

Unravelling the impact of lignin on cell wall mechanics: a comprehensive study on young poplar trees downregulated for CINNAMYL ALCOHOL DEHYDROGENASE (CAD)

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SUMMARY

Lignin engineering is a promising tool to reduce the energy input and the need of chemical pre-treatments for the efficient conversion of plant biomass into fermentable sugars for downstream applications. At the same time, lignin engineering can offer new insight into the structure–function relationships of plant cell walls by combined mechanical, structural and chemical analyses. Here, this comprehensive approach was applied to poplar trees (*Populus tremula* × *Populus alba*) downregulated for *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*) in order to gain insight into the impact of lignin reduction on mechanical properties. The downregulation of *CAD* resulted in a significant decrease in both elastic modulus and yield stress. As wood density and cellulose microfibril angle (MFA) did not show any significant differences between the wild type and the transgenic lines, these structural features could be excluded as influencing factors. Fourier transform infrared spectroscopy (FTIR) and Raman imaging were performed to elucidate changes in the chemical composition directly on the mechanically tested tissue sections. Lignin content was identified as a mechanically relevant factor, as a correlation with a coefficient of determination (r^2) of 0.65 between lignin absorbance (as an indicator of lignin content) and tensile stiffness was found. A comparison of the present results with those of previous investigations shows that the mechanical impact of lignin alteration under tensile stress depends on certain structural conditions, such as a high cellulose MFA, which emphasizes the complex relationship between the chemistry and mechanical properties in plant cell walls.

Keywords: genetic modification, plant cell wall, microtensile test, cell wall mechanics, lignin, cinnamyl alcohol dehydrogenase, *Populus tremula* × *Populus alba*.

INTRODUCTION

Lignocellulosic material potentially represents a very valuable source for biorefinery processes (Vanholme *et al.* 2013). Despite its abundance and availability, the use of this material has been limited so far by the necessity of a large energy input and harsh physicochemical treatments for the breakdown of its recalcitrant structure. These limiting aspects hold especially true for the removal of lignin, an aromatic heteropolymer that limits the access of hydrolytic enzymes to cell wall polysaccharides. In this context, genetic engineering has been employed to produce plants

that either deposit less lignin or deposit lignin with an altered structure that is more easily degradable (Vanholme *et al.*, 2010, 2012a). Such changes have proven to increase the efficiency of downstream processes such as chemical pulping and cellulose-to-glucose conversion in biorefinery processes, which may lead to significant economic and environmental benefits (O'Connell *et al.*, 2002; Pilate *et al.*, 2002; Baucher *et al.*, 2003; Van Acker *et al.*, 2014; Dumitrache *et al.*, 2016). Alterations in lignin content and composition may affect the cross-linking between lignin and other

cell wall components, however, and alter the integral structure of the cell wall. These alterations may lead to changes in cell wall mechanics, with an impact on the growth and robustness of the whole plant. Lignin engineering via downregulation of *CINNAMOYL COA REDUCTASE* (*CCR*) in *Arabidopsis* resulted in a disordering of the cellulose microfibril network (Ruel *et al.*, 2009). Such a loss of cell wall integrity may lead to inferior mechanical properties of the stem (Horvath *et al.*, 2010a), and may in part explain the impaired growth often seen in plants with reduced lignin content (Lep   *et al.*, 2007; Vanholme *et al.*, 2012b), impairing the benefits of reduced recalcitrance obtained by the reduction in lignin content (Van Acker *et al.*, 2014).

As a tool, genetic engineering offers the possibility to gain new insights into cell wall mechanics, if changes in mechanical properties can be correlated with altered structural and biochemical features (Cavali   *et al.* 2008); however, the underlying relationships between lignin chemistry, structural features and mechanical properties of the cell walls are as yet only partially understood. A number of studies have correlated changes in mechanical properties with structural and chemical shifts in the cell walls of lignin-modified poplar (Koehler *et al.*, 2006; Bj  rhager *et al.*, 2010; Horvath *et al.*, 2010b); however, different outcomes regarding the influence of changes in lignin content and composition on the mechanical properties of the cell wall have been reported. Horvath *et al.* (2010b) have found that a reduction in (Klason) lignin content from around 21% in wild type (WT) to around 15–17% led to a significant reduction in bending modulus and compression strength in young quaking aspen, which were downregulated for either *4-COUMARATE-COA LIGASE* (*4CL*) or both *4CL* and *CONIFERALDEHYDE 5-HYDROXYLASE* (*CaId5H*). No significant changes in those mechanical parameters were observed when lignin content was reduced to only 19%. The highest decrease (60%) in elastic modulus was observed for one of the transgenic lines, in which both lignin content and S/G ratio were altered simultaneously (Horvath *et al.*, 2010b). It has been shown that *C4H* downregulation in poplar resulted in a decreased tensile stiffness that could largely be assigned to a decrease in density (Bj  rhager *et al.*, 2010). The reduction of lignin content by 30% in this wood neither influenced the specific (density-normalized) tensile stiffness nor the specific ultimate stress (Bj  rhager *et al.*, 2010).

In the present study, we carry out a comprehensive study using transgenic poplars that were downregulated for *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*) via sense (S) and antisense (AS) gene silencing techniques (Baucher *et al.*, 1996; Lapierre *et al.*, 1999, 2004; Pilate *et al.*, 2002). These transgenic poplars are termed S-CAD and AS-CAD, respectively. The downregulation of *CAD* normally results in minor changes in lignin content, but also leads to a considerably increased incorporation of

hydroxycinnamaldehydes into lignin compared with the lignin of WT plants. The resulting plant biomass shows increased pulping efficiency and fermentable sugar yield (Baucher *et al.*, 1996; Lapierre *et al.*, 1999, 2004; O'Connell *et al.*, 2002). In a theoretical work employing molecular dynamic modelling, it was suggested that such an atypical incorporation of aldehydes reduces lignin interaction with hemicellulose, thereby increasing cell wall porosity and making the resulting biomass degradable more easily (Carmona *et al.*, 2015). In *Nicotiana tabacum* (tobacco), an approximately 30% reduction of elastic modulus was observed upon *CAD* downregulation, which was explained by a reduced cross-link density (Hepworth *et al.*, 1998). Awad *et al.* performed three-point bending tests on stems of AS-CAD, S-CAD and WT poplars. For one AS-CAD line, a significant decrease in bending modulus was observed, whereas the other AS-CAD line and the S-CAD line did not show any significant difference compared with the WT (Awad *et al.*, 2012); however, no specific chemical analysis was provided in these two studies.

Here, we perform a comprehensive study on *CAD*-downregulated poplars, combining mechanical, structural and chemical analysis on the very same samples. The goal is gaining further insights into the basic structure–property relationships of plant cell walls, with a specific focus on the impact of lignin alterations on the mechanical properties. Mechanical properties were obtained by microtensile tests on a large number of thin tissue strips. Density, cellulose microfibril orientation and chemical composition were determined for these mechanically tested strips. The chemical composition was investigated by Fourier transform infrared spectroscopy (FTIR) and Raman microscopy in order to determine changes in lignin content and structure in the native state directly on the mechanically tested samples, and with high spatial resolution at the cell wall level (Raman).

RESULTS AND DISCUSSION

Downregulation of *CAD* affects elastic modulus and yield stress

The mechanical properties of the xylem in AS-CAD, S-CAD and WT plants were obtained by micro-mechanical tensile tests using tissue strips with a size of 30 × 1.5 × 0.1 mm. Table 1 gives an overview of the numbers of biological and technical replicates tested, whereas Figure S1 provides an illustration depicting the determination of the elastic modulus, ultimate stress, yield stress, ultimate strain and toughness from the stress–strain curves obtained. The averaged stress–strain curves for the biological replicates are shown in Figure 1(a). Biphasic behaviour is visible in all curves, which indicates a large cellulose microfibril angle (MFA) (K  hler *et al.*, 2002; Keckes *et al.*, 2003). Despite this overall similar behaviour, significant

Table 1 Names and numbers of biological and technical replicates of the three genotypes tested

Genotype	Biological replicate	Number of technical replicates for each biological replicate			
		Micromechanical tensile test	XRD measurements ^a	FTIR measurements ^{a,b}	Raman ^c
WT	WT4	11	5	11	3
	WT7	10	–	10	–
	WT8	14	–	14	3
	WT9	19	5	19	3
	WT10	12	–	12	–
AS-CAD	AS3	8	5	8	3
	AS6	14	–	14	–
	AS7	17	–	12	–
	AS8	12	5	12	3
	AS10	12	5	12	3
S-CAD	S2	13	5	13	3
	S7	11	–	11	–
	S8	10	–	9	–
	S12	13	5	13	3
	S15	16	5	16	3

^aSame technical replicates as used for the micromechanical tensile test.

^bThree spectra were acquired for each technical replicate and averaged.

^cThree mappings were performed on one cross section taken from each biological replicate.

differences were observed for stiffness and yield stress between the WT and the transgenic plants (Figure 1b–d), whereas the differences between the two transgenic lines were not significant. The average elastic modulus decreased by approximately 15% (AS-CAD) and approximately 10% (S-CAD), and the yield stress decreased by approximately 16% (AS-CAD) and approximately 14% (S-CAD), respectively (details and the variations of the mechanical properties between the biological replicates of WT and transgenic lines are given in Tables S1 and S2). Ultimate stress (Figure 1c) was not significantly affected.

Density and MFA are not affected in CAD-downregulated poplars

In order to investigate whether certain structural changes cause the differences in mechanical behaviour, the density and MFAs of the WT and the transgenic lines were compared. The mechanical properties of wood are largely affected by its tissue density, which integrates structural features in mass per volume. Thus, a comparison of density values is a good indicator for structural differences at the tissue level. The wood of the WT poplar showed a mean density of $0.54 \pm 0.05 \text{ g cm}^{-3}$, whereas that of the AS-CAD and the S-CAD lines had averaged densities of 0.51 ± 0.02 and $0.52 \pm 0.03 \text{ g cm}^{-3}$, respectively (Figure 2a). Although slightly lower, the densities of both transgenic lines did not significantly differ from that of the WT.

In microtensile tests on wood, the cellulose MFA in the S2 layer of the cell wall is a crucial factor determining the mechanical behaviour in terms of elastic modulus, strain to fracture, yield stress and ultimate stress (Cave, 1968; Köhler *et al.*, 2002). A small MFA in the range of 5–15°

results in a stiff and strong but rather brittle material, whereas larger MFAs above 15° lead to a more pliant, weaker but tough material (Cave, 1968; Donaldson, 2008). The X-ray diffraction measurements revealed an average MFA of $27 \pm 3^\circ$ for AS-CAD, $25 \pm 2^\circ$ for S-CAD and $27 \pm 2^\circ$ for the WT (Figure 2b). These high values coincide with typical values measured for juvenile wood (Gorisek *et al.*, 1999), and are in good accordance with the biphasic pattern of the stress–strain curves observed in Figure 1(a) (Köhler *et al.*, 2002; Keckes *et al.*, 2003). It is noteworthy that three of the 45 measurements showed the presence of tension wood (TW) within the respective samples (two samples of AS-CAD and one sample of S-CAD), despite the screening for the presence of gelatinous layers (G-layers), characteristic for tension wood, by optical microscopy. These samples were excluded from the data sets shown in Figure 1. Yet, the average values of the mechanical parameters calculated with and without the TW samples showed no significant differences (Figure S2).

In conclusion, as both density and cellulose MFA did not significantly differ between the WT and the transgenic lines, the differences in elastic modulus and yield stress may not be based on previously described structure–property relationships (Page *et al.*, 1971; Reiterer *et al.*, 1999), but would rather be the result of chemical differences at the cell wall level caused by CAD downregulation.

Fourier transform infrared spectroscopy reveals changes in lignin deposition in CAD downregulated lines

The FTIR spectra were acquired directly on the mechanically tested strips for a non-destructive investigation of changes in cell wall chemistry. By normalizing for the

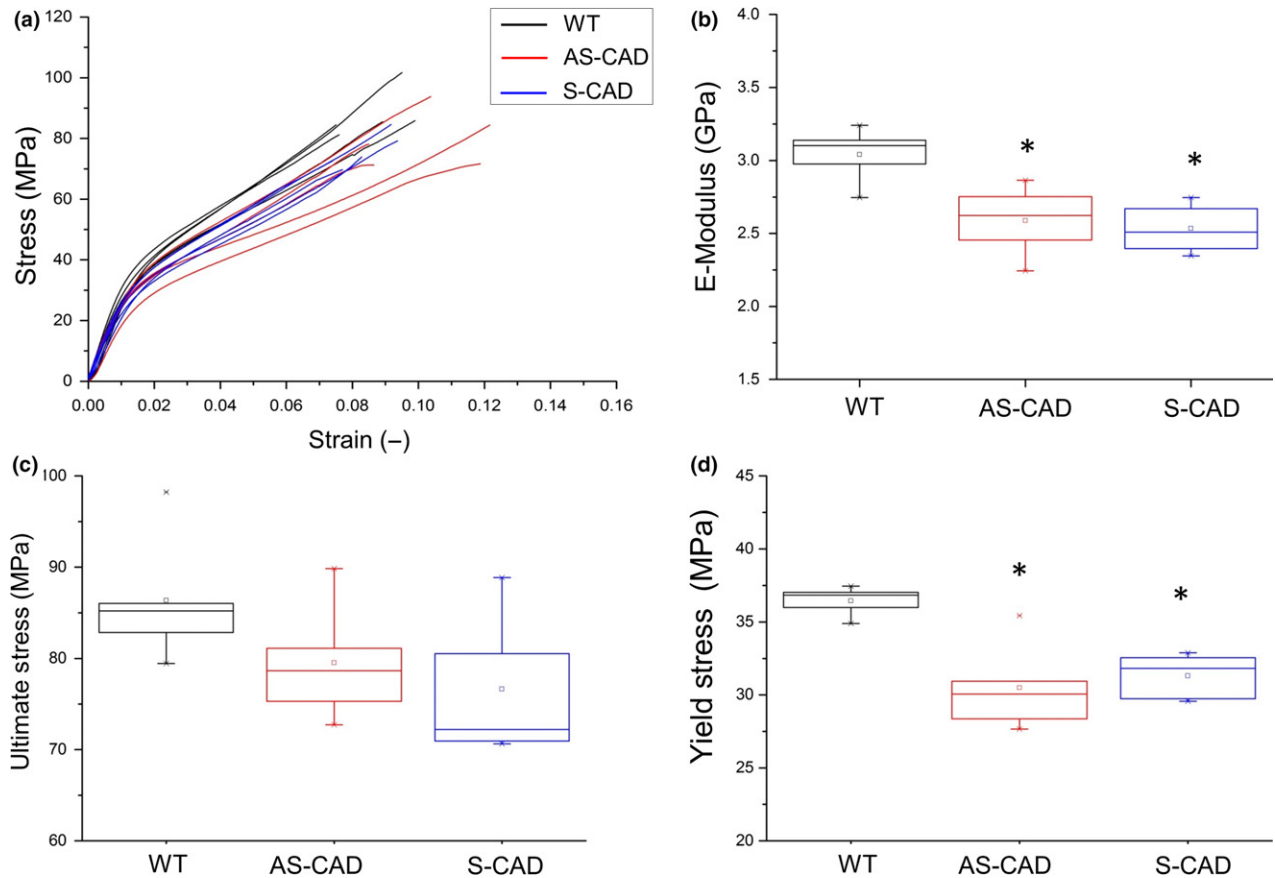


Figure 1. Mechanical properties of wild-type (WT), AS-CAD and S-CAD poplar: (a) Averaged stress-strain curves for the biological replicates; (b) stiffness; (c) ultimate stress; and (d) yield stress. The squares in the box plots show the mean values of the biological replicates, and the lines inside the boxes denote the median values. The boxes mark the 25th and 75th percentiles, whereas the whiskers indicate the 1st and 99th percentiles; outliers are shown as individual points ($\times$). Bold asterisks indicate significant differences between the WT and the transgenic lines at 95% confidence level (ANOVA test).

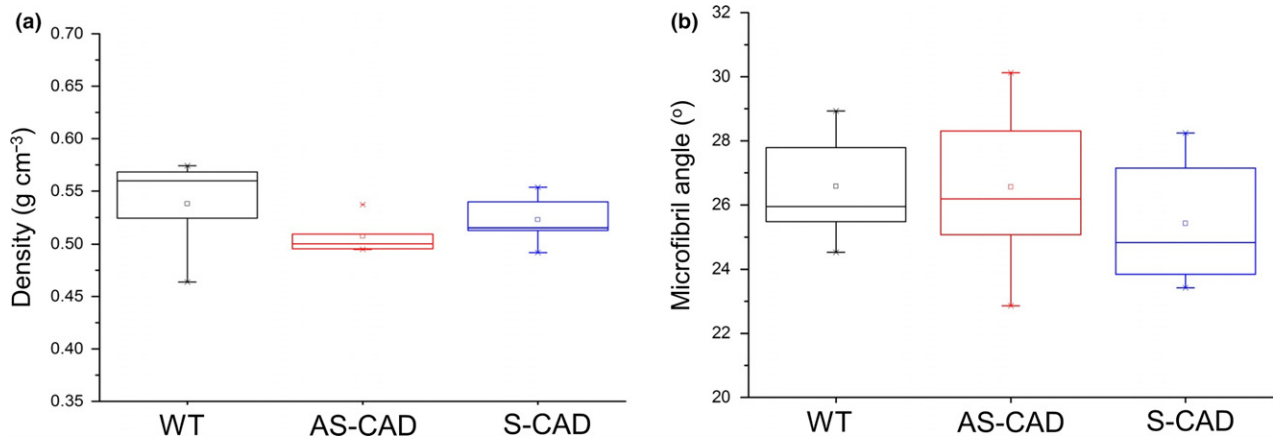


Figure 2. Structural properties of wild-type (WT), AS-CAD and S-CAD poplar: (a) density and (b) microfibril angle (MFA). For an explanation of the box plots, see Figure 1.

strongest cellulose band at 1032 cm^{-1} , changes in lignin content relative to cellulose content can be studied (Figure 3); however, it is very important to measure a sufficient number of spectra as variability within the same section, as well as between consecutive sections of the same

sample, can be very high (Figure S3). This variability is observed because only the surface is measured, and different spectra will be gained if, for example, vessels or rays or fibers are probed. Nevertheless, although high variability was observed within each genotype, the average

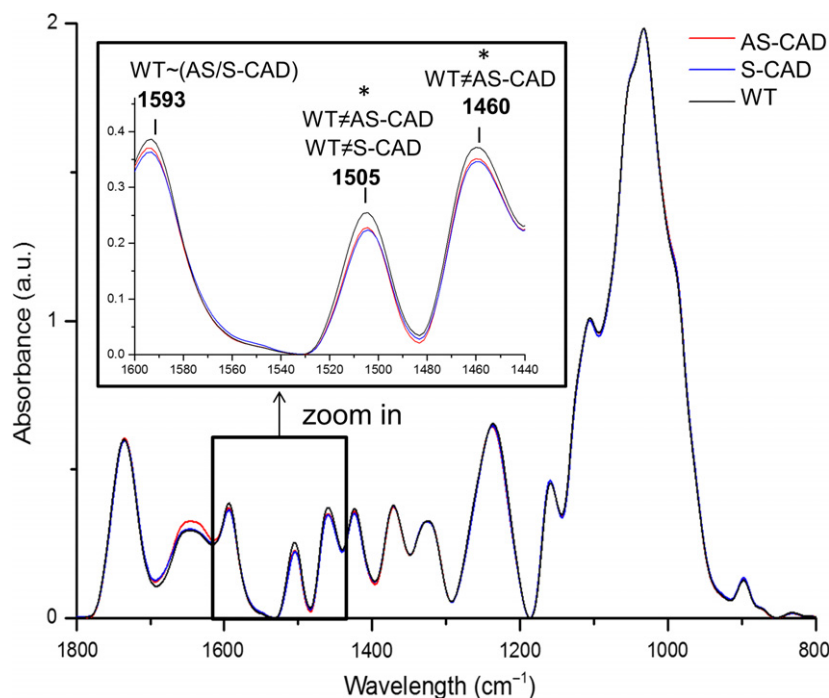


Figure 3. The average Fourier transform infrared spectroscopy (FTIR) spectra of wild-type (WT), AS-CAD and S-CAD in the range of 1800–800 cm^{-1} (baseline corrected and normalized to the highest peak: 1032 cm^{-1}). According to ANOVA analysis ($P = 0.05$), bands marked * show significant differences, whereas bands marked approximately show similarities between WT and transgenic lines. Band assignments: 1593 cm^{-1} , aromatic skeletal stretching plus C=O stretching; 1505 cm^{-1} , C=C stretching of aromatic ring; 1460 cm^{-1} , C–H deformation band. Each spectrum represents the average of at least 150 spectra calculated as three measurements of approximately 10–15 strips with five biological replicates (given in Table 1).

spectra showed slight but significant differences for at least two bands, which can be assigned to lignin (Figure 3).

In the *CAD*-downregulated lines, lower absorption was observed at 1593 cm^{-1} (aromatic skeletal stretching plus C=O stretching) and 1505 cm^{-1} (C=C stretching of the aromatic ring in lignin) (Faix, 1991). Also, the C–H deformation band of lignin at 1460 cm^{-1} (Faix, 1991; Kline *et al.*, 2010) showed lower absorption values for the transgenic plants compared with the WT. ANOVA analysis of the average spectra revealed that the differences are significant at 1505 cm^{-1} (for both AS-CAD and S-CAD) and 1460 cm^{-1} (for only AS-CAD) at the 95% confidence level ($P = 0.05$). For the average spectra (normalized on 1032 cm^{-1}), no changes were observed in the region between 1190 and 850 cm^{-1} for both peak position and absorbance of the bands which is the region characteristic for polysaccharide structures (Sibout *et al.*, 2005). Altogether, these results indicate that *CAD* downregulation affected lignin deposition in terms of a lower lignin content, but neither changed the structure of the cellulose microfibrils nor changed the hemicellulose content to a significant degree. The differences in absorbance at 1635 cm^{-1} are the result of adsorbed water in the wood (Marchessault, 1962).

Micro-scale chemical analysis using Raman microscopy shows increased incorporation of hydroxycinnamaldehydes in *CAD*-downregulated lines

For a more specific and spatially resolved chemical analysis, Raman microscopy was used, which allows for

mapping and separating chemical changes in the different cell wall regions and layers. By integrating the main lignin bands between 1650 and 1550 cm^{-1} , maps of the lignin distribution were obtained for each genotype (AS-CAD, S-CAD and WT; Figure 4). The maps show the highest concentration of lignin in the cell corners (CCs), whereas the cell walls (CWLs) show lower lignin accumulation. A more detailed region of interest (ROI) study was performed separately for CC and CWL, and average spectra were extracted. These average spectra were baseline corrected and normalized at 1598 cm^{-1} (aromatic ring, symmetric stretching of lignin). Figure 4 shows all ROIs for CCs and CWLs for one mapping, and the resulting average spectra. The Raman bands obtained and their assignments are summarized in Table S3 (Edwards *et al.*, 1997; Agarwal, 1999; Saariaho *et al.*, 2003; Ma *et al.*, 2011). In the CWL, the signals of cellulose at 1377, 1147, 1118, 1093, 897 and 519–380 cm^{-1} , and those of hemicelluloses at 1730 and 1039 cm^{-1} , are much more intense, compared with those in the CC, because of the higher concentration of cell wall polysaccharides in the CWL (Agarwal, 1999). In the aromatic lignin region (1700–1550 cm^{-1}), an additional band can be observed for *CAD*-downregulated poplars in comparison with the WT at 1630 cm^{-1} . This band is assigned mainly to ring conjugated C=C stretching of coniferyl and sinapyl aldehydes. It is known that *CAD* downregulation results in a considerably increased incorporation of hydroxycinnamaldehydes into the lignin, compared with WT lignin, and that this gives rise to ring conjugated C=C structures (Kim *et al.*, 2002; Lapierre *et al.*, 2004).

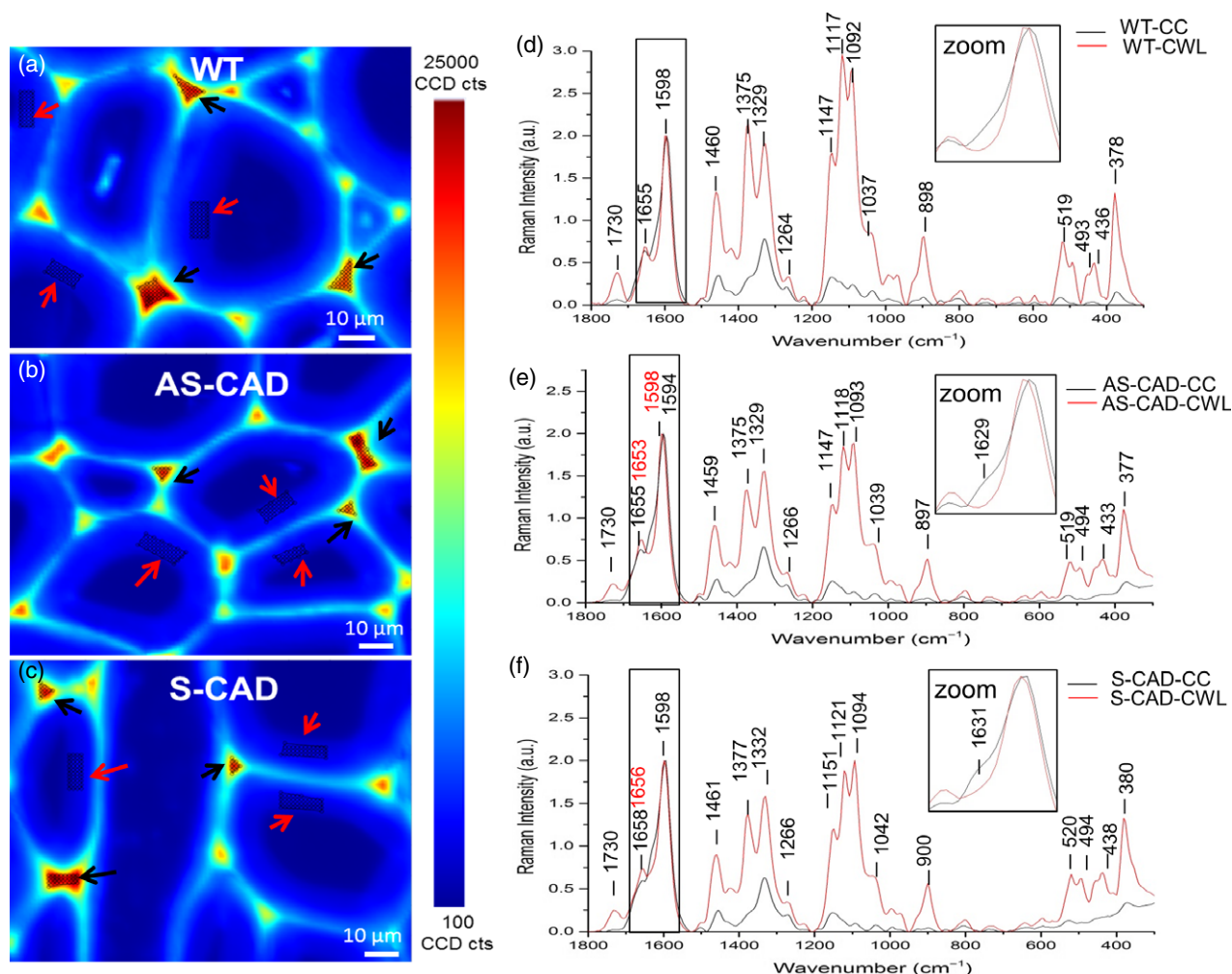


Figure 4. Raman analysis of wild-type (WT), AS-CAD and S-CAD. (a–c) Raman images of representative cross-sections showing the integrated intensity from 1550 to 1650 cm^{-1} (aromatic lignin region), with regions of interest (ROIs) marked for further analysis. Three ROIs were selected from cell corners (CCs, black arrows) and cell walls (CWs, red arrows). (d–f) Extracted average spectra obtained from the ROIs of CCs and CWs. The spectra of the transgenic lines exhibit a shoulder at around 1630 cm^{-1} (assigned to ring conjugated C=C stretching derived from hydroxycinnamaldehyde incorporation).

The average spectra of the ROI from CCs and CWs of all samples in the range 1700–1550 cm^{-1} revealed significant variability in the height of the aldehyde band among the genotypes. Especially for the CC, higher intensities at 1630 cm^{-1} were observed for S-CAD plants compared with AS-CAD and WT plants (Table 2). Although some of the spectra of AS-CAD plants were similar to those of the WT, most of them also showed slightly higher intensity. The variability of the intensity of this peak is quite high for both CC and CWL within each genotype, however. When the aldehyde/coniferyl alcohol (1630 cm^{-1} /1658 cm^{-1}) intensity ratio was compared between the CCs and the CWs of the transgenic lines, it was observed that the CCs show higher ratios than the CWs (Table 2). The intensities of the aldehyde peak in the spectra at 1630 cm^{-1} indicate a higher incorporation of aldehydes for S-CAD compared with AS-CAD and WT genotypes, and a higher incorporation into

the CCs than into the CWs. Recently, higher aldehyde contents have also been imaged by hyperspectral stimulated Raman scattering in CCs of Arabidopsis plants deficient for CAD (Liu *et al.*, 2015).

Table 2 The variability of the Raman intensity of the spectra at 1630 cm^{-1} (aldehydes) and 1658 cm^{-1} (coniferyl alcohol), and the increase in their ratio (1630/1658 cm^{-1}) in AS-CAD and S-CAD with regards to wild type (WT) within cell corners (CCs) and cell walls (CWs)

	Samples	1630 cm^{-1}	1658 cm^{-1}	1630/1658 cm^{-1}
CC	WT	0.792 ± 0.047	0.704 ± 0.036	–
	AS-CAD	0.902 ± 0.067	0.642 ± 0.039	24.88%
	S-CAD	1.052 ± 0.082	0.6156 ± 0.04	51.90%
CWL	WT	0.5702 ± 0.07	0.688 ± 0.043	–
	AS-CAD	0.647 ± 0.081	0.656 ± 0.032	19.1%
	S-CAD	0.783 ± 0.087	0.689 ± 0.044	37.13%

The challenge of unravelling the impact of lignin alteration on tensile stiffness

The FTIR allows the determination of lignin absorbance (as an indicator for lignin content) directly on the mechanically tested samples. Hence, it was possible to correlate elastic modulus and lignin absorbance at the level of biological and technical replicates for gaining further insights into the underlying relationship between chemistry and mechanics. The values for the genotypes and the correlation for the biological replicates are given in Figure 5. Plotting stiffness against lignin absorbance for AS-CAD, S-CAD and WT (Figure 5a) revealed a positive correlation with a coefficient of determination (r^2) of 0.65 for the biological replicates of the three genotypes; however, the strength of correlation between these two parameters varied among the different genotypes (Figure 5b). Although r^2 is 0.925 for AS-CAD, it is 0.01 for S-CAD and 0.54 for the WT. If one records the correlation for the single technical replicates, hardly any correlation can be extracted (values of r^2 for AS-CAD, S-CAD and WT were: 0.120, 0.032 and 0.094, respectively; correlation not shown).

These results indicate a potential impact of lignin alterations on the tensile properties, but it is also obvious that the underlying relationships can hardly be extracted at the individual sample level. On the one hand, this could be the result of methodological limitations. The absorbance values are highly variable, because different types of cells with different lignin contents are measured and the tensile stiffness can vary because of deviations in fibre orientation. On the other hand, the coefficients of determinations are strongly affected by the range of measured values covered. AS-CAD, with the strongest correlation, also possessed the largest range in stiffness and absorbance among the biological replicates, whereas for S-CAD the range of values was much lower.

A comparison of our results with previously published data (Bjurhager *et al.*, 2010; Horvath *et al.*, 2010a; Awad *et al.*, 2012) indicates different findings regarding the impact of alterations in lignin content and/or composition on the mechanical properties of transgenic trees. This may be linked to different test protocols, such as bending or tension tests on either macroscopic or microscopic samples; however, even the same testing protocols on different transgenic lines have resulted in a different assessment of lignin regarding its influence on mechanics.

In this regard, a more detailed comparison of the current study with the investigations by Bjurhager *et al.* reveals that most probably the specific CWL configuration determines the impact of lignin content and structure on the tensile behaviour (Bjurhager *et al.*, 2010). In this study, a 30% reduction in lignin content and a slight, but significant reduction in tensile stiffness for *CINNAMATE 4-HYDROXYLASE* (*C4H*)-downregulated poplars has been reported. Yet, the observed changes in stiffness were accompanied by significant differences in density, which could parallel a possible impact of the reduction in lignin content. In the current study, no significant changes in density between the WT and the transgenic plants were found, which allows us to exclude this important structural feature as an influencing factor.

Next to density, cellulose microfibril orientation has a major impact on the mechanical properties of the cell wall (Cave, 1968; Lichtenegger *et al.*, 1999). In the study of Bjurhager *et al.* (2010), MFAs of less than 16° were reported, whereas in the present study, MFAs of 23–30° were measured. In both studies, no significant differences between the MFAs of the WT and transgenics were found. Hence the genetic alterations did not affect the cellulose orientation, but a high MFA seems to be of crucial importance for a detectable impact of lignin content and composition on

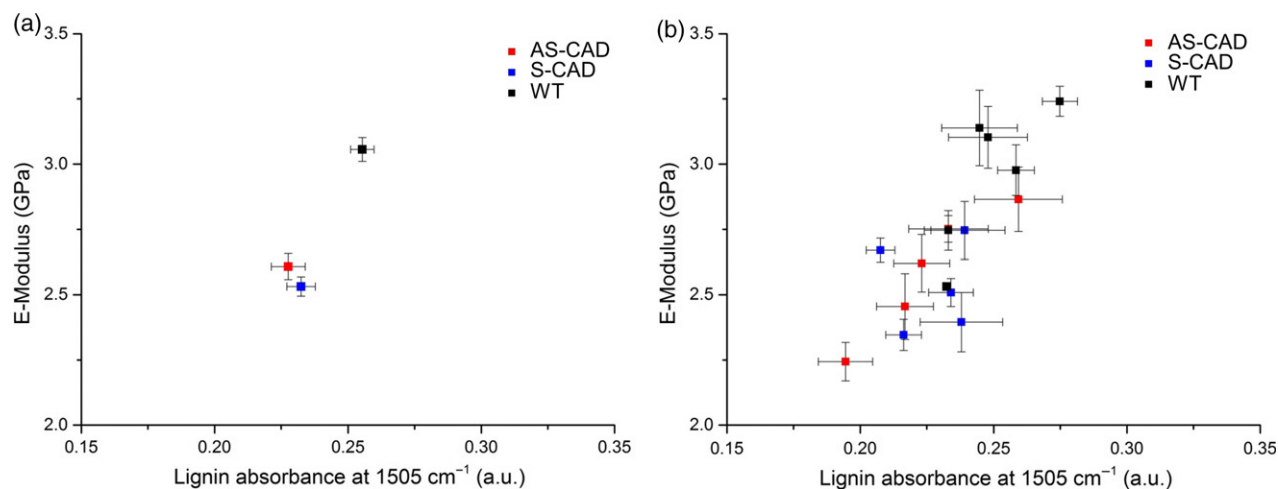


Figure 5. Correlation of stiffness with absorbance (Fourier transform infrared spectroscopy, FTIR) at 1505 cm⁻¹ for (a) genotypes and (b) biological replicates of each genotype; error bars show standard errors.

the mechanical properties. In the case of rather small MFAs, the cellulose microfibrils represent the almost exclusively load-bearing elements in the plant cell wall (Salmén *et al.*, 2009). By contrast, for larger MFAs (greater than approximately 20°) the cell wall matrix and the cellulose fibrils contribute in a more collective manner, which makes the lignin content and composition more relevant for the mechanical properties of the cell wall. The filling and cross-linking of the matrix with lignin is likely to increase the shear modulus and shear strength of the matrix, which in turn results in a higher elastic modulus and yield stress of the plant cell wall when tested in tension.

A similar relationship between chemistry and mechanics has been found in palm trees. Here, a gradient in the stiffness of cell walls in the fiber caps of vascular bundles is formed by a gradual alteration of lignin content for MFAs in the range of 15–40° (Rüggeberg *et al.*, 2008). Thus, besides directly influencing the mechanical properties of the cell wall, the cellulose microfibril orientation further affects the mechanical role of lignin, especially when loaded in tension. Hence, the comparison of the present data with the study by Bjurhager *et al.* (2010) indicates that wood cell walls with sufficiently high MFAs are needed for obtaining a detectable mechanical role of lignin in the plant cell wall under tensile loading.

Another factor to be considered when analysing the impact of lignin modifications is the spatial heterogeneity of the lignin alterations within the cell wall. The in-depth analysis by Raman microscopy revealed that the downregulation of *CAD* differentially affected the lignin deposition in the secondary cell walls and in the cell corners/middle lamella, which might influence the outcome of the tensile tests, as not only the cell wall but also the mechanical connection between the cells matters for the mechanical properties at the tissue level. Hence, although a correlation between lignin absorbance (lignin content) and tensile stiffness was found in the genotypes analysed, this correlation is complex and influenced by various parameters, and therefore remains far from being entirely understood.

Next to the heterogeneity of lignin modification at the cell wall level, the variability of modification found at larger length scales may increase the difficulty to obtain clearly interpretable results. The gene silencing technique, which is used to obtain the *CAD*-downregulated poplars, results in different degrees of gene silencing over the trunk (Baucher *et al.*, 1996). This variability in downregulation has also been observed with other lignin-modified lines (Tsai *et al.*, 1998; Leplé *et al.*, 2007; Van Acker *et al.*, 2014). In the present study, we have performed mechanical, structural and chemical analysis on the very same samples to minimize the influence of spatial heterogeneity. This beneficial aspect comes along with a restriction to semi-quantitative spectroscopical techniques for chemical analysis,

however, because of the very low quantities of material being used. Yet, for lignin content analysis it was demonstrated that FTIR spectroscopy can provide reliable semi-quantitative information on lignin content (Rodrigues *et al.*, 1998; Schwanninger *et al.*, 2004).

In conclusion, a correlation of lignin content with mechanical stiffness was visible for *CAD*-downregulated plants. The impact of lignin content on mechanics seems to depend on the specific structural configuration of the plant cell wall, with high MFAs likely to be a requirement for a visible impact of lignin on tensile stiffness. This interrelationship of structure and impact makes understanding the relationship between chemistry and mechanics complex, and may explain the deviating results for studies performed on other transgenics with structurally different plant cell walls. Next to that, structural variability, such as density or MFA, between transgenics and the WT should be small, whereas differences in lignin content have to be high enough for a detectable influence. Using the new CRISPR/Cas9 gene-editing technology, which has been demonstrated to work efficiently in poplar (Tsai *et al.*, 2015), at least the spatial variability in the efficiency of gene downregulation can be overcome (Zhou *et al.*, 2015).

EXPERIMENTAL PROCEDURES

Plant materials

The downregulation of *CAD* was obtained by transforming poplars with antisense and sense constructs (Baucher *et al.*, 1996). Briefly, the full-length cDNA of poplar *CAD1* (the only *CAD* paralog highly expressed in poplar xylem, according to Shi *et al.*, 2010) was inserted in both sense and antisense orientation into the pGSJ780A vector, containing the cauliflower mosaic virus 35S promoter and the 3' T7 polymerase terminator. The resulting expression plasmids p35SSCAD (sense) and p35SASCAD (antisense) were used for the genetic transformation of poplar (*Populus tremula* × *Populus alba*) using *Agrobacterium* (Leplé *et al.*, 1992). Total *CAD* activity was reduced by approximately 70% in both transgenic lines used in this study, which correspond to the lines ASCAD21 and SCAD1 for antisense and sense, respectively, of the original study of Baucher *et al.* (1996). The three investigated genotypes were coded as WT (wild type), AS-*CAD* (ASCAD21) and S-*CAD* (SCAD1). *CAD* downregulation resulted in red xylem tissue (Figure S4; Baucher *et al.*, 1996). These plants were micropropagated *in vitro*, after which they were transferred to soil. The plants were then grown in the glasshouse for 3 months. Before harvesting, the total length of the stems was measured. The average length was 130 cm, with no significant differences between the WT and the transgenic lines. From a 10-cm stem fragment collected at the base of the plant, suitable 30-mm-long sections were chosen, debarked and taken for further analysis. Stem sections of five individuals (biological replicates) from each genotype (Table 1) were chosen. As tension wood is frequently present in the stems of young poplar trees, the stem sections were visually checked for the presence of G-layers, characteristic for tension wood, on both ends by light microscopy. Only regions without obvious presence of G-layers were taken for the investigations.

First, using a rotary microtome the developing xylem next to the cambium was removed to a depth of approximately 200 μm . Then, 10 consecutive longitudinal-tangential sections (LT sections) of 100- μm thickness were cut from the stem, which were taken as technical replicates for all analysis steps. Then the stem was turned to a new position for cutting the second series of up to 10 sections. The total number of technical replicates successfully tested and analysed for each biological replicate is given in Table 1. The precise xylem region from where the LT sections were taken is shown in a cross-sectional image of the stem in Figure S5. All sectioning was performed in wet conditions and the sections were kept wet until mechanical testing was complete.

For statistical analysis, the mean values of the technical replicates were taken to calculate the mean of the biological replicates ($n = 5$ for AS-CAD, S-CAD and WT). The mean values of the biological replicates were then compared using one-way ANOVA (Tukey's test range) at 95% ($P = 0.05$) confidence level.

Microtensile tests

From each LT microtome section cut from the poplar stems, a 1.5-mm-wide strip was cut using a scalpel to obtain tissue strips for micromechanical testing of the following dimensions: $30 \times 1.5 \times 0.1$ mm ($L \times T \times R$). The strips, called samples hereinafter, were kept in water until mechanical testing. For mechanical testing, a microtensile testing stage was used based on the set-up previously described (Burgert *et al.*, 2003). The number of successful tests for each biological replicate within a given genotype is shown in Table 1. Strain measurement was performed with video extensimetry using a stereomicroscope and a CCD camera. The test length was set to 15 mm and the samples were strained with $10\text{--}15 \mu\text{m s}^{-1}$ (a strain rate of $0.07\text{--}0.11\%$ s^{-1}) until failure. The force was recorded with a 50-N load cell (Sensotec Sensors; Honeywell, <https://www.honeywell.com>). The elastic modulus, ultimate stress and yield stress were calculated.

Density calculation

The density of the mechanically tested samples was calculated based on volume in the fresh state and oven dry mass (Rowell, 2012).

Microfibril angle measurement

For elucidating the cellulose MFA in the cell walls, wide-angle X-ray diffraction (WAXD) measurements were performed on the mechanically tested samples. Fifteen samples from three biological replicates were taken from each genotype (Table 1) and analysed using a Nanostar (Bruker AXS, <https://www.bruker.com>). Measurements were performed under vacuum conditions at a wavelength of 1.54 Å (CuK α radiation). The X-ray beam diameter was approximately 300 μm and the sample-detector distance was set to 8.5 cm. For each sample, one diffraction image was collected with an exposure time of 35 min.

For cellulose orientation analysis, azimuthal intensity profiles of the (200)-Bragg peak of cellulose were generated from the diffraction images by radial integration, according to the method described by Rüggeberg *et al.* (2013). The azimuthal profiles were then used for calculating the microfibril orientation distribution for each measuring spot.

Fourier transform infrared spectroscopy spectroscopy

The FTIR spectra were obtained for all mechanically tested samples (Table 1) with a spectral resolution of 4 cm^{-1} on a TENSOR 27 spectrometer (Bruker) in the range of $4000\text{--}350 \text{ cm}^{-1}$

using an attenuated total reflection (ATR) unit (Platinum; Bruker). Three spectra were recorded from each sample and averaged. The spectra were baseline corrected and normalized, which results in an absorption value of 2 for the highest peak. As carbohydrate content and composition are not expected to be affected (or far less affected) compared with lignin content and composition, bands are normalized on the 1032 cm^{-1} band. As ratios of the aromatic lignin band at 1505 cm^{-1} with different carbohydrate bands (1157 , 1048 and 898 cm^{-1}) have been shown to be highly correlated with lignin content (Grandmaison *et al.*, 1987; Rodrigues *et al.*, 1998; Schwanninger *et al.*, 2004; Zhou *et al.*, 2011), we used the 1505 cm^{-1} band for the semi-quantitative determination of lignin content.

Raman spectroscopy

Raman analysis was performed on 25- μm -thick cross sections of three biological replicates for each of the three genotypes (WT, AS-CAD and S-CAD). The samples were placed with a drop of water on a glass slide and sealed with a coverslip and nail polish. Raman spectra of the sections were acquired using a confocal Raman microscope (InVia; Renishaw, <https://www.renishaw.com>) equipped with a linearly polarized green laser ($\lambda = 532 \text{ nm}$). For high spatial resolution, a $100\times$ oil immersion lens was used. For Raman mapping, the step size was set to $0.3 \mu\text{m}$ and the integration time was set to 0.4 s per spectrum. A grating of $600 \text{ lines mm}^{-1}$ and laser power of approximately 19.6 mW was used. After data acquisition, the cosmic ray background was removed using WIRE 3.7 (Renishaw). The spectra were then exported to CYTOSPEC 2.00.01 for further analysis (CytoSpec, <http://www.cytospec.com>). A more detailed ROI study was performed separately for the CC and the CWL. Three ROIs were chosen on each map for both CC and CWL, and average spectra were calculated from each ROI. These average spectra were baseline corrected and normalized at 1598 cm^{-1} (symmetric stretching of the aromatic ring in lignin).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Stress-strain curve showing the definitions of the mechanical properties.

Figure S2. Calculating the influence of samples with tension wood on averaged mechanical properties.

Figure S3. Variability of FTIR spectra for technical and biological replicates.

Figure S4. Representative debarked stems of each genotype: WT, AS-CAD and S-CAD.

Figure S5. Origin of samples within a stem section.

Table S1. Comparison of micromechanical test results between the genotypes WT, AS-CAD and S-CAD.

Table S2. Comparison of micromechanical test results for the five biological replicates within the three genotypes: WT, AS-CAD and S-CAD.

Table S3. Detected Raman bands and their assignments to functional groups.

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