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Research article

Unraveling the impact of *Pto4CL1* regulation on the cell wall components and wood properties of perennial transgenic *Populus tomentosa*

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ABSTRACT

Cell wall components and structure impact the physical and mechanical properties of plants, thereby affecting wood applications. Lignin is the most abundant biopolymer after cellulose in the wood cell wall and can be modified by certain lignin biosynthesis enzymes. 4-Coumarate: coenzyme A ligase(4CL) is an important lignin biosynthesis enzyme. To demonstrate the impact of the regulation of *Pto4CL1* from poplar on wood properties, we analyzed the composition and anatomy of 5-year-old *Pto4CL1*-modified poplar cell walls, assessing the density, strength, volume shrinkage, and impact toughness of the transgenic trees. These results showed that the up-regulation of *Pto4CL1* increased the lignin content to 46.65% from 33.11% in the control plants, while hydrophilic polysaccharides such as cellulose, hemi-cellulose, and pectin decreased. In contrast, the down-regulation of *Pto4CL1* resulted in a reduction in lignin content to 27.39%, and the content of cellulose and hemicellulose showed compensatory variation. Raman spectroscopy showed that the change in lignin in the transgenic events was embodied in the deposition and concentration of lignin in the secondary cell wall. Moreover, the increased lignin content caused significantly increased wood strength and slightly increased wood density. In contrast, a reduction in lignin content resulted in a significant decrease in wood strength and a slight decrease in wood density. However, the *Pto4CL1*-modified trees had similar stiffness to the control group. We also found a significant decrease in volume shrinkage and increase in impact toughness in the low-lignin events. These results indicate that *Pto4CL1* regulation alters the chemical composition of plant cell walls and these changes affect the physical and mechanical properties of the wood.

1. Introduction

Lignin is one of the main components in plant cell walls and not only provides mechanical support for plants but also plays an important role in the defense against biotic and abiotic stresses. However, in the papermaking process, it is necessary that lignin is removed from the cancelled cell wall and the cellulose is retained; a process that consumes large volumes of chemical reagents and discharges sewage in to the environment. Therefore, researchers have applied gene engineering technology to the enhancement of wood properties in an attempt to improve the utilization of biomass energy and reduce environmental damage through the regulation of lignin. There are two strategies for regulating plant lignin. One is to reduce the lignin content in the plant cell wall; the other is to change the proportion of lignin monomer composition (Chiang, 2006). In previous studies, researchers found that the plant phenotype and/or cell morphology was significantly altered in genetically modified plants. In transgenic poplars, inhibition of the expression of the *CCoAOMT* gene resulted in a significant reduction in the total lignin content, causing the xylem to appear slightly orange. Microscopic observation indicated the malformation of the xylem vessels in the transgenic plants (Morimura et al., 2000). The same phenomenon was observed in birch with down-regulated *CCR* expression (Zhang et al., 2015). Shafrin (Shafrin et al., 2017) found that the total lignin decreased by 13% and 23%, respectively, in jute with down-regulated *C4H* and *COMT* expression, but there was no morphological difference between the transgenic plants and the control group. Microscopic observation showed that lignin was reduced in the epidermal cells of the transgenic plants compared with the control plants.

As perennials, trees are subjected to various environmental pressures during their lifecycles, such as wind, frost, and snow. The effects

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of the variations in cell wall components on structural stability and the mechanical and physical properties of the tree are largely unknown. Researchers have attempted to clarify the effects of the microscopic components of plant cell walls on the macroscopic physical and mechanical properties of trees. It was found that when the side of the tree is subjected to external force, the stem of the tree will simultaneously exhibit pressure on one side and tension on the opposite side. In this process, cellulose mainly exhibits resistance to tension, and lignin mainly exhibits resistance to compression (Niklas, 1992; Gindl and Teischinger, 2002; Horvath et al., 2010a). In previous studies, it was determined that reducing the content of lignin in the cell wall had an inconsistent effect on the physical and mechanical properties of the wood. Gindl and Teischinger (2002) found that when the content of lignin in the cell wall of spruce increased to 190%, the pressure resistance only increased by 10%. However, Horvathet (Horvath et al., 2010b) found that the down-regulation of *4CL* in poplar reduced the Klason lignin concentration by 30%, while the wood density did not change and the modulus of elasticity was reduced by 40%. In poplars with down-regulated *CAD* expression, Özparpucu (Ozparpucu et al., 2017) found that the Fourier transform infrared absorption of lignin decreased by 12% and the tensile strength of the wood decreased by 15%, which maybe due to macroscopic increases or decreases in lignin, with the amount of change required varying (Horvath et al., 2010b; Kohler and Spatz, 2002). While altering the lignin content can change the wood properties, changing the composition and structure of the lignin can also affect the physical and mechanical properties of the wood. Hepworth (Kohler and Spatz, 2002) found that down-regulated *CAD* expression in tobacco exhibited the same Klason lignin content as the wild-type (WT) tobacco, but a decreased S/G ratio caused the modulus of elasticity to decrease in the transgenic tobacco. On the contrary, in *F5H* up-regulated poplar, a significant increase in S/G led to the compressive strength of the wood decreasing significantly (Horvath et al., 2010b).

However, most of the materials selected in these studies were tissue culture plants, green house plants, and some immature trees. Few studies on trees grown for a few years in the field are available. Moreover, the physical and mechanical measurement methods, such as nanoindentation and the mechanical measurement of single wood fiber, which are applied to mature wood, are not suitable for saplings and juvenile woods. Therefore, these microscopic detection methods and data on young and tender materials can not truly reflect the properties of mature trees. Therefore, 5-year-old genetically modified *Populus tomentosa* grown in the field were selected as materials in the present study in an attempt to elucidate the influence of genetic changes on the physical and mechanical properties of the wood, as well as to provide a molecular biological basis for future wood quality improvement.

2. Results

2.1. Effect of altered Pto4CL1 gene expression on the cell wall chemical composition of transgenic P.tomentosa

The cell wall composition was analyzed in transgenic poplars based on the amount of cell wall residues (CWR) in the stem xylem. In the control line (WT), the lignin content was 33.11%. In the *Pto4CL1* up-regulated line (sense line), the lignin content increased to 46.65%. In contrast, the lignin content in the *Pto4CL1* down-regulated line (anti-sense) was decreased to 24.04% and a concomitant increase in cellulose content was observed. It is generally believed that the cellulose content of paper making material should be more than 40% in order to keep pulping costs low. Table 1 indicates that cellulose content of > 40% in the down-regulated transformants, suggesting that the *Pto4CL1* down-regulated lines could be used as pulp material.

Based on the altered lignin and cellulose contents, we analyzed the cell wall polysaccharide content of the transgenic poplars. Table 1 shows that the pectin content in the transgenic lines differed from that in the WT plants. The total pectin II content in the sense transformant was significantly reduced compared to the WT plants. However, the content of the two types of pectin in the antisense transformant was similar to the WT. The pectin I component in the experiment was low-methylated pectin, and pectin II was a high-methylated group that maintain cell shape. In all lines examined in this study, the hemi-cellulose content of the cell wall was approximately 20%. The hemi-cellulose content decreased slightly in the sense-transformant but was higher in all down-regulated lines relative to the WT plants.

2.2. Effect of altered Pto4CL1 gene expression on the cell wall phenolic acid content of transgenic P.tomentosa

As 4CL is responsible for the third step of the phenylpropanoid metabolic pathway and is also the entry enzyme for the monolignol-specific biosynthesis pathway and the flavonoid metabolic pathway, the regulation of the *Pto4CL1* results in variation in the content of substances in the different pathways. These variations in the substance contents may cause positive and negative feedback regulation of different enzymes, ultimately affecting the accumulation of phenolic acids in the cell wall. Analysis of soluble phenolic acids in the cell walls of transgenic poplars in Fig. 1 indicated that there was a significant increase in phenolic acid content, except for cinnamic acid. This may be caused by the inhibition of 4CL enzyme activity by coumaric acid and caffeoyl shikimic acid, and the two intermediates specifically inhibited the formation of 4-coumaricacid-CoA and feruloyl-CoA, resulting in extra 4-coumaric acid and ferulic acid accumulation in the cell wall. In *Pto4CL1* down-regulated transgenic poplar, the accumulation of phenolic acids in the cell wall was due to the decreased expression of the *Pto4CL1* gene. However, in *Pto4CL1* up-regulated plants, specific phenolic acids (caffeicacid, p-coumaric acid, and ferulicacid) accumulated as a result of the specific inhibition of the intermediates.

2.3. Effect of altered Pto4CL1 gene expression on cell wall monolignol content in transgenic P.tomentosa

To determine the composition of monolignol in transgenic poplars, we examined the CWRs from each plant. Lignin is decomposed by thioacidolysis, and the G/S/H composition was analyzed by gas chromatography-mass spectrometry. A decrease in G units and a significant increase in S units were observed in all transgenic poplars, which resulted in increased S/G ratios compared with the control (Table 2). Conversely, the H units were significantly decreased in all transgenic poplars compared with the control.

2.4. Effect of altered Pto4CL1 gene expression on cell wall architectural structure in transgenic P.tomentosa

To determine the effect of modified *Pto4CL1* on the anatomy of *P.tomentosa* micro structures of stem sections were observed under an optical micro scope (Olympus Corp.,Tokyo, Japan) (Fig. 2). According to the anatomical structure, Image-Pro Plus software was used to analyze two characteristics: cell wall thickness and vessel cavity perimeter. In total, 20 sections per line were used as inputs. The results are shown in Table 3. The cell wall thickness and the xylem vessel lumencircum ference of all transgenic trees varied in comparison to the control line. The cell wall thickness in the sense line was thicker than that of the WT, and the vessel cavity perimeter was smaller. The cell wall thickness and the xylem vessel lumencircum ference in the anti-sense *Pto4CL1* gene poplars were significantly reduced by 17.2% and 33.26%, respectively. The reduction in cell wall thickness of transgenic *P.tomentosa* has great application value in the paper industry. Due to the decrease in cell wall thickness of the xylem, the amount of pulping alkali and the time of alkali penetration in to the cell wall were reduced. Therefore, in this experiment, genetic altering of the chemical composition and structure of the wood cell wall resulted in an excellent material for the pulping

Table 1
Composition analysis of cell wall from 5-year old *Pto4CL1* transgenic poplar.

Popolar line	Pectin I Mass%	Pectin II Mass%	Hemicellulose Mass%	Cellulose Mass%*	Lignin Mass%*
WT	1.29 ± 0.52	5.82 ± 0.73	17.79 ± 1.72	42.00 ± 0.62	33.11 ± 1.84
Sense	1.28 ± 0.92	2.37 ± 0.62	16.60 ± 2.63	33.10 ± 0.97	46.65 ± 2.94
Anti-sense	1.32 ± 0.60	5.65 ± 0.58	18.34 ± 2.30	50.65 ± 4.85	24.04 ± 1.10

Significantly different values are represented by asterisk (*) and determined by ANOVA followed by Dunnett's post-hoc test. The mean values are significantly different at $P < 0.05$. Error bars indicated ± SE.

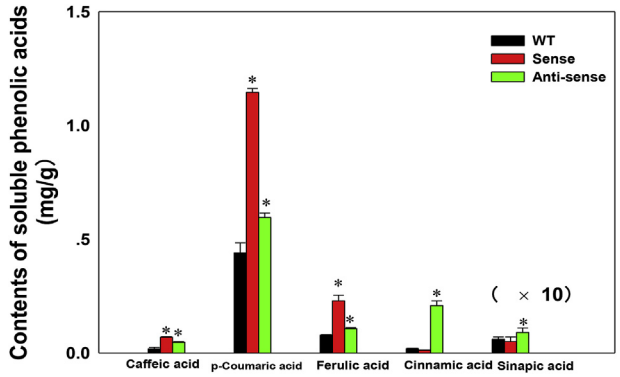

Fig. 1. Soluble phenolic acids contents of cell wall from 5-year old *Pto4CL1* transgenic poplars. The concentration of Caffeic acid, p-coumric acid, Ferulic acid and Cinnamic acid is expressed in mg/g. The concentration of Sinapic acid is expressed in μg/g. Significantly different values are represented by asterisk (*), the mean values are significantly different at $P < 0.05$.

Table 2
Syringyl, guaiacyl, and p-hydroxyphenyl monomer composition of control and transgenic poplar, as determined by thioacidolysis.

Poplar line	monomer composition %			S/G
	H	G	S	
WT	0.63 ± 0.59	33.64 ± 4.78	65.77 ± 3.24	2.01 ± 0.11
Sense	0.28 ± 0.01	30.51 ± 0.36	69.21 ± 0.36*	2.27 ± 0.04
Anti-sense	0.41 ± 0.05	32.10 ± 0.65	67.48 ± 0.69*	2.10 ± 0.06

Significantly different values are represented by asterisk (*) and determined by ANOVA followed by Dunnett's post-hoc test. The mean values are significantly different at $P < 0.05$. Error bars indicated ± SE.

Table 3
The cell wall thickness and vessel cavity perimeter in mature xylem of stem of poplars.

	WT	Sense	Anti-sense
Cell wall thickness	2.50 ± 0.32	2.82 ± 0.35	2.07 ± 0.25
vessel cavity perimeter	199.93 ± 22.27	181.34 ± 11.52	133.44 ± 16.80

Significantly different values are represented by asterisk (*) and determined by ANOVA followed by Dunnett's post-hoc test. The mean values are significantly different at $P < 0.05$. Error bars indicated ± SE.

industry, not only by improving the pulp yield and pulping efficiency but also by reducing the environmental pollution caused by the chemical reagents.

Vessels are an important component of the xylem, serving as a transport corridor for water and inorganic salts. The vessel morphology of transgenic poplars was hypogenetic in the down-regulated lines (Fig. 2). The xylem of the *Pto4CL1* down-regulated plants showed slightly collapsed vessels in contrast to the angular-shaped vessels of the WT plants, and radial multiples of cells in contrast to mostly solitary cells were found in the WT and *Pto4CL1* up-regulated plants. A considerable increase in vessel cavity perimeter in the xylem was observed in the *Pto4CL1* down-regulated lines compared with the WT and up-regulated lines. Interestingly, a smaller vessel with a decreased cavity perimeter was noted in the anti-senseline (Table 3). Auto fluorescence microscopy of transgenic plant cross-sections revealed the absence of tylose in the WT and the *Pto4CL1* up-regulated line. However, tylose was detected in the vessels of the *Pto4CL1* down-regulated plants, with more tylose observed in the lines with collapsed vessels.

For more detailed spatial information of the lignin deposition in the cell wall, cross sections of all poplar lines were observed using Raman spectroscopy scanning. Mapping and separation can reflect the chemical variations in different transgenic poplar lines. Mapping was

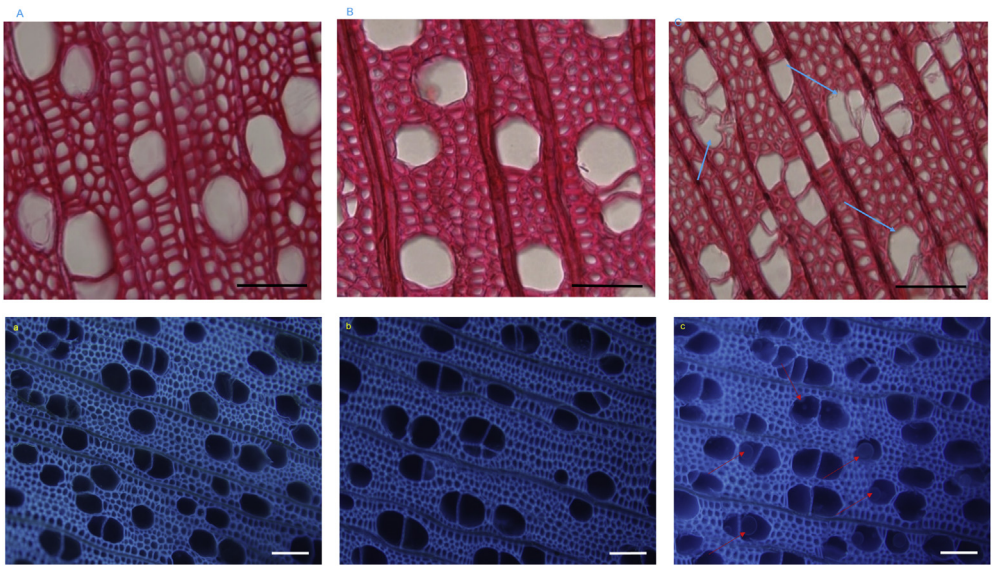

Fig. 2. The transverse anatomy structure of stem with scanning electron microscope from transgenic poplar. (blue arrows point to abnormal vessels, red arrows point to inward thickening in the vessel A, a: WT, B, b:Sense, C, c:Anti-sense) capital letters indicate phloroglucinol staining, minuscule letters indicate autofluorescence. Bar is 100um. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

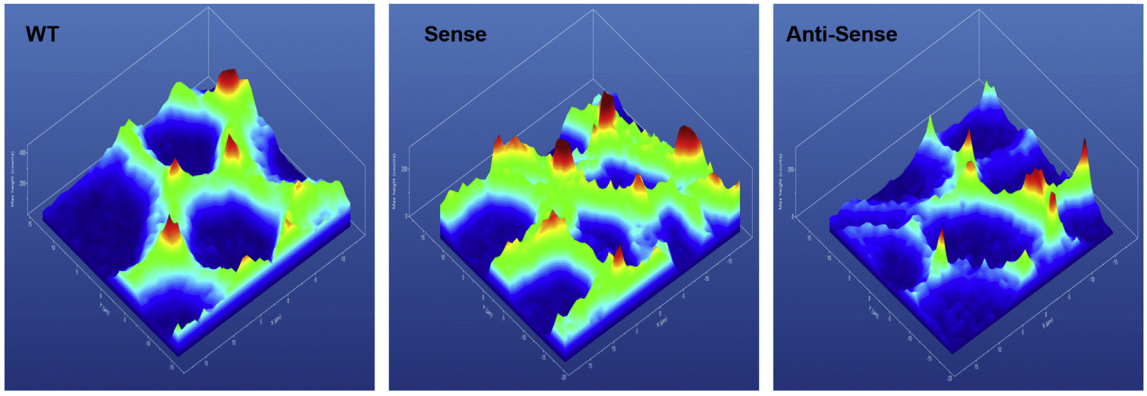

Fig. 3. The 3D anatomy structure of stem transverse section with Raman spectral from transgenic poplar.

carried out by integrating the main lignin bands between 1650 and 1550 cm^{-1}. The 3D maps are shown in Fig. 3, and the images indicated that the lignin was mainly deposited in the secondary cell wall and cell corner, and the lignin concentration was high in the cell corner. It can be seen in Fig. 3 that the changes in lignin were mainly concentrated in the secondary cell wall and were caused by the regulation of *Pto4CL1* gene expression. In the sense *Pto4CL1* transgenic poplar, the amount of lignin deposition in the secondary cell wall increased. In contrast, the amount of lignin deposited in the secondary cell wall was significantly reduced in the anti-sense *Pto4CL1* transgenic lines, but the amount of deposition in the cell corners did not change much.

Analysis was performed on the secondary wall and cell corners of the xylem fibers separately. The signals of cellulose at 1093,1117,1150, and 1374 cm^{-1} were much more intense in the cell walls than cell corners (Fig. 4), which corroborates a previous study (Hongo et al., 2012). In the transgenic line of the sense *Pto4CL1*, the cellulose signal in the cell corner was lower than that of the control and anti-sense lines, which may be due to the decrease in the total amount of cellulose, resulting in a lower cellulose distribution in the cell corner. The signal of hemi-cellulose at 1037 cm^{-1} showed that, in the control and down-regulated *Pto4CL1* plants, the concentration of hemi-cellulose in the cell corner was higher than that of the secondary cell wall. In the transgenic plants with sense *Pto4CL1*, in contrast, the signal intensity of hemi-cellulose was higher than that of the cell corner. According to the analysis of the chemical composition of the cell wall, although the content of hemi-cellulose was only slightly reduced, it varied greatly in distribution. The lignin-specific signals at 1597 and 1659 cm^{-1} revealed that the concentration of lignin in the cell corners of all the plants was much higher than that in the secondary cell walls. In the transgenic plants with sense *Pto4CL1*, the Raman signal intensity of lignin was slightly higher than that of the control, and, unexpectedly, the signal intensity of lignin in the anti-sense *Pto4CL1* lines was higher

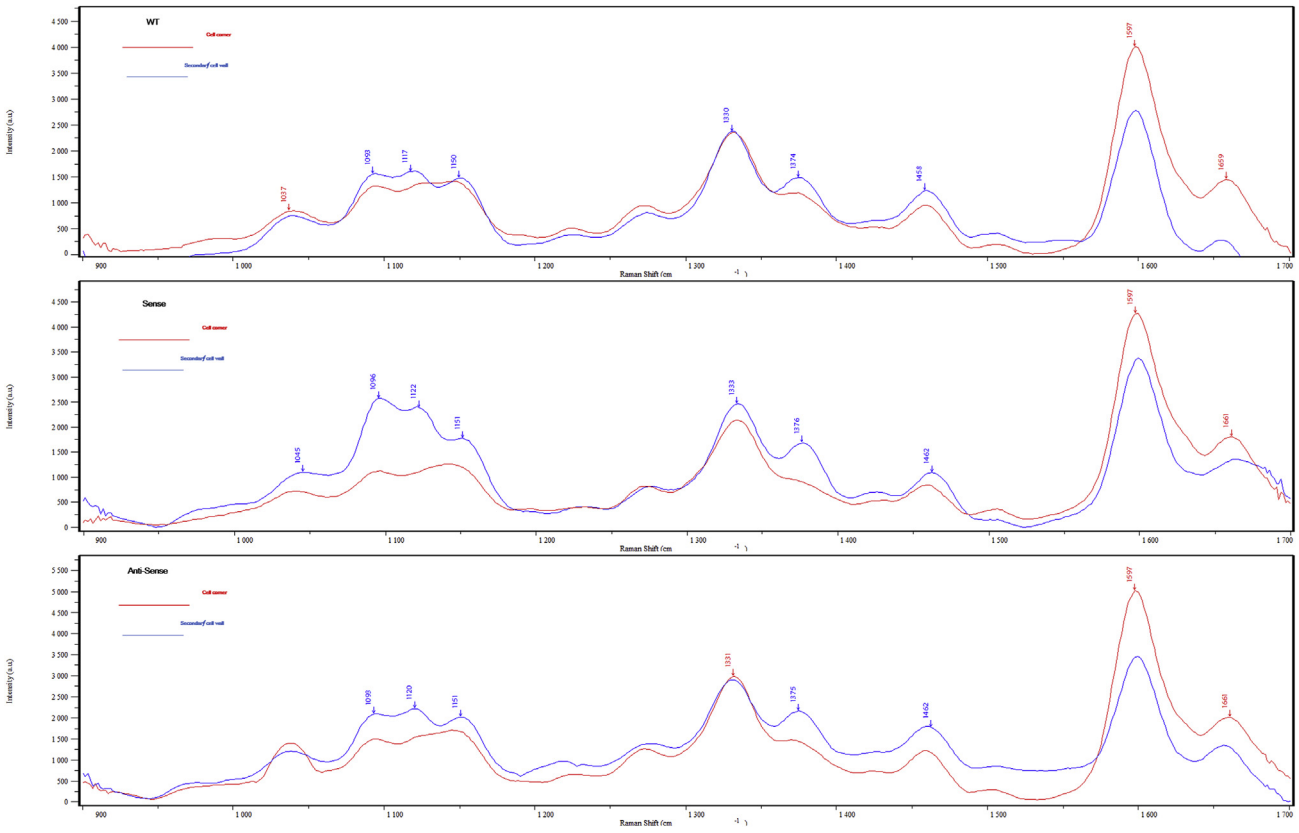

Fig. 4. The transverse anatomy structure of stem with Raman spectral from transgenic poplar.

Table 4
The analysis of physical properties from transgenic poplar.

Poplar line	Impact toughness KJ/m^{2*}	Volume Shrinkage %	MOR Mpa*	MOE Gpa	Density
WT	48.76 ± 1.44	9.04 ± 0.07	71.50 ± 4.55	8.19 ± 0.69	0.34 ± 0.03
Sense	46.02 ± 1.09	9.57 ± 0.05	76.80 ± 11.86	8.32 ± 0.25	0.36 ± 0.08
Anti-sense	49.15 ± 2.43	7.53 ± 0.02	52.73 ± 3.20	8.57 ± 0.41	0.31 ± 0.04

Significantly different values are represented by asterisk (*) and determined by ANOVA followed by Dunnett's post-hoc test. The mean values are significantly different at $P < 0.05$. Error bars indicated ± SE.

Table 5
The coefficient matrix of chemical composition and wood property parameters in transgenic poplar.

	Pectin	Hemi-cellulose	Cellulose	Lignin	Shrinkage	Density	Impact toughness	MOR	MOE
Pectin	1								
Hemi-cellulose	**.727**	1							
Cellulose	**.729**	.581	1						
Lignin	**-.866**	**-.745**	**-.964**	1					
Shrinkage	-.096	-.093	-.407	.320	1				
Density	.092	**.633**	-.248	**.698**	.181	1			
Impact toughness	**.611**	.397	.220	-.365	.070	.183	1		
MOR	-.508	**-.822**	**-.666**	**.721**	.106	-.378	.135	1	
MOE	.065	-.107	.214	-.145	.654	-.387	-.224	-.217	1

Bold indicates significant difference at 5% level, $P < 0.05$.

than that of the sense *Pto4CL1* group. Combined with the previous analysis of lignin content and mapping results, this may explain why the deposition of lignin in the secondary cell wall varied significantly despite the lignin contentbeing reduced much.

2.5. Effects of cell wall composition changes on the physical and mechanical properties of transgenic P.tomentosa

To determine whether the up-or down-regulation of *Pto4CL1* affects wood properties, the physical and mechanical properties of the wood were examined. Descriptive statistics for shrinkage, density, impact toughness, bending strength, modulus of elasticity, and modulus of rupture of the transgenic poplars are provided in Table 4.

Volumetric shrinkage varied from 7.53% to 9.57% in the transgenic events. In the *Pto4CL1* up-regulated line, the volumetric shrinkage increased. In contrast, the volumetric shrinkage of the *Pto4CL1* down-regulated line significantly decreased. The impact toughness of the trees with up-regulated expression of the *Pto4CL1* gene decreased by 5.62%; the impact toughness of the antisense *Pto4CL1* transgenic plants increased slightly (0.79%); while the impact toughness of the lines transfected with antisense *Pto4CL1* was unchanged. The results indicated that brittleness increased in the transgenic poplars with up-regulated *Pto4CL1* expression. This may be due to the decrease in the amount of pectin present in the compound middle lamella and the cell corner. High methyl ester pectin can absorb more impact force and increase the toughness of the plant. This result is consistent with the results of Shoko Hongo in the mutant *Arabidopsis thaliana* inhibiting PME35, where by the degree of pectin methylation was higher than that of the control, and the resistance to deformation was increased (Hongo et al., 2012).

As observed in Table 4, in the *Pto4CL1* up-regulated poplars with greater lignin content, the modulus of rupture was significantly increased. Conversely, the modulus of rupture decreased to 52.73 and 62.27 MPa, in the *Pto4CL1* down-regulated trees. Correlation analysis also showed a strong positive correlation between lignin and modulus of rupture and a strong negative correlation with carbohydrate content in the cell wall. The result is also consistent with the theory that lignin acts as a mechanical support for wood cell walls. The modulus of elasticity of all transgenic lines showed no significant change compared with the control line, indicating that the stiffness of the transgenic lines did not change by altering the chemical components in the cell wall. In terms of changing the physical properties of the poplar, the volumetric shrinkage of the transgenic sense line was increased, and conversely, the volumetric shrinkage of the anti-sense line was reduced to 7.53%. The basic density of all transgenic lines did not differ significantly from the control. This indicated that the regulation of poplar cell wall components affects the dimensional stability of the wood but does not influence the biomass accumulation of the wood.

The chemical composition of the wood cell wall is an important factor in the wood quality and wood processing determines all the physical and mechanical properties of the wood. For transgenic poplars, the genetic improvement of wood chemical properties is mainly concentrated on the content and composition of cellulose and lignin, but pectin and hemi-cellulose also play an important role in the composition and structural stability of plant cell walls. The correlation between cell wall chemical composition and wood quality of transgenic *P.tomentosa* was analyzed. The results are shown in Table 5. It can be seen that wood density was significantly positively correlated with the content of lignin and hemi-cellulose, while the MOR of the wood was negatively correlated with the content of cellulose and hemi-cellulose. The correlation coefficients were −0.666 and −0.822, and the correlation coefficient with lignin content was 0.721.

3. Discussion

Altering the expression of 4CL, which is an enzyme located at the intersection of the phenylpropionic acid pathway and the lignin-specific biosynthetic pathway, causes changes in the deposition and content of lignin in the plant cell wall (Wei et al., 2015; Voelker et al., 2011). The content and location of lignin crucially influence the mechanical and physical properties of the wood cell wall, and the variations in microscopic cell walls will be reflected in the macroscopic physical and mechanical properties of the wood (Donaldson, 2001; Chen and Sarkanen, 2010).

To reveal the effects of individual lignin biosynthesis gene regulation on cell wall components and wood anatomy, 5-year-old field-grown poplars harboring the sense and antisense *Pto4CL1* gene were selected as experimental materials. In the down-regulated *Pto4CL1* line, Raman observation showed that the variation in lignin deposition, which was caused by a change in the cell wall microstructure, was

mainly embodied in the secondary wall of the wood fiber cells, rather than in the cell corner. This result indicated that the lignin in the compound middle lamella of the cell wall is more than three times denser than that in the secondary wall. Similar to reports in lignin-deficient plants with the antisense 4CL gene (Kitin et al., 2010; Gerdenitsch, 1984; Hosam et al., 2012; Beauzamy et al., 2015; Hura et al., 2015), we confirmed that the cell wall thickness of lignin-deficient poplar fiber cells and vessel cells decreased to varying degrees, and the vessels collapsed slightly. With the variation in lignin, the carbon flow showed compensatory changes in cell wall component contents in genetically modified trees, but the haul of carbon flow is not alternative from lignin to polysaccharides (Bjurhager et al., 2010; Voelker et al., 2010; Van et al., 2013). A case in point is that the flavonoid content increased in lignin-modified alfalfa (Van et al., 2013). In addition, the accumulation of corresponding aldehydes was observed in *CCR*-and *CAD*-inhibited maize and tobacco and these extra aldehydes discolored the xylem (Prashant et al., 2011; Tamasloukht et al., 2011; Provan et al., 2015). It is indicated that regulating individual enzymes in the lignin biosynthesis pathway affects not only the lignin content but also the lignin composition. In this study, extra phenolic acids accumulated in the cell wall of the transgenic poplars (Fig. 2). Lin (Lin et al., 2015) indicated that 4-coumaroyl shikimic acid could inhibit the catalysis of 4-coumaric acid and caffeicacid to 4-coumaroyl-CoA and caffeoyl-CoA by 4CL. However, the modified expression of *Pto4CL1* did not affect C4H expression. This might explain the accumulation of p-coumaric acid and caffeic acid in the cell walls of *Pto4CL1* up-regulated poplar. In the *Pto4CL1* down-regulated trees, the 4CL enzyme was deficient and the consumption of 4-coumaric acid was reduced. This suggests that 4-coumaric acid inhibited the catalysis of cinnamicacidto4-coumaricacid by C4H, resulting in the accumulation of cinnamic acid in the cell wall. However, this phenomenon was observed in the *Pto4CL1* up-regulated poplar. For the accumulation of sinapic acid, the reduced *Pto4CL1* might have resulted in an increase in the expression of *PtoF5H*, and thus more phenolic acid was catalyzed to sinapic acid, and sinapic acid cannot be consumed by the 4CL enzyme. Kitin (Kitin et al., 2010) demonstrated that extra non-lignin phenolic acids in transgenic poplars were deposited in the cell lumen, and the deposited phenolic acid could impede water transport. Voelker (Voelker et al., 2011) revealed that the brown wood in *4CL1* down-regulated poplars exhibited irregular vessels that strongly impeded water transport, resulting in shoot dieback. These results also accord with our observations, which showed that the vessels were thickened inwards due to the down-regulation of *Pto4CL1*.

There is no consistent conclusion about the S/G ratio increases or decreases by the disturbed gene in longitudinal of lignin monomer biosynthesis. In different species, the regulation of different genes has different effects on the S/G ratio (Davison et al., 2006; Tolbert et al., 2016). The results of this study showed that both the up-regulation and down-regulation of the *Pto4CL1* gene ultimately led to an increase in S/G. However, *in vitro* experiments showed that the 4CL protein cannot catalyze sinapic acid to the corresponding coenzyme A ester, but the analysis of lignin biosynthesis gene expression showed that *PtoF5H* was significantly increased in1-year-old down-regulated *Pto4CL1* poplar, and the expression of *PtoCCR* and *PtoCAD* was simultaneously inhibited, which resulted in an increase in S monolignol.

All alterations in the cell wall have an impact on the microscopic morphology of the wood, but it is unclear whether these alterations influence the properties of the whole tree. Wood density is used for describing biomass accumulation in per unit volume of wood. This experiment did not detect any significant changes of the basic density in the transgenic trees, suggesting that the down-regulation of *Pto4CL1* gene expression did not influence the accumulation of wood biomass. This is in agreement with Özparpucu's findings that the lignin content was reduced in *CAD* down-regulated poplars, while wood density and cellulose microfibril angles were unaffected (Ozparpucu et al., 2017).

The wood will shrink when it loses moisture, therefore wood shrinkage is an important indicator of wood dimensional stability. This study has identified that the content of non-lignin phenolic acids in transgenic poplars was significantly higher than that of the control group, and thus it is possible that these additional phenolic acids for meda chemical bond with the cell wall and remained in the cell cavity. This was similar to Ermeydan's study, they soaked a block of locust in 3-hydroxy flavone and found that although the 3-hydroxy flavone could not be inserted into the cell wall, it could form a chemical bond with the component sin the cell wall and fill into the cell lumen, thereby increasing dimensional stability of the wood block (Ermeydan et al., 2012). It might be the non-lignin phenolic acids instead of the water bonding the polysaccharides in the cell wall, and thus when all the plants reached the same moisture content in the experiment, the low lignin trees lost less water, resulting in a decrease in volumetric shrinkage and an increase in dimensional stability. The research has also shown that when the lignin was reduced and the volume shrinkage of the wood was decreased after the cellulose content was increased, the free water content lost in the cell wall in these trees was lower than that of the control group.

In this experiment, the trees with up-regulated *Pto4CL1* and down-regulated *Pto4CL1* expression both showed a slight increase in the modulus of elasticity (MOE), but no statistically significant were found. However, Özparpucu (Ozparpucu et al., 2017) showed that the MOE of CAD transgenic poplars was reduced, possibly because the cell wall contained more aldehydes. Wang found that an increase in cinnamaldehyde content caused a decrease in MOE in Populus but with no change in total lignin content. A possible explanation for this might be the accumulation of aldehydes in the lignin biosynthesis pathway. Horvath (Horvath et al., 2010b) found that wood of different ages had a great influence on MOE. In his experimental samples, there were1-year-old, 2-year-old, and 2.5-year-old poplar trees with MOE values ranging from 1422 to 6283 MPa. As Horvath noted, the MOE of trees should be affected by the multiple effects of cell wall aldehydes, environment, damage, but these extra phenolic acids should not affect the MOE of the tree.

Plants with low lignin content exhibited reduced MOR (Horvath et al., 2010a; Ozparpucu et al., 2017; Bjurhager et al., 2010), which could be attributed to the more complex structure of lignin in comparison to cellulose (Heredia et al., 1993). As can be seen from Table 4, the modulus of rapture (MOR) and impact toughness of transgenic poplar reported significantly different from WT. When the wood was fractured by an external force, many covalent bonds and non-covalent bonds between the molecules were fractured. Since the cellulose chain has a rigid structure, which makes it difficult to twist, the cellulose chain can be broken mainly by breaking its glycosidic bond. On the other hand, the network structure formed by lignin, in addition to covalent bonds (e.g. C−C and C−O), is also designed for hydrogen bonding, π-π stacking, and so forth (Scalbert et al., 1985; Balakshin et al., 2011). Therefore, increased lignin content enhances the chemical linkage of the wood, while the macroscopic realization can withstand greater external forces, causing an increase in MOR. However, the impact toughness is a dynamic process relative to the static force.The internal structure of the wood, such as its polysaccharide component, plays a very important role in buffering or diluting powerful external forces (Sexton et al., 2012).

In conclusion, the present study revel that it is possible for researchers to use biotechnology to alter the content and structure of cell wall component by regulation of lignin biosynthesis gene (Thevenina et al., 2011; Anderson et al., 2015), and in our research the regulation of lignin can affect the properties of the wood without affecting the normal grown of trees. Moreover, the samples we selected for this experiment were 5-year-old poplars, which were closer to the raw materials used directly in production compared with tissue culture or green house trees. The results showed the conjunct effect of genetic modification and natural environmental factors on transgenic trees, it can provide a direct molecular basis for the improvement of production

materials.

4. Methods

In this experiment, wild-type, GRP1.8-sense *4CL1*, GRP1.8-antipartal *4CL1* 5-year-old 741 *Populus tomentosa* grown in the field were used as experimental materials to analysis the cell wall main components, morphological and mechanical properties.We have previously measured the growth of 1-year-old transgenic *Populus tomentosa*. In one growth season, all transgenic poplars grew better than WT, and relevant data have been published (Tian et al., 2013). In order to analyze whether the modification of the gene still affects the trees after a long period of accumulation, we selected the expanded trees of the 1-year-old trees that have been determined as the materials of this experiment.

4.1. Determination of transgenic poplar cell wall components

According to the method of Zhong (Zhong et al., 2005, 2010), cell wall residue (CWR) was prepared, then 100 mg of CWR powder was accurately weighed and removed into a 15 ml centrifuge tube, and three replicates of each sample were taken (Arne et al., 2007); 4 ml of trifluoroacetic acid (TFA 2 mol/L) was added and hydrolyzed at 120 °C for 2 h. After cooling to room temperature, 6000 r/min, centrifugation for 15 min, the precipitate was collected then washed3 times with distilled water and dried in an oven. The dried residues were treated with 2 ml of 72% sulfuric acid for 2 h at 120 °C. After cooling, the supernatant was collected for determination of cellulose content. The residue was washed with distilled water at 50 °C to remove the residual acids and dried to a constant weight at 100 °C, and the dried crucibles were weighed for calculated lignin content.

Add 10 mL pectinase reaction solution (sodium acetate 50 mM, acetic acid, pH 5.0, 2 μL pectinase) to each sample; mix the sample and solution with vortexed, place the tube on a shaker at 24 °C, 200 rpm 24 h; centrifugation, 3000 rpm, 15 min; collect the supernatant in a 50 mL polypropylene centrifuge tube; the pellet was resuspended with pectinase, vortexed again, digested for 24 h; centrifuged and washed with 2 mL ddH_2O for 3 times, with eddy current during washing The device was shaken up, centrifuged as above, and the supernatant was combined. This was the pectin I (acidic pectin) component; 10 mL of Na_2CO_3 solution (50 mM Na_2CO_3, EGTA 5 mM) was added to the precipitate, and after vortex precipitation, it was placed on the shaker. In the bed, 25 °C, 12 h. The supernatant was collected in a 50 mL centrifuge tube, and then added with 10 mL of Na2CO3 solution, vortexed and placed in a shaker, digested for 12 h. Centrifuged and washed with 3 mL of ddH2O for 3 times. The mixture was shaken with a vortexer, centrifuged, and the supernatant was combined, which was a component of pectin II (alkaline pectin).

4.2. Determination of phenolic acid content in transgenic 741 poplar cell wall

Chromatographically grade acetonitrile and formic acid were obtained from Fisher; cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, salicylic acid, syringic acid, o-coumaric acid (purity > 99%, Sigma Company) Chromatographically grade, the rest of the organic reagents were analytical grade. Ethyl acetate, sodium hydroxide and hydrochloric acid were analytical grade. The liquid chromatograph was obtained from Themo-Finnigan Scientific, Waltham, MA, USA. HPLC was performed using a C18 column (3.5 mm × 150 mm, 2.1 μm,Agilent), detector: ion trap mass spectrometer, Millipore ultrapure water meter (Millipore); BRANSON 3510 ultrasonic generator (BRANSON).

The phenolic acid extraction method was done as follows: 0.5 g of fresh tissue is weighed, and grind with liquid nitrogen, moved into a 50 mL polyethylene tube. Add 5 mL of 80% methanol (methanol extract contains 50 ng of internal standard o-coumaric acid) and a little antioxidant into the tube, sonicate for 30 min, and leaching the phenolic acid overnight at 4 °C. The next day, the mixture was centrifuged at low temperature, 10000 g, 20min, then the supernatant was collected, subsequently, the supernatant was extracted with 80% methanol for 1–2 h, centrifuged at low temperature, 10000 g, 20 min, the precipitate was removed. The two obtained supernatants were combined, a little activated carbon was added, and the mixture was placed at 4 °C for 30 min, and then the activated carbon was removed by low temperature centrifugation. The supernatant was distilled at 45 °C, the organic phase was removed, and the aqueous phase was placed in a 15 mL tipped centrifuge tube, 1.2 mL of 1 mol/L NaOH was added, sealed, placed in a 20 °C shaker, and digested at 100 r/min. h. After filtration, the filtrate was extracted three times with ethyl acetate. The ethyl acetate phase was evaporated, the aqueous phase was acidified with 1 mol/L HCl, and ethyl acetate was extracted three times. After the organic phase was combined, the organic phase was evaporated under reduced pressure. Finally, it was reconstituted with 100 μl of 10% methanol, passed through a 0.22 μm micropore filter, and subjected to HPLC-MS.

4.3. Determination of monolignols content in transgenic 741 poplar cell wall

2 mg of CWR powder was accurately weighed and putted into an ampoule, then 200 μL of freshly prepared reaction solution was added into the same ampoule (final concentration of 2.5% BF3 and 10% EtSH in dioxane), 0.2 mL of internal standard (tetradecane) at a concentration of 1 mg/mL was added for quantification and place the ampoule in a constant temperature oven at 100 °C. The reaction was carried out for 4 h, and the ampoule was shaken every 1 h. After the reaction was completed, the reaction mixture was putted at −20 °C for 5 min to cool down. Then add 1 mL of ultrapure water to stop the reaction. Adjust the pH to 3–4 with 0.4 mol/L NaHCO3, extract with 500 μL of ethyl acetate. The organic phase was removed to a new tube, and the organic phase was evaporated in a constant temperature oven at 45 °C. The obtained dry product was redissolved in 0.4 mL of ethyl acetate, 50 μL of pyridine and 100 μL of BSA were added into the same tube, the mixture was placed at 50 °C for 1 h. The 2 μL reaction solution was analyzed by GC/MS (Thermo Finnigan) (column: DB 5MS, 30 m × 0.25 mm, 0.25 μm Agilent USA). Each sample was repeated 3 times and averaged.

4.4. Microstructure observation of transgenic 741 Populus tomentosa

The section near the ground of air-dried wood is sawn, then the sections of the xylem were processed into 2 × 2 × 2 cm small blocks; the treated small blocks were placed in clean water and pumped to soften for 2 weeks. The softened wood was sliced with a microtome, the thickness of the slices were adjusted to 25 μm; The slices were sequentially dehydrated by using 25%-50%-75%-80%-95%–100% series concentration of ethanol solution, dehydrated in each concentration gradient ethanol solution for 5–10 min; the dehydrated wood slices were dyed in the ethanol solution of Safranin, the dyeing time is 5–10 min; the dyed material is placed in absolute ethanol for 20–30 min, and then increased transparency gradually by busing 1/2 absolute ethanol +1/2 xylene - Pure xylene-pure xylene, each transparent processing has different time. When it contains ethanol, the processing time is 5min, the first xylene time is 5min, and the second xylene time is 15 min; place the transparent material on clean paper, place the material in the center of a clean glass slide after the xylene evaporated, Canadian resin was added on the material, cover the cover glass. The temporary sample sections were observed under bright field and UV (Ultraviolet light) with high magnification Nikon E200 optical microscope: the structure of the vessel, vessel cavity perimeter and the cell wall thickness of the wood was measured by Image pro plus software.

4.5. Raman spectroscopy of transgenic 741 Populus tomentosa

Raman analysis was performed on 25-lm-thick cross sections of three biological replicates for each of the three genotypes (WT, Sense and Anti-sense). 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 equipped with a linearly polarized green laser (k = 532 nm). For Raman mapping, the step size was set to 0.8 μm and the integration time was set to 0.4 s per spectrum. A grating of 600 lines mm and laser power of approximately 19.6 mW was used. The spectra were then exported to labspecs 6.0 for further analysis. 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).

4.6. Physical and mechanical properties determination of transgenic 741 Populus tomentosa

The 300 mm of the wood section at 1.3 m from the basilar part of raw material was observed, the physical and mechanical properties are determined according to the requirements of GB/T 1929–2009 wood physical mechanics test and each sample was repeated 3 times.

The density determination in this experiment is carried out according to the national standard GB1933-91 "Method for Measuring Wood Density". The operation is as follows: the size of the transverse, radial longitudinal, tangential longitudinal were measured by a micrometer respectively accurate to 0.001 cm, then put the sample into the oven, the initial temperature at 60 °C for 6 h, then increase the temperature to 103 ± 2 °C for 10 h. Then selected 2–3 samples for test weighing every 2 h. Until the difference between the last two weights did not exceed 0.5%, the sample was absolute dry, then calculated the absolute dry density of the sample. The wood shrinkage rate is divided into air dry and full dry; both are divided into volume shrinkage rate, longitudinal shrinkage rate (in the direction of wood grain), tangential shrinkage rate and radial shrinkage rate (direction of cross grain). This experiment was carried out in accordance with GB1932-91 "Method for Measuring Drying Properties of Wood". The test piece was placed in an oven at 103 °C ± 3 °C for 4 h and then weighed for the first time, and then weighed once every l hour until the difference between the last two weights did not exceed 0.0029. The shrinkage ratio is calculated according to the dimensions before and after drying. The mechanical properties of wood are main indicators to measure the ability of wood to resist external forces. Among them, the compressive strength, tensile strength, impact toughness, and elastic modulus are the most important. In this experiment, the indicators were tested and calculated according to the relevant provisions of the Test Method for Physical and Mechanical Properties of Wood (GB/T 1927-43-92).

Author's contributions

JQ Hu and Q Qi performed experiments and analyzed data; YL Zhao, H Lu and XM Tian provided the experimental trees; XN Jiang and Y Gai designed and supervised the study. All authorsread and approved the final manuscript, and dedared no competing interest.

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