RESEARCH PAPER

Significant influence of lignin on axial elastic modulus of poplar wood at low microfibril angles under wet conditions

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Abstract

Wood is extensively used as a construction material. Despite increasing knowledge of its mechanical properties, the contribution of the cell-wall matrix polymers to wood mechanics is still not well understood. Previous studies have shown that axial stiffness correlates with lignin content only for cellulose microfibril angles larger than around 20°, while no influence is found for smaller angles. Here, by analysing the wood of poplar with reduced lignin content due to down-regulation of *CAFFEOYL SHIKIMATE ESTERASE*, we show that lignin content also influences axial stiffness at smaller angles. Micro-tensile tests of the xylem revealed that axial stiffness was strongly reduced in the low-lignin transgenic lines. Strikingly, microfibril angles were around 15° for both wild-type and transgenic poplars, suggesting that cellulose orientation is not responsible for the observed changes in mechanical behavior. Multiple linear regression analysis showed that the decrease in stiffness was almost completely related to the variation in both density and lignin content. We suggest that the influence of lignin content on axial stiffness may gradually increase as a function of the microfibril angle. Our results may help in building up comprehensive models of the cell wall that can unravel the individual roles of the matrix polymers.

Keywords: CAFFEOYL SHIKIMATE ESTERASE (CSE), cell-wall mechanics, lignin, lignin engineering, micromechanics, *Populus tremula×Populus alba*.

Introduction

Wood has been widely used as a sustainable construction material due to its excellent mechanical properties. Although understanding the structural–mechanical relationships at the cell-wall level is challenging, this knowledge is essential for the efficient and advanced utilization of wood, as these relationships are the basis of its macroscopic mechanical behavior. Cellulose microfibrils function as load–bearing elements within the cell wall due to their very high axial stiffness in comparison to that of the matrix polymers hemicelluloses and lignin (Cousins, 1976, 1978). Both experimental and

simulation data have shown the significant influence on axial stiffness of the orientation of the cellulose microfibrils, which is usually referred to as microfibril angle (MFA) (Cave, 1968; Lichtenegger *et al.*, 1999).

The contribution of lignin to the mechanical behavior of cell walls and tissues is less well understood. Simulation studies suggest that lignin content may only influence transverse mechanical properties and not axial ones (Bergander and Salmen, 2002). It has been challenging to validate the mechanical function of lignin experimentally. Tensile tests on single spruce fibres and tissue strips combined with Raman microscopy have revealed tensile loading of the cellulose molecule, as wavenumber shifts of peaks assigned to cellulose are observed (Gierlinger *et al.*, 2006). However, no wavenumber shifts can be detected for lignin-assigned peaks. In a more recent study, tensile tests combined with dynamic Fourier-transform infrared (FTIR) spectroscopy have indicated a contribution of lignin to viscoelastic but not to elastic behavior (Salmen *et al.*, 2016).

In addition to these *in situ* studies, there are a few reports on the mechanical functions of lignin in untreated (native) plant tissues and fibres that have naturally occurring variability in lignin content. The majority of these studies have been conducted on either chemically delignified wood or genetically engineered wood with reduced lignin content. Considering native plant tissues, Grozdits and Ifju (1969) performed tensile tests on the developing xylem of an adult hemlock and found no influence of lignin content on strength. Rünger and Klauditz (1953) found a correlation between (axial) compression stiffness and lignin content for three different poplar species, whereas no correlation was found for lignin content and (axial) tensile stiffness. However, lignin content varied only within a range of 23–27%, and other parameters such as density and cellulose content varied as well, which precludes more definitive conclusions on the contribution of lignin to mechanical behavior. All these experiments were performed in dry conditions. In contrast, axial tensile stiffness could be correlated to lignin content for the fibre caps of the Mexican fan palm tested in a wet state (Rüggeberg *et al.*, 2008, 2009).

Removal of lignin by chemical treatments allows the mechanical properties of wood with varying lignin contents to be tested. Mechanical tests of chemically delignified pine, beech, and poplar wood and of single pine-wood fibres have shown that there is a pronounced decrease of wood strength in comparison to native wood when tested in a wet state (Klauditz, 1952); in a dry state, the strength is either unchanged or even increased for the delignified wood and the fibres. Tamburini (1970) mechanically tested alkaline-treated beech wood with partially removed lignin and hemicelluloses in a dry state and obtained similar patterns for wood stiffness and strength. In addition, Zhang *et al.* (2013) performed single-fibre tests on delignified Chinese fir wood in a dry state and did not find any decreases in stiffness and strength. The decrease in strength of delignified wood tissues tested in a wet state can be explained by the erosion of the middle lamella in the delignification process, which leads to a disintegration of the sample when tested in wet conditions. In dry conditions, the connections between the fibres will be retained. The (few) tests that have been conducted on single fibres may also point towards an influence

of water on the mechanical function of lignin at the cell–wall level. However, the artificial removal of lignin most likely results in a non-representative state of the cell wall, which makes it generally difficult to derive insights on the mechanical function of lignin for native wood cell walls and tissues.

Advances in genetic engineering have allowed for more specific manipulation of lignin content with less effects on other cell-wall components. Thus, studies with engineered plant material can help to increase our understanding of the structural–mechanical relationships of wood at the cell-wall level. Previous studies have produced contrasting results with respect to the influence of lignin content on axial stiffness. In poplar down-regulated in *CINNAMIC ACID 4-HYDROXYLASE* (*C4H*), the resulting decrease in axial stiffness is not attributable to the reduced lignin content but is instead explained by the decrease in density (Bjurhager *et al.*, 2010). In poplar down-regulated in *4-coumarate-CoA LIGASE* (*4CL*), a 30% reduction in lignin content as measured by the Klason method is accompanied by a 40% reduction in bending stiffness, while density is reduced by 10% (Horvath *et al.*, 2010). For transgenic poplars down-regulated in *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*), a decrease of axial tensile stiffness of 10–15% can be correlated with a decrease of lignin content, measured as a 10–15% decrease in FTIR absorbance (Özparpucu *et al.*, 2017). In a recent study, Miller *et al.* (2018) showed a positive correlation of lignin content with bending stiffness for genetically engineered *Poplar trichocarpa* plants down- or up-regulated in *CAD1*, *CAD 2*, *C3H3* (*P-COUMARIC ACID 3-HYDROXYLASE*), *C4H1*, or *C4H2*, either individually or in different combinations.

These seemingly contradictory results on the influence of lignin content on the elastic modulus underline the complexity of the structural–mechanical relationships in the cell wall, especially for matrix polymers. Given the results of the studies on chemically delignified wood fibres that show that the water status may be important for the mechanical relevance of lignin, the distinct anatomical, structural, and chemical set-up of the plant material may significantly influence the mechanical functions of lignin in the cell wall as well. In particular, the orientation of the cellulose microfibrils (the MFA) may be a crucial structural feature that influences the importance of lignin content for the axial mechanical properties. In those studies that have reported a correlation between stiffness and lignin content (Rüggeberg *et al.*, 2008; Özparpucu *et al.*, 2017) and have provided further information on mechanically relevant parameters, the MFA has always been larger than 20°. From studies on palm tissue, Rüggeberg *et al.* (2008) and Eder *et al.* (2009) have suggested that for large MFAs, which induce shear stresses in the matrix due to off-axis loading at the cell-wall level (Hull and Clyne, 1996; Fratzl *et al.*, 2004), lignin might contribute to the axial stiffness by increasing the shear stiffness of the cell-wall matrix. At low MFAs axial loads are mostly carried by the stiff cellulose microfibrils, which renders the influence of the matrix as marginal for axial stiffness (Bergander and Salmen, 2002). Further comprehensive studies, ideally on wood of the same plant species with different MFAs and pronounced changes in lignin content, are needed to support this hypothesis or to

reveal other factors for the correlation between lignin content and stiffness.

Recently, CAFFEOYL SHIKIMATE ESTERASE (CSE) has been described as a novel enzyme central to the lignin biosynthetic pathway in some plant species. *CSE* loss–of–function in Arabidopsis results in 17–36% less lignin (Vanholme *et al.*, 2013), whilst an even more severe reduction is observed in *Medicago truncatula*, indicating that CSE is essential for normal lignification in these species (Ha *et al.*, 2016). Notably, *CSE* loss–of–function in both Arabidopsis and *M. truncatula* results in negative effects on plant growth and development. In poplar, down–regulation of *CSE* results in up to 25% less lignin and a relative increase in cellulose content of 8–13% (Saleme *et al.*, 2017). Because these poplars show pronounced changes in lignin content whilst being morphologically indistinguishable from the wild-type, we have used these plants in the present study in order to further evaluate the role of lignin in the mechanical properties of wood. We performed mechanical tests in a wet state, as this closely resembles natural conditions and may highlight the mechanical contribution of the wall matrix, and in particular lignin. Lignin content, mechanical properties, density, and the orientation of cellulose microfibrils were all obtained from the same samples, allowing individual parameters to be correlated and thus providing new insights into the mechanical functions of lignin in wood cell walls.

Materials and methods

Plant materials and sample preparation

Poplar plants with down–regulation of *CSE* were produced as previously described by Saleme *et al.* (2017). Briefly, a 120-bp fragment of the *PtxaCSE2* coding sequence (corresponding to *Populus trichocarpa* Potri.003G059200) was PCR–amplified from cDNA obtained from stems of *P. tremula* × *P. alba* (INRA 717-1B4) and cloned into the pDONR221 vector. After confirming sequence identity by sequencing, the fragment was subcloned into the pK7GWIWG2II destination vector suited for *CaMV 35S*-driven intron-spliced hairpin RNA-mediated gene silencing. *Agrobacterium tumefaciens* strain C58C1 PMP90 was transformed with the resulting recombinant vector, and *Agrobacterium*-mediated transformation of *P. tremula* × *P. alba* was performed according to Leple *et al.* (1992). The transgenic lines analysed in this study were named as *hpCSE#1* and *hpCSE#2* and were the same lines as those subjected to multiple-level phenotyping in the work of Saleme *et al.* (2017).

The *CSE* down–regulated transgenic plants were grown under greenhouse conditions along with the corresponding wild-type (WT) for 3.5 months. Representative samples from each genotype are shown in Supplementary Fig. S1 at *JXB* online, and the numbers of biological and technical replicates used for analyses are provided in Supplementary Table S1. After 3.5 months, stems from each genotype were cut 10 cm above the soil, and the basal 10 cm of this stem cutting was debarked and used for subsequent sampling. Stem sections were cut to a length of ~30 mm. The cross-sectional surface of both ends of the sections were checked using light microscopy for the presence of tension wood. Sections containing tension wood were discarded. Using a rotary microtome, the xylem next to the cambium was removed to a depth of 200 µm. Seven consecutive longitudinal-tangential (LT) sections with a thickness of 100 µm were cut in wet conditions. The stem was then turned and a different side was used to obtain a second series of seven sections. Thus, 14 sections were potentially available for the different analyses (Supplementary Table S1). These sections were considered as technical replicates, as they were derived from a single piece of stem. The sampling from a stem cross-section is illustrated in Supplementary Fig. S2.

Micro-mechanical tensile tests

For mechanical tests, LT-sections (strips) were cut in wet conditions with a width of 1.5 mm using a scalpel. The sections were kept in water until testing, which was also performed in wet conditions. A custom-built micro-tensile testing stage was used as previously described by Burgert *et al.* (2003). The LT-sections were screw-clamped into the sample holders: the two ends of the sections were reinforced by gluing microtome-cut 150-µm thick spruce-wood sections to them on both sides in order to avoid damage due to the clamping. The force was recorded with a 50 N load cell (Honeywell/Sensotec Sensors) and the strips were strained with a strain rate of 0.067% s^{-1} (with a span length of 15 mm) until failure. The data from samples that failed at the edges were excluded from further evaluation. The displacement was tracked by video-extensometry using a stereo-microscope, a CCD camera, and contrasting black lines on white background on the holders close to the ends of the samples. Force–deflection curves were converted into stress–strain curves and mechanical properties such as tensile elastic modulus, ultimate stress, ultimate strain, and yield stress were calculated, as previously reported (Özparpucu *et al.*, 2017). The mean values of three biological replicates per genotype were compared using one-way ANOVA (Tukey's test range) at a 95% (P=0.05) confidence level.

Density calculation

The density of the mechanically tested samples was calculated based on their green volume and dry mass (Rowell, 2013). The dry mass of the tissues was measured after drying samples at 65 °C for 2 d. For volume calculation, the thickness of tissues was measured using a micrometer screw, whilst the width and the length of the sample were measured from images taken by a CCD camera mounted on an optical light microscope.

X-ray diffraction

The cellulose microfibril orientation of the mechanically tested strips was measured by wide-angle X-ray diffraction (WAXD) using a Nanostar (Bruker AXS, Germany) and CuKα radiation with a wavelength of 0.154 nm. The X-ray beam diameter was ~300 µm and the sample–detector distance was set to 8.5 cm. For each sample, one diffraction image was taken with a 35-min exposure time. Three biological replicates of each genotype were used for X-ray analysis and at least 10 technical replicates, which had been tested mechanically, were measured for each biological replicate (Supplementary Table S1). To determine the cellulose orientation, azimuthal intensity profiles of the (200)-Bragg peak of cellulose were generated from the diffraction images by radial integration within the *q*-range of the (200)-Bragg peak. After baseline subtraction (amorphous phase), simulated azimuthal intensity profiles were fitted to the measured profiles and the fit parameters revealed the microfibril orientation distribution for each measuring spot. For this simulation routine, which was developed by Rüggeberg *et al.* (2013), a representative cell–wall orientation is calculated from a sample cross-section and incorporated in the simulation of the azimuthal intensity distributions. Technical replicates in which tension wood was found were omitted from statistical analysis. In addition, the cellulose structure was analysed by powder X-ray diffraction (XRD), with samples being cut into very small pieces manually with a scalpel to resemble powder conditions (see Supplementary Methods S1).

Fourier-transform infrared spectroscopy

FTIR spectra were acquired for all the mechanically tested samples (Supplementary Table S1) using a Platinum ATR (attenuated total reflection) unit on a TENSOR 27 spectrometer (both Bruker, Germany) in the range 4000–350 cm^{-1} with a spectral resolution of 4 cm^{-1}. Three spectra were recorded for each sample and a mean was calculated. The spectra were baseline-corrected and normalized at the highest peak (1032 cm^{-1}, cellulose) to an absorption value of 2 using the Opus v.7 software (Bruker, Germany) before detailed evaluation.

Results

Down-regulation of CSE *affects wood mechanical properties*

Micro–mechanical tensile tests were used to determine the axial stiffness (measured as elastic modulus) of tissue strips that had not been subject to drying. Compared to the WT, the elastic modulus was significantly decreased ($P=5.03\times10^{-4}$) by 36% and 53% in *hpCSE#1* and *hpCSE#2*, respectively, and ultimate stress was decreased by ~15% ($P=0.083$) in both lines (Fig. 1a, b). The individual stress–strain curves are shown in Supplementary Fig. S3. The ultimate strain was significantly higher for *hpCSE#1* and *hpCSE#2* than for the WT ($P=0.04$; Fig. 1c), but no significant differences were observed for toughness ($P=0.59$; Fig. 1d).

FTIR spectroscopy confirms reduced lignin content in lines with down-regulation of CSE

The two transgenic lines that we used, *hpCSE#1* and *hpCSE#2,* have been characterized previously and shown to deposit lower amounts of lignin, mildly increased levels of H–units in the lignin polymer, and slightly higher amounts of cellulose in stems (Saleme *et al.*, 2017). In order to confirm these previous results, FTIR spectra were acquired directly on the tissue strips of all genotypes after mechanical testing. Three FTIR spectra were acquired for each technical replicate ($n=10$–13, Supplementary Table S1). These spectra were averaged and normalized to the absorption band of cellulose at 1032 cm^{-1} (C-O stretching) (Marechal and Chanzy, 2000) in order to evaluate the changes in lignin content relative to the cellulose content. In the average spectra of both transgenic lines, pronounced and significant decreases were observed in the absorbance of the characteristic lignin bands at 1593 cm^{-1} (aromatic skeletal stretching plus C=O stretching), at 1505 cm^{-1} (C=C stretching of the aromatic ring in lignin, which can be directly correlated with lignin content), at 1460 cm^{-1} (C-H deformation of lignin, plus C-H2 bending of hemicellulose), and at 1423 cm^{-1} (aromatic skeletal combined C-H deformation, plus hemicellulose) (Fig. 2, Supplementary Table S2) (Marchessault, 1962; Faix, 1991; Kacurakova *et al.*,
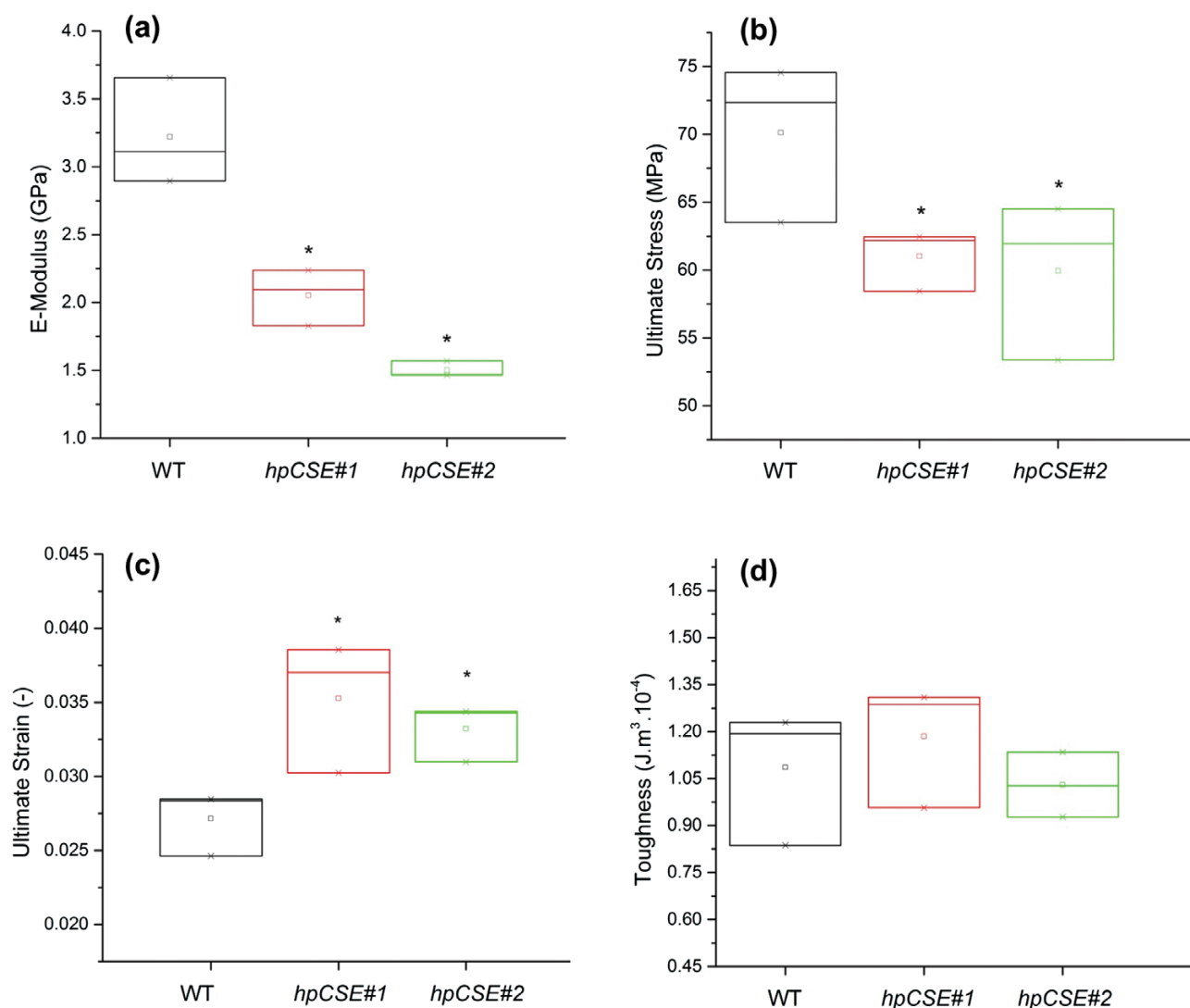
Fig. 1. Tensile properties of wood of wild-type (WT) poplar and the transgenic lines *hpCSE#1* and *hpCSE#2*. (a) Elastic modulus, (b) ultimate stress, (c) ultimate strain, and (d) toughness. The squares within the box plots indicate the mean values of the biological replicates and the lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. Significant differences between the WT and the transgenic lines were determined using ANOVA (*$P<0.05$). For the number of technical replicates see Supplementary Table S1.

1998), which suggested a lower lignin content. In addition, a significant difference in the region of 1350–1330 cm^{-1} was observed for both the *hpCSE* lines. Whereas most of the WT spectra exhibited a single broad peak at 1325 cm^{-1}, a peak at 1318 cm^{-1} was observed in the transgenic lines with an additional shoulder at 1332 cm^{-1} (Fig. 2). The band at 1325 cm^{-1} is assigned to S ring plus G ring condensed (Faix, 1991) and the decrease in absorbance of this band in the average spectra of the transgenic lines also reflected the reduction in lignin abundance. The small band shifts observed in the FTIR spectra also reflected small changes in lignin composition. Accordingly, higher proportions of H-units have been observed in the *hpCSE* lines (Saleme *et al.*, 2017). Our FTIR results confirmed that down-regulation of *CSE* in poplar led to lower amounts of lignin and a mild shift in lignin composition in the stems.

Lower density can partly explain lower elastic modulus in lines with down-regulation of *CSE*

Wood density is one of the most important parameters that influences macro-mechanical properties such as stiffness and strength (Gibson and Ashby, 1997). We measured density as oven-dry weight per green volume for the tissue strips that were mechanically tested. WT plants exhibited a density of 0.35±0.03 g cm^{-3}, while *hpCSE#1* and *hpCSE#2* had significantly lower values of 0.32±0.04 g cm^{-3} and 0.30±0.03 g cm^{-3}, respectively (P=0.035; Fig. 3a). In order to exclude the potential effects of density on elastic modulus, the specific elastic modulus (E/ρ, elastic modulus normalized to density) was calculated and compared among the different genotypes. The specific elastic modulus was significantly lower (reduction of 30–44%) for both the transgenic lines compared to that of WT (P=5.43×10^{-4}; Fig. 3b). The correlation of the elastic modulus with density was relatively low for the technical replicates (R^2=0.13; Supplementary Fig. S4) due to the
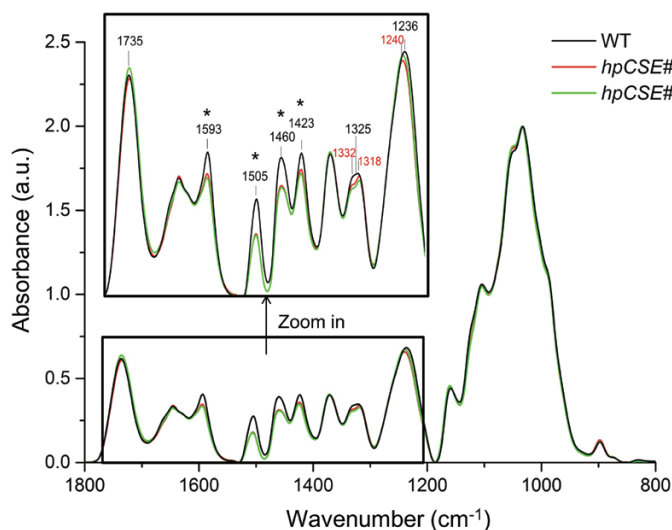
rather high variation of both parameters, whereas a much higher correlation was obtained when the calculations were based on the biological replicates and averaged values (R^2=0.71; Fig. 3c). Because the elastic modulus was still significantly different between the transgenic lines and the corresponding WT even after normalization to density, the decrease in elastic modulus could only be partly explained by the decrease in density.
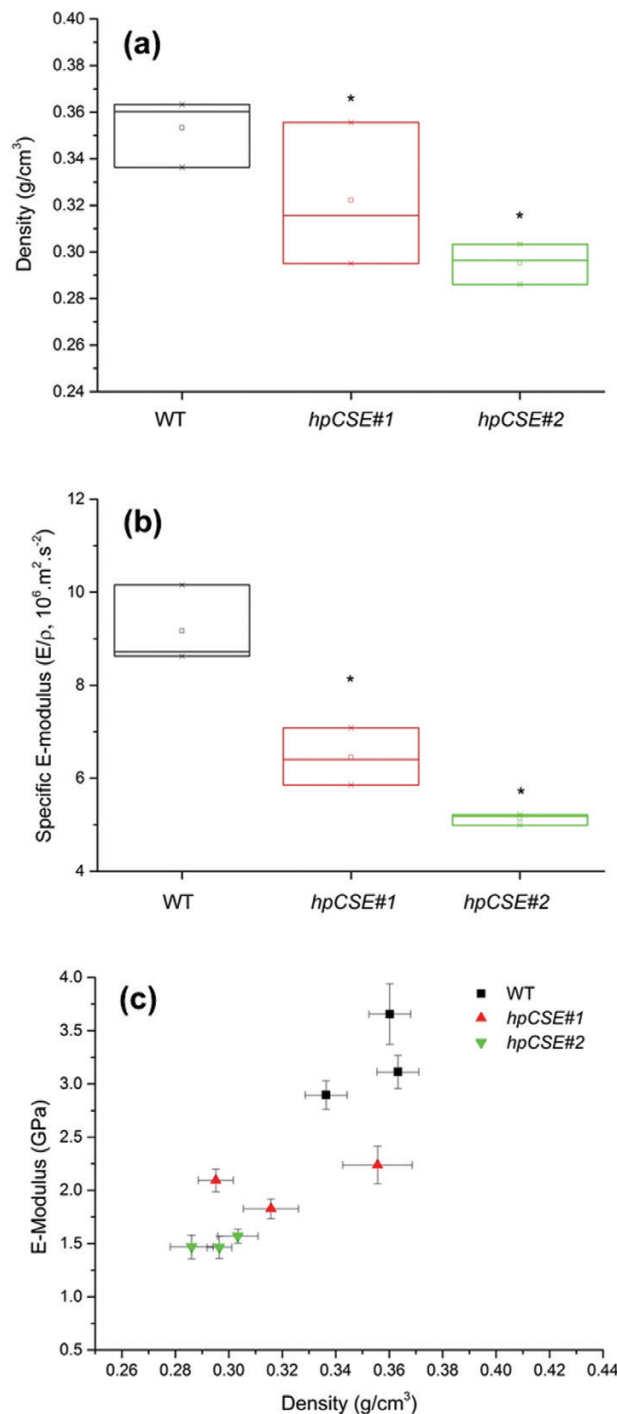

Fig. 2. Average Fourier-transform infrared spectra of wood of wild-type (WT) poplar and the transgenic lines *hpCSE#1* and *hpCSE#2* in the range of 1800–800 cm^{-1} (baseline-corrected and normalized to the highest peak at 1032 cm^{-1}). Significant differences were determined using ANOVA (*P=0.05). Each line represents the average of 90–117 spectra calculated as 3 measurements × ~10–13 sample strips × 3 biological replicates.

Fig. 3. Density–stiffness relationships for wood of wild-type (WT) poplar and the transgenic lines *hpCSE#1* and *hpCSE#2*. (a) Density values, (b) specific elastic modulus (E/ρ, density-normalized), and (c) correlation between density and elastic modulus (R^2=0.71), based on biological replicates (±SE). For an explanation of the box plots see Fig. 1.

Lines with down-regulation of CSE *show similar cellulose orientation and structure*

The MFA of the S2 cell-wall layer, a crucial factor for mechanical behavior, was determined by wide-angle X-ray diffraction (WAXD) (Cave, 1968; Köhler and Spatz, 2002). This revealed a similar average MFA of ~15±1° for both the WT and the transgenic lines (*P*=0.23; Fig 4.a) and a correlation was found between elastic modulus and the MFA neither for the technical (*R*²<0.01; Supplementary Fig. S4) nor for the biological replicates (*R*²=0.05; Fig. 4b). This suggested that the cellulose microfibril orientation was not responsible for the changes in mechanical behavior found in the wood of plants with down-regulation of *CSE* .

To further evaluate potential changes in cellulose structure, samples were cut into very small pieces and cellulose crystallinity and crystallite size were determined by powder X-ray diffraction (XRD). The 2Θ-profiles obtained (Supplementary Fig. S5a) were very similar in terms of peak-to-background ratio and peak width, which points to comparable crystallinity and crystallite size between the WT and *hpCSE* lines. We calculated a crystallinity index (CI) of ~42% and a crystallite diameter perpendicular to the *c*-axis of the cellulose microfibrils of ~3 nm. The variation within the biological replicates of each genotype was higher than the variation in the average 2Θ-profiles between the genotypes (Supplementary Fig. S5b). These results suggested that, despite the relative increase in cellulose content that had previously been observed (Saleme *et al.*, 2017), the cellulose orientation and structure were unaffected in the *hpCSE* lines.

Lignin absorbance is positively correlated with elastic modulus

It has been shown that the aromatic stretching vibration of lignin at 1505 cm⁻¹ in FTIR experiments strongly correlates with lignin content as estimated by different wet chemistry techniques (Rodrigues *et al.*, 1998; Pandey and Pitman, 2004; Schwanninger *et al.*, 2004). Because the FTIR spectra were acquired directly on the samples used for the mechanical tests,

the correlation between elastic modulus and lignin absorbance at 1505 cm⁻¹ might reveal a potential relationship between these two parameters. When calculated for biological replicates, high and significant correlations between lignin content and both elastic modulus (*R*²=0.82) and specific elastic modulus (*R*²=0.83) were found (Fig. 5), suggesting a strong influence of lignin content on the elastic modulus.

Density and lignin content influence elastic modulus

The elastic modulus showed a good correlation with density and with lignin absorbance, and multiple linear regression (MLR) was employed to determine which of these two parameters had the strongest effect. For completeness, MFA was also included in the analysis. A stepwise approach (with forwarding selection) was followed, in which density was chosen as the first variable and MFA and lignin absorbance were then added, with the regression model being made and evaluated at each step. The regression results based on the biological replicates showed that density explained ~70% of the variation in the elastic modulus (*R*²=0.71; *P*=0.024; Supplementary Table S3a). By adding MFA as the second variable, *R*² increased slightly to 0.77 but this parameter was not found to contribute significantly (*P*=0.68), whereas density did (*P*=0.04). When lignin absorbance was added as a third variable, *R*² increased significantly from 0.71 to 0.98. Therefore, almost all the variation in elastic modulus was explained by variation in density and lignin content ($P_{density}$=0.03, P_{lignin}=0.0005). The final step in the MLR (i.e. when all three parameters were included) was performed by including the interaction of density and lignin; however, this approach did not improve the model (*R*²=0.95) and led to a correlation coefficient that was even lower than that obtained when the interaction of density and lignin absorbance was not included (Supplementary Table S4).

In addition, MLR was also performed using the values of the technical replicates, and the same trend was obtained. Accordingly, density and lignin content were both found to significantly influence the tensile modulus. Nevertheless, due to the higher variation in these parameters at the level of the technical replicates, lower *R*² values were found in comparison
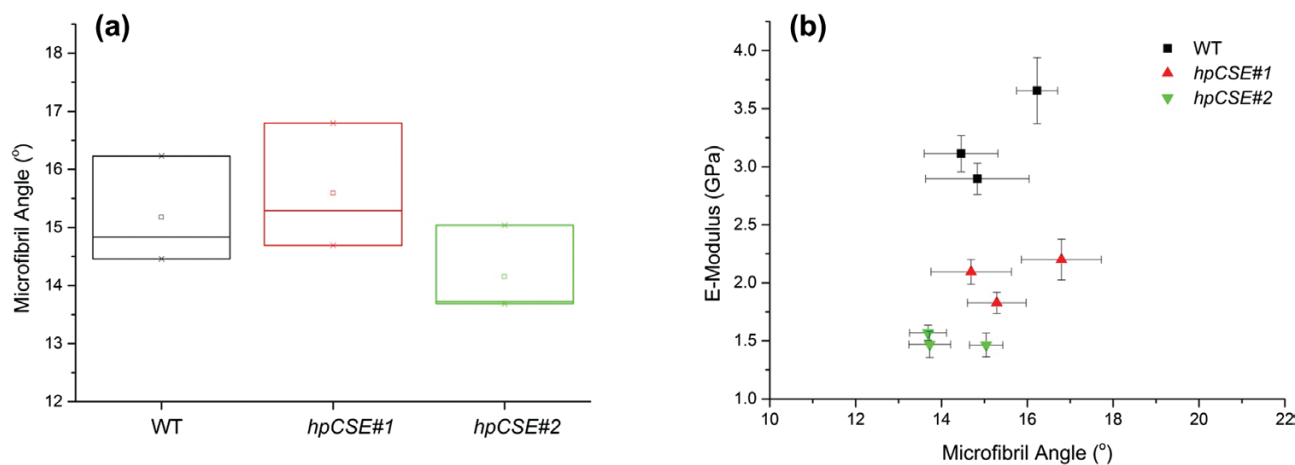

Fig. 4. Structural properties of wood of wild-type WT poplar and the transgenic lines *hpCSE#1* and *hpCSE#2*. (a) Cellulose microfibril angle (MFA) and (b) correlation between elastic modulus and MFA for biological replicates (±SE). For an explanation of the box plots see Fig. 1.
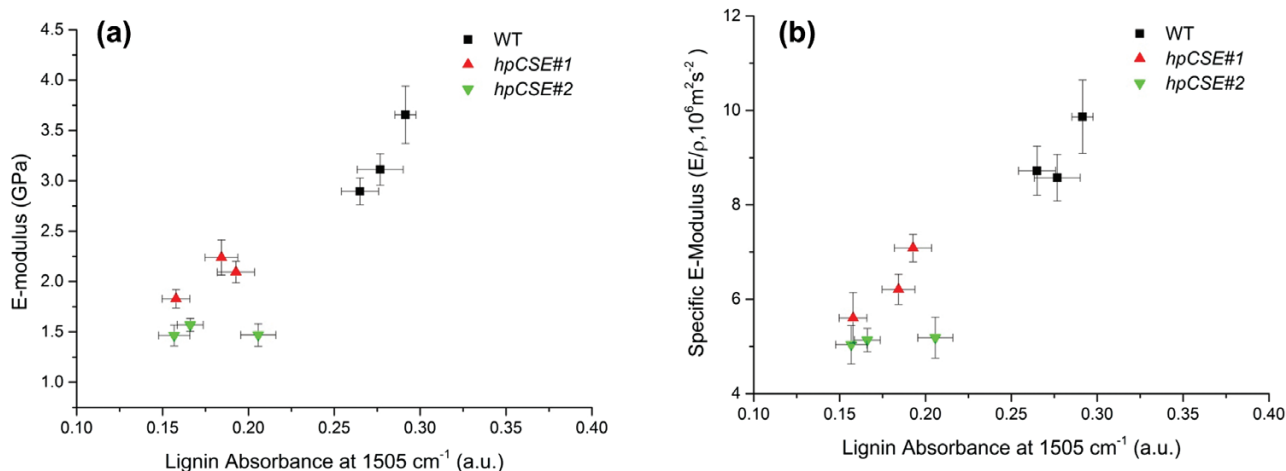
Fig. 5. Correlations of lignin absorbance at 1505 cm^{-1} with (a) elastic modulus and (b) specific elastic modulus calculated for biological replicates (±SE) for wood of wild-type (WT) poplar and the transgenic lines *hpCSE#1* and *hpCSE#2*. For the number of replicates see Supplementary Table S1.

to the biological replicates. A coefficient of 0.39 was found in the final step of MLR when density, MFA, and lignin absorbance were included in the analysis (Supplementary Table S3b).

Discussion

Genetic engineering has proved to be a valuable tool for revealing the mechanical functions of lignin; however, the reductions in lignin deposition that are obtained often result in negative effects on plant growth and development (Leplé *et al.*, 2007; Voelker *et al.*, 2011; Van Acker *et al.*, 2014), which precludes the assessment of the individual contribution of lignin to the mechanical properties of the wood. Because down-regulation of *CSE* in poplar has previously been shown to cause significant changes in lignin content and composition whilst plant yield remains unchanged (Saleme *et al.*, 2017), we anticipated that mechanical characterization of the *hpCSE* lines would help to elucidate the role of lignin in the wood mechanical properties. A reduction in FTIR absorbance at 1505 cm^{-1} of ~35% was observed for the *hpCSE* lines when compared to the WT (Fig. 2), which largely agrees with previously observed changes in Klason lignin content (18–25% reduction; Saleme *et al.*, 2017). The difference in the extent of lignin reductions as measured by FTIR absorbance and wet chemistry might be due to methodological differences, but also to biological ones, as the poplar samples with down-regulation of *CSE* that were studied by Saleme *et al.* (2017) originated from a different batch of plants as those examined in the present study.

Our micro-mechanical tensile tests revealed a significant decrease in elastic modulus of more than 30% in the transgenic lines compared to the WT (Fig. 1a), which was shown to correlate with a decrease in FTIR absorbance at 1505 cm^{-1} and, therefore, with lignin content (Fig. 5). Very similar values of MFA (Fig. 4) and crystallinity index (Supplementary Fig. S5) were found for the WT and the transgenic lines. Thus, despite the fact that cellulose microfibrils represent the main load-bearing elements of the cell wall due to their much higher elastic modulus compared to the matrix, it is unlikely

that microfibril orientation was the cause of the observed decrease in elastic modulus. In addition, the 10% decrease in density that we found in the transgenic lines could only partly explain the decrease in stiffness, as the specific elastic modulus was still significantly different between the genotypes (Fig. 3). These results suggest that the changes in lignin content play a major role in the altered mechanical behavior observed in the wood of poplars with down-regulation of *CSE*. Next to this, Saleme *et al.* (2017) reported a slight alteration of lignin composition in the *hpCSE* lines with a significant increase in the relative content of p-hydroxyphenyl units (H-units) from 0.6% to 1.2% and a slight reduction in the syringyl to guaiacyl unit ratio (S/G ratio) of ~9% from an initial ratio of ~2. However, it has been shown that even large changes in the S/G ratio due to misregulation of lignin biosynthetic genes do not influence the mechanical properties of poplar wood (Horvath *et al.*, 2010; Özparpucu *et al.*, 2018). It is rather unlikely that the small changes in lignin composition observed in the *hpCSE* plants had any significant influence on the mechanical properties. In addition to the cell-wall matrix, the structure and mechanical properties of the middle lamella may also have been affected, as it largely consists of lignin. However, due its very small volume fraction compared to that of the cell wall, any changes in the mechanical properties of the middle lamella probably would not significantly affect the behavior in the elastic range and, thus, the stiffness of the wood tissue. Taken together, our results demonstrate a correlation between lignin content and axial stiffness (measured as elastic modulus) under wet conditions for a lower value of MFA (15°) than has been reported in previous studies.

The results that we found for poplars with down-regulation of *CSE* are in contrast to those obtained by Bjurhager *et al.* (2010) who studied poplars silenced for *C4H*. Although similar MFAs were found for the WT and the transgenic lines in both studies, the reduction in lignin content found by Bjurhager *et al.* did not seem to have any influence on cell-wall stiffness, and the observed significant decrease in axial stiffness could be completely explained by the decrease in density. It should be noted that different enzymes were targeted in the two studies, which

may have resulted in different anatomical or structural changes in the cell walls that, in turn, may have superimposed the effects of the lower lignin content on axial stiffness. However, identifying and in particular measuring the contribution of such secondary effects on wood mechanics is difficult. These effects could include changes in the degree of cross-linking among the cell-wall polymers that affect the fibre–matrix interface or in the lengths of the cellulose microfibrils. These potential secondary effects were not captured either in our present study or in any previous studies. The well-known compensatory effect of an increased cellulose content, which has also been reported for the *hpCSE* lines (Saleme *et al.*, 2017), would theoretically lead to an increase in stiffness. Assuming this is the case, it would make the influence of lignin content on stiffness more pronounced. However, our measurements using powder X-ray diffraction did not reveal an increase in crystalline cellulose content (Supplementary Fig. S5).

Based on the observation that lignin content and axial stiffness correlate in the case of large MFAs, an explanation for this correlation has been suggested by Rüggeberg *et al.* (2008) and Eder *et al.* (2009). This is based on the assumption that lignin content influences the shear stiffness of the matrix, with a lower content resulting in a lower shear stiffness. In case of uniaxial loading in a macroscopic longitudinal direction, large MFAs result in off-axis loading in respect to the cellulose microfibril axis. According to composite theory, off-axis loading of fiber-reinforced composites induces shear stresses in the matrix (Hull and Clyne, 1996). A decrease in the shear stiffness of the matrix would then result in a lower axial stiffness of the cell wall in the case of off-axis loading (i.e. large MFAs). Assuming that the composite theory can be applied to a complex hierarchical material such as wood (Fratzl *et al.*, 2004), a gradually increasing influence of lignin content on axial stiffness as a function of MFA could be postulated. In this respect, our present study provides valuable new data, as it has revealed a measurable influence of lignin content on axial stiffness at a lower MFAs (~15°) compared to previous studies (MFAs >20°).

Despite this new contribution of our present study and the possible explanation provided by composite theory, deriving a causal relationship between these factors remains challenging because of the following considerations. It remains to be determined whether and how lignin content actually influences the shear stiffness of the cell-wall matrix. Despite increasing knowledge of the molecular arrangements of the polymers in the secondary cell wall (Dupree *et al.*, 2015; Kang *et al.*, 2019), the stress-transfer mechanisms at this level have not been resolved. Closer examination of the mechanics at this level would certainly reveal the importance of structural and dimensional changes of the cell matrix as a function of lignin content. Furthermore, a reduced lignin content could also lead to a tighter arrangement of the cellulose microfibrils or even the macrofibrils, if the rate of matrix production was reduced to adapt to the lower lignin availability. In this case, a similar lignin concentration would be retained within the matrix and the shear stiffness of the matrix would not necessarily change.

In conclusion, down-regulation of *CSE* in poplar resulted in a significant reduction in lignin content, density, and mechanical stiffness and strength. The significant reduction in mechanical stiffness could be partly explained by the reduction in density and was correlated with lignin content. In contrast to previous reports, our data revealed for the first time that lignin correlates with mechanical properties of cell walls at comparatively low MFAs (~15°). These results suggest that shear stiffening by lignin might be relevant for axial cell-wall mechanics at lower MFAs than previously reported. This further supports the hypothesis that lignin content becomes relevant for off-axis loading of the cell wall because it induces shear stresses in the wall matrix under such loading. Overall, this would lead to a gradual increase of the influence of lignin content on axial mechanics as a function of MFA. The results of our study may provide an important input for future simulation studies based on cell-wall models that incorporate chemical composition (lignin content) and structure (cellulose orientation and structure). Confirming the gradually increasing influence of lignin content on axial stiffness as function of the MFA and, ultimately, deriving a causal relationship between lignin content and mechanical properties are important objectives for future research.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Representative images of debarked samples for each genotype.

Fig. S2. Illustration of location of samples within a representative poplar stem section.

Fig. S3. Stress–strain curves of the technical replicates.

Fig. S4. Correlations of elastic modulus with density, MFA, and lignin absorbance, based on the technical replicates.

Fig. S5. Curves obtained by X-ray diffraction analysis of powdered samples.

Fig. S6. Representation of the peak-fitting method for calculation of the crystallinity index.

Methods S1. Protocol for powder X-ray diffraction (XRD).

Table S1. Number of biological and technical replicates used in the various analyses.

Table S2. Comparison of genotypes by Fourier-transform infrared (FTIR) spectroscopy.

Table S3. Results of stepwise multiple linear regression analyses for biological and technical replicates.

Table S4. Multiple linear regression analysis including the interaction of density and lignin absorbance.

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