter ratio of free-standing field-grown and staked glasshouse-grown hybrid poplar trees. Open squares, field-grown brown wood transgenic; open circles, field-grown normal transgenic; open triangles, field control; closed squares, staked brown wood transgenic; closed circles, staked normal transgenic; closed triangles, staked control.



**Fig. 3** Examples of variation in stem architecture between the field trial (a) and the staked poplar trees (b). The largest free-standing field-grown tree from event 640 was photographed from late August 2007 (a) and staked trees from events 224 and 225, pictured in center left and right, respectively, were photographed in early August 2007 (b).

**Table 1** Selected analysis of variance (ANOVA) results for traits compared within and between field-grown free-standing poplar trees and staked glasshouse-grown trees

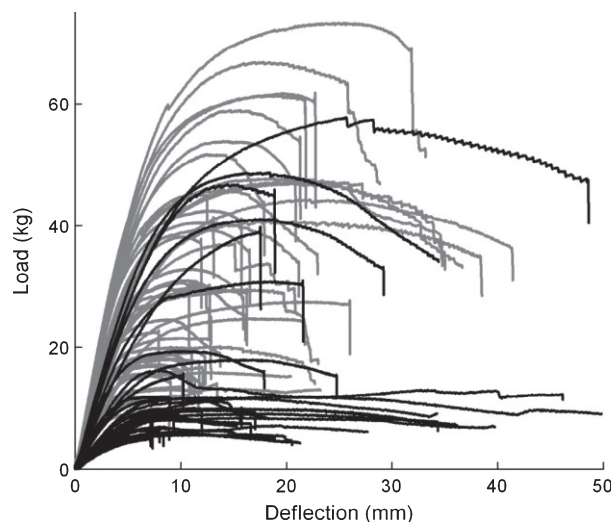
Trait	Source	df	F ratio	P value
Height : diameter ratio	Treatment	1	894.93	< 0.01
	Line	14	14.33	< 0.01
	Trt. × Line	13	4.47	< 0.01
Safety factor	Treatment	1	43.05	< 0.01
	Line	14	1.18	0.39
	Trt. × Line	13	0.53	0.87
MOR	Treatment	1	44.00	< 0.01
	Line	14	21.43	< 0.01
	Trt. × Line	12	5.94	0.01
Height	Treatment	1	61.88	< 0.01
	Line	14	8.88	< 0.01
	Trt. × Line	13	0.81	0.65
Branch biomass	Treatment	1	122.20	< 0.01
	Line	14	2.50	0.01
	Trt. × Line	13	0.69	0.76
MOE	Treatment	1	17.52	< 0.01
	Line	14	18.57	< 0.01
	Trt. × Line	13	5.34	< 0.01
Tension wood	Treatment	1	0.72	0.40
	Line	14	4.76	< 0.01
	Trt. × Line	13	0.76	0.70
Diameter	Treatment	1	19.46	< 0.01
	Line	14	2.52	< 0.01
	Trt. × Line	13	0.37	0.98

The 'treatment' effect relates the variation as a result of growing environment (staked vs free-standing), whereas the 'line' effect relates the variation induced by changes in lignin content among the transgenic events and the control line. MOR, modulus of rupture; MOE, modulus of elasticity.

two-fold variation in branch biomass associated with changes in the height : diameter ratio, and thus to lignin content (Fig. 2, Table S2). There was no treatment × line interaction for branch biomass proportion (Table 1).

To account for the effects of stem size, as well as the patchy nature of brown wood and associated lignin reductions, load and deflection data (Fig. 4) were combined with individual sample dimensions to calculate wood stiffness (MOE) and wood strength (MOR) across a range of stem diameters for each event and the control line (Fig. 5). MOE values of controls and normal transgenics declined with diameter ( $P < 0.003$ , Fig. 5), but there was no detectable trend for the brown wood transgenics ( $P = 0.15$ , Fig. 5). MOR values declined with diameter for each group ( $P < 0.03$ , Fig. 5). For a given diameter, the values for controls were greatest, followed by the normal transgenics and then the brown wood transgenics (Fig. 5). Wood density was similar among the three groups (Fig. 6), with the exception of a trend towards lower minimum wood density in the smallest diameter samples and higher maximum wood density in slightly larger stems of the brown wood transgenics.

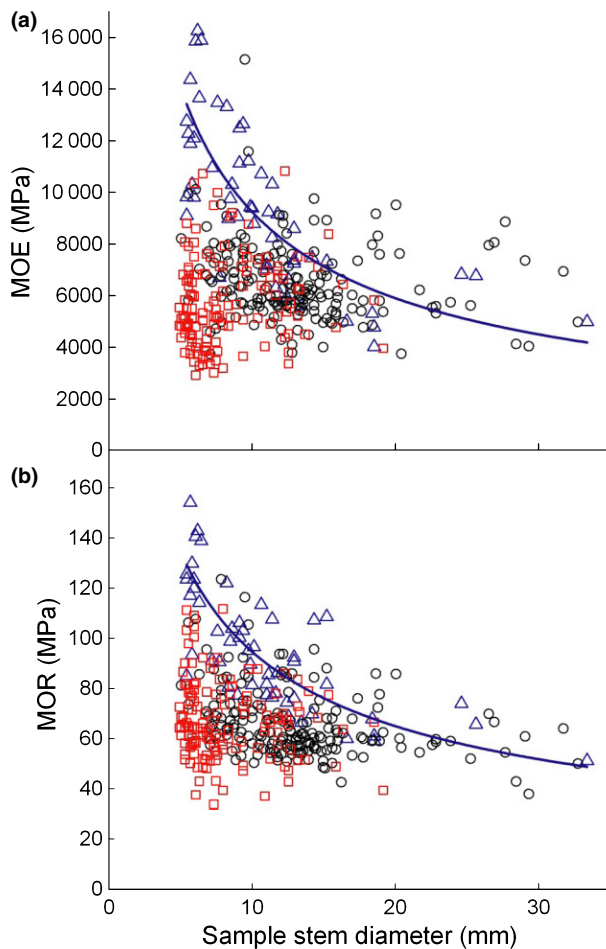
Reductions in lignin content of up to 40% were associated with reductions in stem diameter-standardized MOR



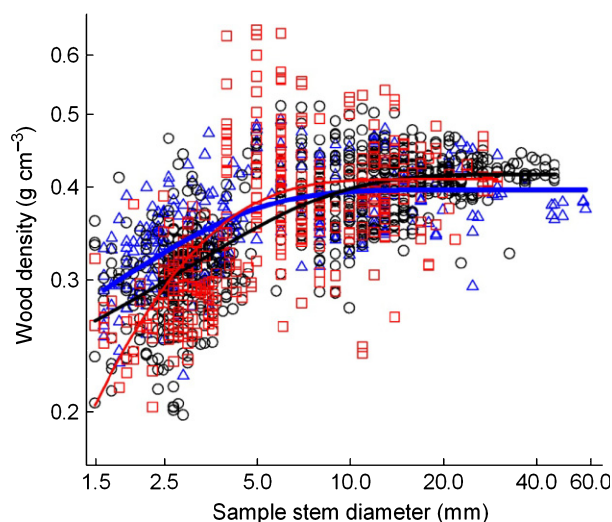
**Fig. 4** Examples of data from bending tests used to calculate modulus of elasticity (MOE) and modulus of rupture (MOR). Data shown here were from stem sections of the control line (gray lines,  $n = 47$ ) and transgenic event 712 (black lines,  $n = 35$ ) that ranged in diameter from 24 to 60 mm and from 21 to 73 mm, respectively. Note that the six samples from event 712 that supported the greatest loads were all larger stem sections that had no brown wood apparent.

of *c.* 40% ( $P = 0.0004$ , Fig. 7a) and MOE by *c.* 50% ( $P < 0.0001$ , Fig. 7b). Among the control line and transgenic events, MOE did not vary across the small range of average wood densities that were sampled (Fig. 8). Tree height : diameter ratios were positively correlated with lignin content ( $P = 0.0004$ , Fig. 9a), but wood stiffness explained more of the variation in this trait ( $P = 0.0001$ , Fig. 9c). Similarly, the percentage of xylem area in tension wood fibers was correlated with lignin content ( $P = 0.0017$ , Fig. 9b), but the relationship with wood stiffness was stronger ( $P < 0.0001$ , Fig. 9d). Lignin content and MOE were also both inversely correlated ( $P < 0.002$ ) with the ratio of radial to apical cell division rates (Fig. S3). Although the height : diameter ratios were greatly affected by whether plants were grown in the field or staked in the glasshouse, no significant effect of staking was found for the incidence of tension wood (Tables 1, S2). The proportion of biomass in branches showed a relatively weak inverse relationship to lignin content, whereas the effect of staking on this trait was very strong, with the proportional biomass in branches in staked trees being less than half of that in free-standing trees (Tables 1, S2).

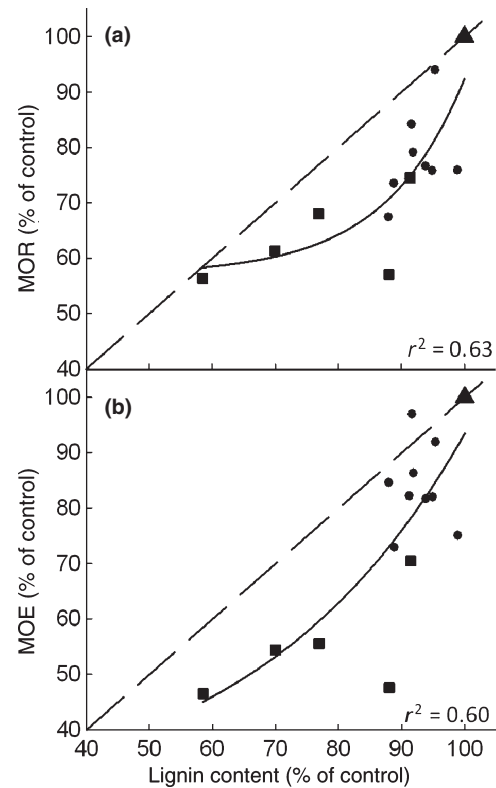
Safety factors against elastic buckling were calculated to account for the co-dependence of whole-tree biomechanical stability on tree form and wood properties (Fig. 10). These calculations showed that free-standing controls and normal transgenics varied around the angiosperm tree average estimated at 4.66 (Niklas, 1994). Free-standing brown wood transgenics all had lower safety factors than the angiosperm average, but were still in no danger of buckling under



**Fig. 5** Modulus of elasticity (MOE) (a) and modulus of rupture (MOR) (b) of control (triangles), normal transgenics (circles) and brown wood transgenics (squares) by sample diameter. MOE is a measure of wood stiffness and MOR is a measure of wood strength.



**Fig. 6** Wood density of control (triangles), normal transgenics (circles) and brown wood transgenics (squares) by sample diameter. Data are plotted on a logarithmic scale.

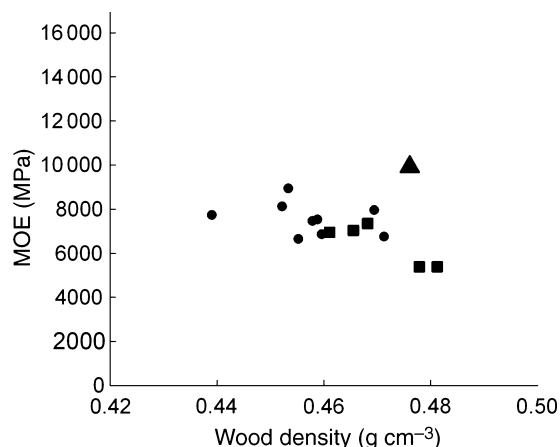


**Fig. 7** Variation among transgenic events and the control line in modulus of rupture (MOR) (a) and modulus of elasticity (MOE) (b) as related to lignin content. Control (triangle), normal transgenics (circles) and brown wood transgenics (squares).

their own weight (Fig. 10, Table S2). By contrast, safety factors of trees grown staked in a glasshouse ranged from 0.96 to 1.53, placing some trees below the predicted limit for plant self-support. These calculations indicate that most of the staked trees would have buckled under even modest wind loading if left unsupported. Indeed, a number of trees did buckle under their own weight, including leaf and branch mass, on being released from their stakes during harvest.

## Discussion

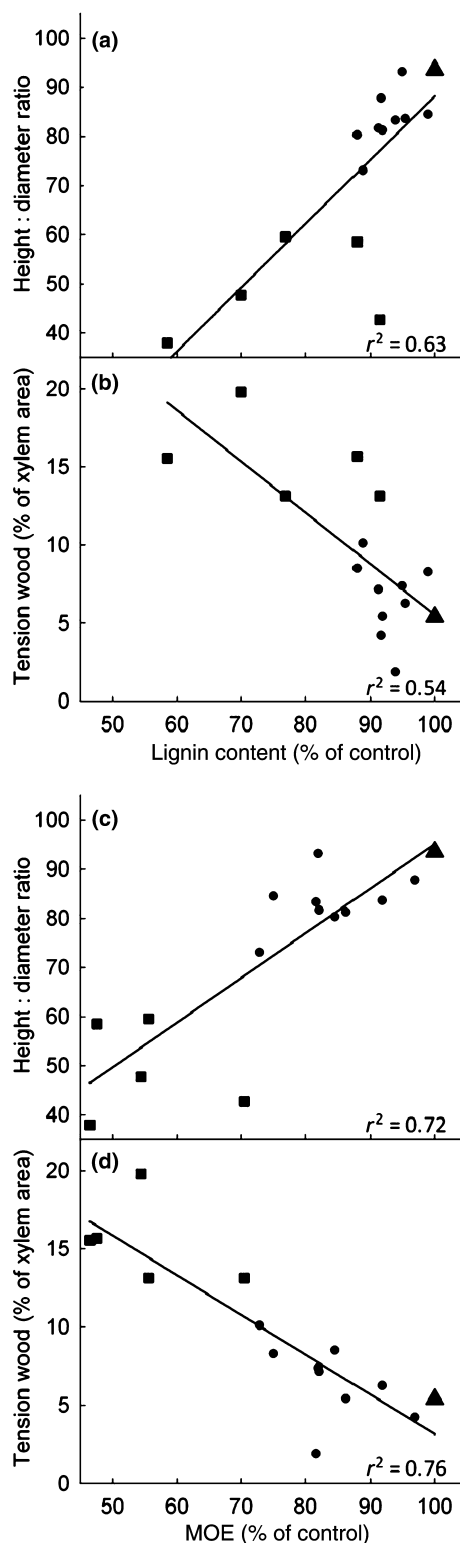
Differences in plant form arise from meristematic cells sensing both physical and biochemical signals that locally alter the specification of cell identity, division and expansion (Hamant & Traas, 2009; Jönsson & Krupinski, 2009). Physical stresses that elicit changes in developing cells, and thus alter plant shape, are modulated in part by the mechanical properties of the previously formed plant tissues (Mouliya & Fournier, 2010). Lignification, the last step in the assembly of secondary cell walls, rigidifies cells and causes them to cease expansion (Panshin & De Zeeuw, 1980; Donaldson, 2001). Thereby, a variation in lignin content, which implies a change in the rigidity of previously



**Fig. 8** Variation among transgenic events and the control line in modulus of elasticity (MOE) as related to wood density. Control (triangle), normal transgenics (circles) and brown wood transgenics (squares).

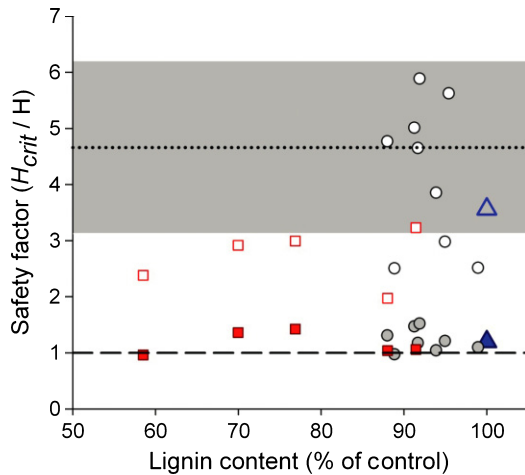
formed tissues, will alter the stresses experienced by a developing cell.

For the poplars investigated here, reductions in lignin content, in combination with increased tension wood incidence, were associated with increases in average cellulose content from *c.* 432 to 486 mg g<sup>-1</sup> (Voelker *et al.*, 2010). Light microscopy showed that the largest reductions in lignin content appeared to be in fiber cell walls and, to a lesser extent, within the middle lamellae and cell wall corners (Voelker *et al.*, 2010). Lignin was not as strongly reduced in vessel cell walls in comparison with fibers. Despite increases in cellulose content, low-lignin wood was weaker and more flexible (Figs 4, 5, 7). Low-lignin transgenic poplars compensated for reduced tissue stiffness by altering their stem form and mechanical stability (Fig. 1). Diagnostic of the many complex changes in stem form were the coordinated changes in proportional branch biomass and height : diameter ratios (Fig. 2), which, in turn, were related to lignin content and wood stiffness (Fig. 9a,c). Because of the substantial differences in tree height between growth environments, there was a significant treatment × diameter interaction for the height : diameter ratio (Table 1). For low-lignin trees with weaker wood, greater radial growth near stem bases with respect to tree height growth was caused by changes in rates of cell division, rather than cell expansion, because fiber cell sizes did not differ among the control line and transgenic events (Table S3). If it is further assumed that fiber length scales with fiber width, the ratio of radial cell division to apical cell division correlates with both lignin content and MOE (Fig. S3). In addition, low-lignin trees showed three-fold increases in tension wood incidence (Fig. 9b,d; Table S2). Indeed, these local changes in cell division rates and cell identity would be predicted by the uniform stress hypothesis if lower wood



**Fig. 9** Variation among transgenic events and the control line in height : diameter ratio and tension wood occurrence as related to modulus of elasticity (MOE) or lignin contents for field-grown trees. Control (triangle), normal transgenics (circles) and brown wood transgenics (squares).





**Fig. 10** Safety factors of free-standing and staked trees vs lignin content. The safety factor is the critical height ( $H_{crit}$ ) of buckling for a given stem shape and material properties divided by the actual height of the tree. The mean  $H_{crit}$  value (dotted line) and standard error (gray shading) are shown for the results of a large survey of trees (Niklas, 1994). The dashed line equal to 1.0 is the safety factor where tree height =  $H_{crit}$ , the point at which a tree would be likely to buckle under its own mass as a result of mechanical instability. Open squares, field-grown brown wood transgenic; open circles, field-grown normal transgenic; open triangles, field control; closed squares, staked brown wood transgenic; closed circles, staked normal transgenic; closed triangles, staked control.

stiffness transmits greater bending stresses to the cambial zone for a given force applied (Morgan & Cannell, 1994; Gartner, 1995; Telewski, 2006; Coutand *et al.*, 2009).

### Low-lignin wood has reduced strength and stiffness

Across hardwood species, there is a loss in wood stiffness of *c.* 28% (Green *et al.*, 1999) for mature wood tested at 12% moisture content compared with that exceeding 30% moisture content (i.e. the fiber saturation point). A similar pattern is found for lignin and hemicelluloses (Cousins, 1976, 1978). Because we tested our samples at 12% moisture content, it could be argued that, if lignin contents interacted with moisture content by 'shielding' structural polysaccharides from interactions with water, we may have underestimated the importance of lignin content for *in vivo* plant biomechanics. In a parallel study, *4CL*-downregulated aspen was tested green, above the fiber saturation point (Horvath, 2009, 2010; Horvath *et al.*, 2010). A comparison of MOE values from static bending tests (Horvath, 2009) with MOE values of stems from our study (with a similar wood density, diameter and range of lignin contents) found that samples tested green had a 24% lower stiffness than those tested at 12% moisture content. This is similar to the expected relationship for mature wood across hardwood species (Green *et al.*, 1999), but opposite in sign to that expected if lignin  $\times$  moisture content interactions are an important determinant of wood stiffness. It is uncertain

whether this pattern holds across the juvenile wood tested here and elsewhere (Horvath, 2009, 2010; Horvath *et al.*, 2010). Nevertheless, the hypothesis that lignin is important for 'protecting' or 'shielding' structural polysaccharides from interactions with water may not be as important as once thought (Klauditz *et al.*, 1947; Klauditz, 1952; Köhler & Spatz, 2002). Thereby, the effects of reduced lignin content on mechanical properties documented by our study (Fig. 7), and other recent data (Horvath, 2009, 2010; Horvath *et al.*, 2010), should provide an improved estimate of the role of lignin in biomechanics compared with tests of chemically de-lignified wood (Burgert *et al.*, 2005a,b; Jungnickl *et al.*, 2008).

The variation in MOE was not driven by differences in wood density (Fig. 8). Indeed, only the smallest diameter stems of the brown wood transgenics showed a trend towards lower wood densities than the control line (Fig. 6), and samples of this diameter range were not included for the mean MOE values reported here. The reduced initial wood densities in brown wood transgenics may be indicative of *4CL* suppression causing a progressive reduction in rates of lignification relative to cell division, as previously demonstrated in mutants exhibiting altered lignin biosynthesis (Blee *et al.*, 2001; Patten *et al.*, 2005; Laskar *et al.*, 2006). Despite the lower initial wood density of brown wood transgenics, it is likely that cell collapse and the deposition of phenolic compounds contributed to greater maximum wood density values as stems developed further (Fig. 6). The greater wood density of collapsed wood should increase mechanical properties if we assume that changes in wood ultrastructure do not affect mechanical properties significantly. Although it is conceivable that cell collapse could cause fractures of the cell wall and affect material properties, no fractures could be observed in scanning electron micrographs of collapsed wood from other transgenic low-lignin poplars (Coleman *et al.*, 2008). This leads us to believe that the chemical composition and arrangement within cell walls have a stronger influence on wood properties than does the occurrence of cell collapse or cell wall fractures.

Apart from the influence of wood density, much of the variation in wood mechanical properties depends on the properties of cellulose within secondary cell walls (Mark, 1967; Keckes *et al.*, 2003; Salmén & Burgert, 2009; Lachenbruch *et al.*, 2010). Although *4CL* down-regulation should not have affected the properties of cellulose directly, it is possible that a variation in cellulose crystallinity and microfibril angle may have affected the wood properties of transgenics (Horvath, 2010). Cellulose and hemicelluloses have been estimated to be *c.* 60- and three-fold as stiff as lignin, respectively, in the longitudinal plane, *c.* 1.6- and 12-fold as stiff in the transverse plane and *c.* 2.3 and 5.8-fold stronger in shear (Bergander & Salmén, 2000). If wood mechanical properties were more strongly influenced by the

amount and orientation of cellulose rather than the interaction of cellulose with hemicelluloses and lignin, we would expect equivalent or greater stiffness of low-lignin wood, as tension wood fibers are nearly pure cellulose oriented close to the vertical axis, and were up to three-fold more common in transgenics with the lowest lignin contents (Fig. 9b,d; Table S2). It appears that increasing the lignin content beyond the species' average has little effect on the wood strength (Gindl & Teischinger, 2002), whereas our data suggest that decreasing the lignin content below the species' average can exacerbate the role of lignin as the 'weakest link' in the composite cell wall, and thus disproportionately affect wood stiffness and strength.

There are several possible mechanisms responsible for the reduced wood stiffness and strength of low-lignin wood. The cell layers in which lignin was most reduced were not determined, but an overall reduction in lignin content by up to 40% must cause a decrease in bonding or the physical interface between lignin and hemicelluloses necessary to transfer shear stresses between cells and to the stiffer cellulose microfibril components within the cell wall. Reduced cross-linking and stress transfer between cells or cell wall components have been thought to promote fractures within and between cells on the tension side of the stem (Donaldson, 1995, 1997). This process may occur at lower loads if the lignin content is insufficient to adequately constrain transverse expansion of the cell wall in compression, or if shear failure occurs more readily between cell wall lamellae. Both scenarios could result in cell walls crumpling on the compression side of the stem and transferring additional stress to the tension side of the stem, where reduced linkages between lignin and hemicelluloses may allow the hydrogen bonds to cellulose microfibrils to more easily slide past one another and re-form at a new point. Indeed, ultimate compressive strength and MOE both showed severe reductions in low-lignin aspen (Horvath, 2010). Hence, the weakened compressive properties of low-lignin wood can help to explain the reduced initial slopes of the load–deflection curves for transgenic event 712 relative to the control line (Fig. 4). Meanwhile, an increased 'slip–stick behavior' of cellulose microfibrils (Keckes *et al.*, 2003) would help to explain the greater deflection distances of low-lignin wood for a given stem diameter (Fig. 4).

#### Tree form and safety from buckling is affected by lignin content and growth environment

Because bending stresses would have been reduced drastically in staked compared with unstaked trees, we expected that there would be less tension wood in staked trees, but this difference was not detected (Table 1). Staked transgenics had significantly lower MOR values than the same transgenic events grown in the field, but MOE showed no such change (Tables 1, S2). Thus, the extent to which

tension wood affected wood mechanical properties is uncertain.

Similar to the patterns in MOR, stem taper was less in staked vs free-standing trees. Within a growth environment (i.e. treatment), the majority of staked transgenics had lower height : diameter ratios compared with the control line (Table S2), resulting in a significant treatment  $\times$  line interaction for height : diameter ratios (Table 1). It is known that reduced bending stresses can increase tree height growth at the expense of diameter growth (Holbrook & Putz, 1989; Telewski, 1995; Meng *et al.*, 2006), but it was surprising that lower lignin and reduced height : diameter ratios were also strongly related to proportional allocation to branches (Fig. 2). It is uncertain whether these changes could be associated with a difference in the flow of hormones, such as auxin or gibberellin, or whether stresses acting on the cambial zone may contribute to the initiation of sylleptic branch formation. The relationship of sylleptic branching to stresses in the cambial zone seems to be more consistent with the changes that occurred across both growth environments and gradients in wood stiffness.

One measure of tree structural stability can be obtained by comparing the height of a tree to  $H_{crit}$ , the height at which an untapered column of wood of the same diameter and stiffness will buckle if bent from vertical because of elastic instability. Although the calculation of  $H_{crit}$  (see the Materials and Methods section) is undoubtedly a simple representation for the complex growth forms of trees, it has been demonstrated that trees become unstable as their heights approach  $H_{crit}$  (Holbrook & Putz, 1989). Because of the need to support branches and leaves under mechanical loading by wind, snow, ice, lianas and other loads, evolutionary pressures have resulted in trees being over-engineered by a certain 'safety factor' to avoid buckling. Niklas (1994) found that trees have a large range of safety factors, but, on average, they have a value of *c.* 4.66 (i.e.  $H_{crit}$ /total tree height = 4.66). There is a propensity for subcanopy saplings or trees to risk low safety factors when they experience both light limitation and low exposure to wind-sway (Jacobs, 1954; King, 1986; Holbrook & Putz, 1989; King *et al.*, 2008). The safety factors for the free-standing control line and normal transgenics were centered just below the overall average of 4.66 (Fig. 9). Brown wood transgenics greatly increased their taper to compensate for reduced wood stiffness, but were on the low end of the safety factors observed. By contrast, staked trees all approached or exceeded the  $H_{crit}$  threshold, all having safety factors less than those documented for trees typical of shaded and protected growth environments (King, 1986; Niklas, 1994).

#### Are low-lignin poplars mechanically unstable?

The Greenhill equation for  $H_{crit}$  predicts that the mechanical stability of trees of the same height is less sensitive to

material properties than to the basal stem diameter. In other words, much of the variation in resistance to mechanical loading is conferred by a tree's height : diameter ratio. For the free-standing and staked poplars, height : diameter ratios ranged from 38 to 94 and from 95 to 199, respectively (Table S2). We found that all staked trees approached and occasionally exceeded  $H_{crit}$  (Fig. 9). Holbrook & Putz (1989) were able to grow wild-type trees that substantially exceeded  $H_{crit}$  by staking combined with shading out of 90% of the light to the lateral branches along the length of the trees, whereas the same trees without shading only approached  $H_{crit}$ . Provisionally, these data suggest that, when bending stresses are minimized, there may be a distinct limit to the extent of growth form modulation that roughly corresponds with  $H_{crit}$ , but that trees are more likely to exceed  $H_{crit}$  when light limitation is combined with minimal bending stresses.

Differing environmental demands on the xylem can lead to great plasticity in stem form and resulting patterns of carbon allocation in woody plants (Figs 1–3). Tree species requiring moderate to high-light conditions tend to grow more slender stems when forest canopy closure occurs as a result of both light limitation and diminished bending stresses, as the canopy protects individuals from excessive wind loading (Jacobs, 1954; Holbrook & Putz, 1989; Oliver & Larson, 1996; Meng *et al.*, 2006). For naturally regenerated cottonwood and trembling aspen stands, average height : diameter ratios often peak above 100 after canopy closure (King, 1981; Colbert *et al.*, 2002), and can attain values as great as 150–200 when poplars are planted very close together (Harrington & DeBell, 1996; DeBell *et al.*, 1997). This range for poplar stands overlaps that observed for the staked trees in this study (95–194, respectively) and for other transgenic low-lignin vs control poplars grown in a glasshouse (148–195, respectively) (Coleman *et al.*, 2008). The much lower height : diameter ratios of our free-standing poplars grown in the field trial (50–90) probably represents a compensation for reduced wood stiffness by developing stems with reduced bending moments and greater taper. Mechanical testing of whole trees has demonstrated that birch (*Betula* spp.), with a height : diameter ratio of 120, incurs stem breakage at half the critical wind speed of trees with a height : diameter ratio of 80 (Peltola *et al.*, 1999). These data suggest that height : diameter ratios approaching 160 would result in stem failure at a wind speed of  $< 1 \text{ m s}^{-1}$  without the shelter of an accompanying canopy to deflect and diffuse wind gusts. Hence, there are strong selective pressures towards maximizing height : diameter ratios, or 'risking' a minimal safety factor, in crowded stands where bending stresses from wind are lower and competition for light is greater.

Our data suggest that lignin has an important influence on the resistance to bending of woody stems. Reduced lignin content promotes increased stem taper, which apparently

alleviates some of the risk of catastrophic stem failure for low-lignin trees. However, if competition forces low-lignin trees to attain height : diameter ratios equivalent to those expected for normal biomass plantings, they may be at a substantially greater risk for mechanical failure. Reduced lignin content also shifts carbon allocation away from height growth and towards stem bases and lateral branches, in both unsupported and supported growing environments. Because height growth is such a valuable trait in light-competitive environments, this apparent trade-off between lignin content (i.e. wood stiffness) and height growth argues strongly for why certain minimum lignin contents are necessary for woody cell walls. Both stand- and tree-level characteristics are important for predicting the likelihood of wind-toppling in poplar plantations (Harrington & DeBell, 1996). Therefore, a remaining uncertainty for low-lignin trees is how tree survival and stand-level biomass production may vary under differing planting densities in response to competition for light if these trees tend to allocate more growth towards lateral crown expansion at the expense of tree height growth. A full appreciation of the consequences of transgenic modification for wood properties, structural stability, product performance, adaptation to abiotic stresses and overall tree fitness will require expanded field trials conducted in diverse environments.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** The empirical relationship for relative lignin contents predicted from total thioacidolysis yield.

**Fig. S2** Example of methods used for tension wood estimates.

**Fig. S3** Line/event mean ratios of the rates of radial cell division to apical cell division vs lignin content and modulus of elasticity (MOE).

**Table S1** Total thioacidolysis yields and associated relative lignin contents after corrections for 70% reaction yield

**Table S2** Selected trait means for field-grown and staked trees

**Table S3** Mean characteristics of fiber cells for each line/event

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