Significant influence of lignin on axial stiffness of poplar wood at low microfibril angle under wet conditions

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HIGHLIGHT: Lignin engineered poplar wood reveals that lignin content significantly influences axial stiffness at lower microfibril angles than previously reported.

ABSTRACT

Wood is extensively used as construction material. Despite accumulating knowledge on 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 was found for smaller angles. Here, by analyzing the wood of poplar plants with reduced lignin content due to down-regulation of CAFFEOYL SHIKIMATE ESTERASE, we showed that lignin content influences axial stiffness also 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. The obtained results may help setting up comprehensive models of the cell wall for unraveling the individual role of the cell wall matrix polymers.

Keywords: cell wall mechanics, lignin, CAFFEOYL SHIKIMATE ESTERASE (CSE), 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 structure-mechanic relationship 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 of the cellulose microfibrils orientation, which is usually given as microfibril angle (MFA), on axial stiffness (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 not influence axial mechanical properties but transverse properties only (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 revealed tensile loading of the cellulose molecule as wavenumber shifts of peaks assigned to cellulose were observed (Gierlinger et al., 2006). However, no wavenumber shifts could be detected for lignin-assigned peaks. In a more recent study, tensile tests combined with dynamic Fourier Transform Infrared Spectroscopy (FTIR) revealed a contribution of lignin to viscoelastic, but not to the elastic behavior (Salmen et al., 2016).

Besides these in-situ studies, only a few studies report on the mechanical function of lignin from native plant tissues and fibres with naturally occurring variability in lignin content. The majority of studies on this topic were 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. No influence of lignin content on tensile strength was found. Rünger & 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 assertive conclusions on the contribution of lignin to mechanical behavior. All these experiments were performed in dry conditions. Conversely, axial tensile stiffness could be correlated to lignin content for the fibre caps of the Mexican fan palm. (Rüggeberg et al., 2009; Rüggeberg et al., 2008).
Lignin removal by chemical treatments allows testing mechanical properties of wood with varying lignin contents. Mechanical tests of chemically delignified pine, beech and poplar wood and single pine wood fibres revealed a pronounced decrease of wood strength in comparison to native wood when tested in wet state (Klauditz, 1952). In dry state, strength was either not changed or even increased for the delignified wood and the wood fibres. Tamburini (1970) mechanically analyzed alkaline treated beech wood with partially removed lignin and hemicelluloses in dry state and obtained a similar pattern for wood stiffness and strength compared to the results of Klauditz (1952). Additionally, Zhang et al. (Zhang et al., 2013) performed single fibre tests of delignified Chinese fir wood in dry state and did not find any decrease in stiffness and strength. The decrease in strength of delignified wood tissues tested in wet state was 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, connection could have been retained. The (few) tests on single fibres may point towards an influence of water state on mechanical function of lignin also at cell wall level. However, the artificial removal of lignin most likely results in a non-representative state of the cell wall, which makes it in general difficult to derive insights on the mechanical function of lignin for native wood cell walls and tissues.

Eventually, genetic engineering allows for a more specific manipulation of lignin content with less effect on other cell wall components. Therefore, studies with engineered plant material may deepen our understanding on the structure-mechanics relationship of wood at the cell wall level. Previous studies reveal contrasting results in respect to the influence of lignin content on axial stiffness. In poplar plants downregulated for CINNAMIC ACID 4-HYDROXYLASE (C4H), the decrease in axial stiffness was not attributed to the reduced lignin content but explained by the decrease in density (Bjurhager et al., 2010). In 4-COUMARATE-COA LIGASE (4CL) down-regulated poplars, a 30% reduction in Klason lignin content was accompanied by a 40% reduction in bending stiffness, while density was reduced by 10% (Horvath et al., 2010). For transgenic poplar plants downregulated for CINNAMYL ALCOHOL DEHYDROGENASE (CAD), a decrease of axial tensile stiffness of 10-15% could be correlated to a decrease of lignin content measured as 10-15% decrease in FTIR absorbance (Özparpucu et al., 2017). In a very recent study (Miller et al., 2018) showed a (positive) correlation of lignin content with bending stiffness for genetically engineered Poplar trichocarpa downregulated or upregulated for either CAD1, CAD 2, C3H3 (P-COUMARIC ACID 3-HYDROXYLASE), C4H1, C4H2, or combinations.
These seemingly contradictory results on the influence of lignin content on mechanical stiffness underline the complexity of structure-mechanics relationship in the cell wall especially for matrix polymers. Next to the outcome of the studies on chemically delignified wood fibres that the water status may matter for the mechanical relevance of lignin, the distinct anatomical, structural and chemical setup of the plant material may significantly influence the mechanical function of lignin in the cell wall as well. In particular, the orientation of the cellulose microfibrils (MFA) may be a crucial structural feature, which influences the importance of lignin content for axial mechanical properties. In those studies, which report a correlation between stiffness and lignin content (Özparpucu et al., 2017; Rüggeberg et al., 2008) and provide further information on mechanically relevant parameters, the MFA was always larger than 20°. From the study on palm tissue, Rüggeberg et al. (2008) and Eder et al. (2009) suggested that, for large MFAs, which induce shear stresses in the matrix due to off-axis loading at cell wall level (Fratzl et al., 2004; Hull and Clyne, 1996), 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 rendering 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 reveal alternative causes for the correlation between lignin content and stiffness.

Recently, CAFFEOYL SHIKIMATE ESTERASE (CSE) was described as a novel enzyme central to the lignin biosynthetic pathway in some plant species. CSE loss-of-function in Arabidopsis thaliana resulted in 17-36% less lignin (Vanholme et al., 2013), whereas an even more severe reduction was observed in Medicago truncatula, indicating that CSE is essential for normal lignification in these species (Ha et al., 2016). Noteworthy, in both Arabidopsis and M. truncatula, CSE loss-of-function resulted in negative effects on plant growth and development. In poplar, CSE down-regulation resulted in up to 25% less lignin and a relative increase in cellulose content of 8% to 13% (Saleme et al., 2017). Because the CSE down-regulated poplars showed pronounced changes in lignin content while being morphologically indistinguishable from the wild type, these plants have been taken in the present study for further evaluating the role of lignin in the mechanical properties of wood. Hereby, mechanical tests were performed at wet state, as this closely resembles natural conditions and may put more emphasis on a mechanical contribution of the wall matrix, in particular lignin. Lignin content, mechanical properties, density, and cellulose microfibril orientation were extracted from the very same samples, allowing the correlation of single
parameters and, thus, providing new insights into the mechanical function of lignin in wood cell walls.

Materials and Methods

**Plant Materials and Sample Preparation**

*CSE* down-regulated poplar plants were produced as previously described (Saleme *et al.*, 2017). Briefly, a 120 bp fragment of the *PtrxA*CSE2 coding sequence (corresponding to *P. trichocarpa* Potri.003G059200) was PCR-amplified from cDNA obtained from *P. tremula × P. alba* (INRA 717-1B4) stems and cloned into the pDONR221 vector. After confirming sequence identity by sequencing, the fragment was subcloned into the pK7GWIG2II 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 analyzed in this study were named as *hpCSE#1* and *hpCSE#2* and were the same lines as subjected to multiple-level phenotyping in the work of (Saleme *et al.*, 2017).

*CSE* down-regulated transgenic plants were grown in greenhouse conditions along with the corresponding WT for three and a half months. Representative samples from each genotype are shown in Fig. S1, whereas the number of biological and technical replicates for further analyses is provided in Table S1. After three and a half months, stems from each genotype were cut 10 cm above the soil and a basal 10-cm stem segment was debarked and used for sample preparation. The stem sections were cut to a length of approximately 30 mm. The cross-sectional surface of both ends of the stem sections was checked for the presence of tension wood by light microscopy. 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. Then, the stem was turned and another side was used for the second series of seven sections. Therefore, approximately 14 sections were available for the different analyses (Table S1). These sections were considered as technical replicates, as they were derived from a single stem piece. The sampling from a stem cross-section is illustrated in Fig. S2.

**Micromechanical Tensile Test**

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 mechanical testing, which was
also performed in wet conditions. For mechanical testing, a custom-built micro-tensile testing stage was used as previously described (Burgert et al., 2003). The LT-sections were screw clamped into the sample holders. Before testing, the two ends of the LT sections were reinforced by gluing 150 µm thick microtome cut wood (spruce) sections to them on both sides in order to avoid damages due to the clamping. The force was recorded with a 50N load cell (Honeywell, Sensotec Sensors) and the strips were strained with a strain rate of 0.067%/s (with a span length of 15 mm) until failure. The data of those samples, which failed at the edges, were excluded from further evaluation. The displacement was recorded via video extensometry using a stereo microscope and a CCD camera. Foliar frames with black lines on white background were mounted close to the sample ends on the sample holders for the videoextensometry. Force-deflection curves were converted into the stress-strain curves and mechanical properties such as tensile elastic modulus, ultimate stress, ultimate strain and yield stress were calculated. The mechanical properties obtained from the micromechanical tensile test were calculated as previously reported (Özparpucu et al., 2017). The mean values of three biological replicates per genotype were compared using One Way-ANOVA analysis (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). Dry mass of the tissues was measured after drying samples at 65°C for two days. For volume calculation, the thickness of tissues was measured by a micrometer screw, whereas 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

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 1.54 Å. 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 35 min exposure time. Three biological replicates of each genotype were chosen for X-ray analysis, and at least ten technical replicates, which had been tested mechanically, were measured for each biological replicate (Table S1). For cellulose orientation analysis, 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 with the fit parameters revealing the microfibril orientation distribution for each measuring spot. For this simulation routine 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 not taken into account for statistical analysis. In addition, the cellulose structure was analyzed by powder X-Ray diffraction (XRD) with samples being cut into very small pieces manually with a scalpel to resemble powder conditions (see Methods S1).

**Fourier Transform Infrared (FTIR) Spectroscopy**

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

**Results**

**CSE silencing affects wood mechanical properties**

Axial stiffness of never-dried tissue strips of all genotypes was determined by micromechanical tensile tests. The elastic modulus, as a measure of axial stiffness, significantly decreased \((F_{2,8}=34.70, \ p=5.03\times10^{-4})\) by 36% and 53% in \(hpCSE#1\) and \(hpCSE#2\), respectively, and ultimate stress decreased by around 15% \((F_{2,8}=3.85, \ p=0.083)\) in both lines when compared to the WT (Fig. 1a,b). The individual stress-strain curves are shown in Fig. S3 (see supplementary data). The ultimate strain was significantly higher for \(hpCSE#1\) and \(hpCSE#2\) than for the WT \((F_{2,8}=5.73, \ p=0.04, \text{Fig. 1c})\), whereas toughness was not statistically different \((F_{2,8}=0.57, \ p=0.59, \text{Fig. 1d})\).

**FTIR experiments confirm the reduced lignin content in CSE down-regulated lines**

The two transgenic lines employed in this work, \(hpCSE#1\) and \(hpCSE#2\), were previously characterized 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
In order to confirm these previous results, FTIR spectra were acquired directly on the tissue strips of all genotypes after mechanically testing. Three FTIR spectra were acquired for each technical replicate (n=10-13, 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) to evaluate the changes in lignin content relative to the cellulose content. In the average spectra of both transgenic lines, a pronounced and significant decrease was 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, Table S2) (Faix, 1991; Kacurakova et al., 1998; Marchessault, 1962) suggesting a lower lignin content. Additionally, a significant difference in the region of 1350-1330 cm\(^{-1}\) was observed for both 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. 4). 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 reflects the reduction in lignin abundance. The small band shifts observed in the FTIR spectra also reflect small changes in lignin composition. Accordingly, higher proportions of H-units have been observed in the hpCSE lines (Saleme et al., 2017). The results obtained with the FTIR analysis confirm that CSE down-regulation led to lower amounts of lignin and a mild shift in lignin composition in poplar stems.

**Lower density can partly explain lower stiffness of CSE down-regulated lines**

Wood density is one of the most important parameters influencing wood macromechanical properties such as stiffness and strength (Gibson and Ashby, 1997). In the present study, density was measured as oven dry weight per green volume for the mechanically tested tissue strips. WT plants exhibit a density of 0.35 ± 0.03 g/cm\(^3\), while hpCSE#1 and hpCSE#2 had significantly lower densities with mean values of 0.32 ± 0.04 g/cm\(^3\) and 0.30 ± 0.03 g/cm\(^3\), respectively (\(F_{2,8}=6.11, p=0.035\), Fig. 3a). In order to exclude the potential effects of density on stiffness, specific elastic modulus (E/\(\rho\), elastic modulus normalized to density) was calculated and compared among the different genotypes. Specific elastic modulus was significantly lower (reduction of 30-44\%) for both transgenic lines compared to that of WT (\(F_{2,8}=33.75, p=5.43\times10^{-4}\), Fig. 3b). The correlation of the elastic
modulus with density was relatively low ($R^2 = 0.13$) for the technical replicates (Fig. S4) due to rather high variation of both parameters, whereas a much higher correlation ($R^2 = 0.71$) was obtained when the calculations were based on the biological replicates and averaged values (Fig. 3c). Because elastic modulus was still significantly different between the transgenic lines and the corresponding WT even after the normalization to density, the decrease in stiffness can only be partly explained by the decrease in density.

**CSE down-regulated lines showed similar cellulose orientation and structure**

The MFA of the S2 cell wall layer, a crucial factor for the mechanical behavior, was determined by wide-angle X-ray diffraction (WAXD) (Cave, 1968; Köhler and Spatz, 2002). WAXD measurements revealed similar average MFA of around 15±1° for WT and the transgenic lines ($F_{2,8}=1.85, p=0.23$, Fig 4.a). These results suggest that cellulose microfibril orientation is not responsible for the changes in mechanical behavior found in the wood of CSE down-regulated poplars (Fig. 4b). Because the different genotypes had similar MFAs, no correlation was found between elastic modulus and the MFA, neither when based on technical ($R^2$ of 0.00, Fig. S2) nor on biological replicates ($R^2$ of 0.05, Fig. 4b).

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

**Lignin absorbance was 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 (Pandey and Pitman, 2004; Rodrigues et al., 1998; 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 amount influence tensile stiffness**

The elastic modulus showed a good correlation with density and with lignin absorbance. Multiple linear regression (MLR) was employed to determine which of these two parameters had the strongest effect on the elastic modulus. 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 whereas MFA and lignin absorbance were stepwise added. Accordingly, the regression model was made and evaluated at each step. The regression results based on the biological replicates (Table S3a) showed that density explained approximately 70% of the variation in the elastic modulus values ($R^2$ of 0.71; $p=0.024<0.05$). By adding the MFA as the second variable, $R^2$ increased slightly to 0.77, but this parameter was not found to contribute significantly ($p=0.68>0.05$), whereas density did ($p=0.04<0.05$). When lignin absorbance was added as a third parameter, $R^2$ increased significantly from 0.71 to 0.98. Therefore, almost all variation in elastic modulus was explained by variation in density and lignin content ($p_{\text{density}}=0.03<0.05$, $p_{\text{lignin}}=0.0005<0.05$). In addition, the last step in 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 ($R^2$ of 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 (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 influence the tensile modulus significantly. Nevertheless, due to the higher variation in these parameters at the technical replicates, lower $R^2$ values were found in comparison 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 (Table S3b).

**Discussion**

Genetic engineering has proven to be a valuable tool for revealing the mechanical function of lignin. Nevertheless, reductions in lignin deposition through genetic engineering often result in negative effects on plant growth and development (Leple et al., 2007; Van
Acker et al., 2014; Voelker et al., 2011) precluding the assessment of the individual contribution of lignin to wood mechanical properties. Because down-regulation of CSE in poplar was previously shown to cause significant changes in lignin amount and composition whereas plant yield remained unchanged (Saleme et al., 2017), we anticipated that mechanical characterization of the hpCSE lines will help further elucidating the role of lignin in wood mechanical properties. A reduction in FTIR absorbance at 1505 cm\(^{-1}\) of around 35% was observed for the hpCSE lines when compared to the WT, which largely agrees with the changes in Klason lignin content (18-25% reduction) (Saleme et al., 2017). The difference in the extend of lignin reductions measured by FTIR absorbance and wet chemistry might be due to methodological but also biological differences, as the CSE down-regulated poplar samples studied by Saleme et al. (2017) originated from a different batch of plants than those analyzed in the present study.

The micromechanical tensile tests revealed a significant decrease in mechanical stiffness by more than 30% in the transgenics compared to WT plants, which has been shown to correlate with the decrease in FTIR absorbance and, therefore, with lignin content. Very similar values of MFA and crystallinity index were found for 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 stiffness compared to the matrix, it is unlikely that microfibril orientation is the cause for the observed decrease in stiffness. In addition, the decrease in density by 10% could only partly explain the decrease in stiffness, as elastic modulus was still significantly different between the genotypes after the normalization to density. These results suggest that the changes in lignin content play a major role in the altered mechanical behavior observed in the wood of CSE down-regulated poplars. Saleme et al. (2017) reported a slight alteration of lignin composition in the hpCSE lines, with a significant increase in the relative content of H-units from 0.6% to 1.2% and a slight reduction in S/G ratio (approximately 9% decrease from an initial ratio of around 2). Nevertheless, it has been shown that even strong changes in 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 slight changes in lignin composition observed in the hpCSE plants had any significant influence on its mechanical properties. Next to the cell wall matrix, also structure and mechanical properties of the middle lamella may have become affected, as it largely consists of lignin. However, due its very small volume fraction compared to that of the cell wall, any change in the mechanical properties of the middle lamella should not significantly affect the overall stiffness of the
wood tissue. Altogether, this study reports a correlation between lignin content and axial stiffness in wet condition for a lower MFA (15°) than reported in previous studies.

The results found in this study for CSE downregulated poplars are in contrast to those obtained by Bjurhager et al. (2010) working on poplar plants silenced for C4H. Despite similar MFAs were found for both the WT and the transgenic lines in both studies, the reduction in lignin content found in the study of Bjurhager et al. did not seem to have any influence on cell wall stiffness. The observed significant decrease in axial stiffness could be exclusively explained by the decrease in density. Noteworthy, different enzymes have been targeted in the two studies, which possibly resulted in different anatomical or structural changes in the cell walls of the respective modified poplar plants that, in turn, may have superimposed the effects of the lower lignin content on axial stiffness. However, identifying and, especially, measuring the contribution of such secondary effects on wood mechanics is difficult. These effects could include changes in the degree of cross-linking among cell wall polymers affecting the fiber-matrix interface, or cellulose microfibril lengths. These potential secondary effects were captured neither in the present study, nor in any previous study. 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 of stiffness. Assuming such an influence, this would render the influence of lignin content on stiffness more pronounced. However, the measurements on the samples of the present study with powder X-ray diffraction could not reveal an increase in crystalline cellulose content.

Based on the observation that lignin content and axial stiffness correlate in the case of large MFAs, an explanation of how lignin content may influence axial stiffness as a function of MFA has been suggested by Rüggeberg et al. (2008) and Eder et al. (2009). Their explanation is based on the assumption that lignin content influences the shear stiffness of the matrix, with a lower lignin content resulting in a lower shear stiffness. In case of uniaxial loading in 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 case of off-axis loading (i.e. large MFA). 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, the present study provides valuable new input, as it has revealed a
measurable influence of lignin content on axial stiffness at a lower MFA (around 15°) compared to previous studies (MFAs larger than 20°).

Despite the new contribution of the present study and the possible explanation by composite theory, deriving a causal relationship between these factors remains challenging considering the following aspects. It remains to be investigated whether and how lignin content actually influences the shear stiffness of the cell wall matrix. Despite increasing knowledge on 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. Insights on 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 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 strength could be partly explained by the reduction in density and correlated to lignin content. As a new finding and in contrast to previous reports, our data reveal that lignin correlates with mechanical properties of cell walls at comparatively low MFAs (approx. 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 at off-axis loading of the cell wall due to inducing shear stresses in the cell wall matrix because of the off-axis loading, which would overall lead to a gradual rise of the influence of lignin content on axial mechanics as a function of MFA. The results obtained from the present study may give precious input for future simulation studies based on cell wall models, which incorporate chemical composition (lignin content) and structure (cellulose orientation and structure). Validating a gradual influence of lignin content on axial stiffness as function of the MFA and, as ultimate goal, deriving a causal relationship of lignin content and mechanical properties are important tasks for future research.

Supplementary Information

Figure S1 Representative debarked samples for each genotype.

Figure S2 Origin of samples within a representative poplar stem section.

Figure S3 Stress-strain curves of the technical replicates.
**Figure S4** The correlation of elastic modulus with a) density, b) MFA and c) lignin absorbance, based on the technical replicates level.

**Figure S5.** The XRD patterns

**Figure S6.** Representation of the crystallinity index (%) calculation

**Methods S1** Powder X-Ray Diffraction (XRD)

**Table S1** Number of characterized biological and technical replicates.

**Table S2** FTIR-absorbance comparison of genotypes

**Table S3** Stepwise multiple linear regression results

**Table S4.** Multiple linear regression with the interaction of density and lignin absorbance

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**References**


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Figure Legends

Figure 1. Tensile properties of WT, hpCSE#1 and hpCSE#2. a) Elastic modulus, b) ultimate stress, c) ultimate strain, d) toughness. The squares in the box plots show 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. Asterisks indicate significant differences between the WT and the transgenic lines at 95% confidence level (ANOVA). For the number of technical replicates, see Table S1.

Figure 2. The average FTIR spectra of WT, 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). Asterisks indicate significant differences (ANOVA, p=0.05). Each spectrum represents the average of 90-117 spectra calculated as 3 measurements*~10-13 strips*3 biological replicates.

Figure 3. Density-stiffness relations: a) density values, b) specific elastic modulus (E/ρ, density normalized) of WT, hpCSE#1 and hpCSE#2, c) correlation of density with elastic modulus (R²=0.71), based on biological replicates (error bars show standard errors). For the explanation of the box plots, see Fig. 1.

Figure 4. Structural properties of wood from WT, hpCSE#1 and hpCSE#2 poplars. a) Cellulose microfibril angle (MFA) values and b) correlation between E-Modulus and MFA for biological replicates. For the explanation of the box plots see Fig. 1. Error bars show standard errors.

Figure 5. The correlation of lignin absorbance at 1505 cm⁻¹ with a) elastic modulus and b) specific elastic modulus calculated for biological replicates. Error bars in the scatter plots show standard errors.