Wood forming tissue-specific expression of PdSuSy and HCHL increases holocellulose content and improves saccharification in Populus

Yang Zhang1,2,3 · Hua Xu2,3 · Yingzhen Kong4 · Jiawen Hua2,3,5 · Xianfeng Tang2,3 · Yamei Zhuang1,2,3 · Yue Bai5 · Gongke Zhou2,3 · Guohua Chai2,3,6

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Abstract  Development of strategies to deconstruct lignocellulosic biomass in tree species is essential for biofuels and biomaterials production. We applied a wood forming tissue-specific system in a hybrid poplar to express both PdSuSy (a sucrose synthase gene from Populus deltoides × P. euramericana that has not been functionally characterized) and HCHL (the hydroxycinnamoyl-CoA hydratase-lyase gene from Pseudomonas fluorescens, which inhibits lignin polymerization in Arabidopsis). The PdSuSy-HCHL overexpression poplars correspondingly driven by the promoters of Arabidopsis AtCesA7 and AtC4H resulted in a significant increase in cellulose (> 8%), xylan (> 12%) and glucose (> 29%) content, accompanying a reduction in galacturonic acid (> 36%) content, compared to control plants. The saccharification efficiency of these overexpression poplars was dramatically increased by up to 27%, but total lignin content was unaffected. These transgenic poplars showed inhibited growth characteristics, including > 16% reduced plant height, > 10% reduced number of internodes, and > 18% reduced fresh weight after growth of 4 months, possibly due to relatively low expression of HCHL in secondary xylem.

Our results demonstrate the structural complexity and interaction of the cell wall polymers in wood tissue and outline a potential method to increase biomass saccharification in woody species.

Keywords Saccharification · Biomass · Cell wall composition · Growth · Poplar
Introduction

Tree species accumulate a major portion of lignocellulosic biomass, which is widely used as a raw material for pulp, paper and biofuel industries. Hardwood biomass is mainly composed of cellulose, hemicellulose (primarily xylan), and lignin along with small amounts of pectin (Mellerowicz and Sundberg 2008; Li et al. 2014). Currently, a major challenge for use of hardwoods as biofuel feedstocks is to remove lignin and convert the polysaccharides (particularly cellulose) to sugars (Pauly and Keegstra 2010; Chundawat et al. 2011). Therefore, understanding the functionality and interaction of cellulose and lignin in trees is essential to genetic manipulation of biomass with reduced recalcitrance.

Cellulose accounts for 40–50% of the secondary xylem in woody plants (Chundawat et al. 2011; Li et al. 2014). Cellulose synthase (CesA) is the catalytic subunit of the cellulose synthase complex. Sucrose synthase (SuSy) is strongly associated with CesA and participates in cellulose biosynthesis by directly supplying UDP-glucose to the cellulose synthase complex during secondary xylem development and deposition (Amor et al. 1995; Hertzberg et al. 2001; Song et al. 2010; Fujii et al. 2010). Altering the expression of SuSy from several species causes changes in structural and storage carbohydrates. For instance, natural variations in SuSy levels in wheat alter amounts of cell wall polysaccharides (Xue et al. 2007). Over-expression of a mutant form of the mung bean SuSy in poplar (Populus alba) results in higher catalytic efficiency toward sucrose (Konishi 2004). Over-expression of cotton SuSy in poplar (P. alba ×grandidentata) causes an increase in cellulose content and alteration in the ultrastructure of secondary cell walls (Coleman et al. 2009). In Populus, gene expression pattern analysis reveals that SuSy may be associated with cellulose biosynthesis and tension wood formation (Andersson-Gunnerås et al. 2006). However, genetic evidence is lacking to justly investigate whether poplar SuSy functions in cellulose biosynthesis.

Lignin is a heterozygous polymer composed of phenylpropyl derivatives that is covalently bonded to cellulose and hemicellulose in the secondary cell wall of vascular plants (Chundawat et al. 2011; Li et al. 2014). Down-regulation of genes involved in particular steps of lignin biosynthesis in different species including poplar reduces lignin content, promotes saccharification, but produces undesired phenotypes including sterility, dwarfism and increased susceptibility to environmental stresses (Chen and Dixon 2007; Leplé et al. 2007; Bonawitz and Chapple 2010; Voelker et al. 2011). In contrast, increased accumulation of hydroxycinnamoyl-CoA hydratase-lyase (HCHL) from Pseudomonas fluorescens in Arabidopsis stems promotes saccharification but does not significantly affect biomass yield (Eudes et al. 2012). HCHL is essential for producing hydroxybenzaldehydes by cleaving the propanoid side-chain of hydroxycinnamoyl-CoA lignin precursors. Engineered plants with relatively high HCHL activity show no reduction in total lignin levels but do show increased amount of unusual C₆C₁ lignin monomers. Thus, elevated expression of HCHL represents a promising strategy to reduce cell wall recalcitrance to enzymatic hydrolysis.

Poplar is a tree model for woody plant biology because of its rapid and perennial growth, moderate genome size, biomass-related traits and relatively easy transformation (Jansson and Douglas 2007). In this study, we used a secondary xylem-specific system to express both poplar PdSuSy and bacterial HCHL to increase cellulose levels and enhance saccharification. Transgenic poplar lines overexpressing PdSuSy driven by the Arabidopsis CesA7 promoter (Smith et al. 2013) and HCHL driven by the Arabidopsis CINNA-MATE-4-HYDROXYLASE (C4H) promoter (Weng et al. 2008) showed increased cellulose and xylan contents, and enhanced saccharification efficiency. This finding adds to our understanding of the structural complexity of the cell wall polymers in tree species.

Materials and methods

Generation of the overexpression construction and poplar transgenic lines

The coding sequence of PdSuSy (Potri.006G136700) was amplified by PCR from cDNA of the stems of 3-month-old Populus deltoides × P. euramericana cv ‘nanlin895’ plants. The promoter fragments of secondary cell wall-specific cellulose synthase gene (ArCesA7, AT5G17420, 1127 bp) and lignin synthase gene (ArC4H, AT2G30490, 2977 bp) were amplified from genome DNA of Arabidopsis seedlings. Gene specific primers were designed with Beacon Designer v7.0 (Premier Biosoft International, San Francisco, USA). The primer sequences were as follow: PdSuSy-cDNA (forward, 5′-ATGGTGCTCTTACTCTGTGTTCCAAAGC-3′; reverse, 5′-TTACCTCAGATGAAGAACTGATCGCC-3′), ArCesA7pro (forward, 5′-AGTAAAGATCTTTATGTGTTTGC-3′; reverse, 5′-AGGACCGGCAGGATTAACGC-3′), ArC4Hpro (forward, 5′-AGGACACTGAGGAACTGAGAAT-3′; reverse, 5′-TATATTGGTGATCCGCAGTATA-3′). A HCHL codon-optimized nucleotide sequence from Pseudomonas fluorescens AN103 (GenBank accession number CAA73502) was synthesized by Beijing Genomics Institution (BGI, Beijing, China). The PdSuSy and HCHL coding regions were ligated to the Gateway entry vector pGWC-T and pEN-L4-2-L3 (Invitrogen, Thermo Fisher Scientific, Waltham MA, USA), respectively, and then transferred into the Gateway binary vector pK7m34GW2-8m21GW3 downstream of the ArCesA7 and ArC4H promoters (Fig. 1a) following the method described previously (Qi et al. 2015). After sequence validation, the
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resulting construct was introduced into ‘nanlin895’ via the leaf disc method (Chai et al. 2014).

The transgenic poplar plantlets were selected on a medium containing 50 mg l⁻¹ Kanamycin and identified via PCR at the DNA level and RT-qPCR at the mRNA level. Primers were as follows: \textsc{PdSuSy}-\textsc{PCR} (forward, 5′-ATG TCTGATCTTACCTGGTCTCAAAGC-3′; reverse, 5′-TTA TCTGATCTTACCTGGTCTCAAAGC-3′), \textsc{HCHL}-\textsc{PCR} (forward, 5′-ATGTCTTACCTGGTCTCAAAGC-3′; reverse, 5′-TTA TCTTACCTGGTCTCAAAGC-3′), \textsc{PdSuSy}-\textsc{qPCR} (forward, 5′-ATCGGAGATA TTGGCCGG-3′; reverse, 5′-TATGCTTACCTGGTCTCAAAGC-3′), \textsc{HCHL}-\textsc{qPCR} (forward, 5′-GGTGCTGGTCTCGATTTATCTTACCTGGTCTCAAAGC-3′; reverse, 5′-TATGCTTACCTGGTCTCAAAGC-3′).

Plantlets were acclimatized in a mist chamber for 30 d, and then transferred to a greenhouse with a 16-h-light/8-h-dark cycle at 25–30 °C.

**Quantitative real time PCR (RT-qPCR)**

RNA isolation and first-strand cDNA synthesis were conducted following the method described previously (Chai et al. 2014). RT-qPCR reactions were performed using the Power 2× SYBR Real-time PCR pre-mixture (TransGen Biotech, Beijing, China) in triplicate on a LightCycler®480 Detection System (Roche Holding AG, Basel, Switzerland). \textsc{PdUBQ} (BU879229, forward, 5′-GTT GAT TTT TGC TGG GAA GC; reverse, 5′-GAT CTT GGC CTT CAC GTT GT) was used as the reference gene. The relative transcript expression was determined by the 2⁻ΔΔCT method (Livak and Schmittgen, 2001).

**Microscopy**

Basal stems of 4-month-old transgenic poplars were sampled for microscopy analyses. Tissue fixation and embedding, maceration of xylem, and microscopy observations were performed as described previously (Chai et al. 2014). Briefly, 0.5-cm stem segments were submerged in 4% paraformaldehyde for 3 days, then dehydrated in a graded ethanol series, and finally incubated in pure paraplast. The 7-μm stem sections were cut with a Leica RM 2235 microtome (Leica Camera AG, Wetzlar, Germany) and adhered to Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham MA, USA). The stem sections were stained with toluidine blue-O (TBO, 1% w: v) for 30 s, and then observed using an Olympus DX51 light microscope (Olympus Corp., Tokyo, Japan).

**Cell wall composition analyses**

The basal stems of 4-month-old transgenic poplars were sampled for extraction of alcohol-insoluble residues (AIRs). The stems of 5 individual plants were pooled and assayed for each biological replicate. Monosaccharide composition of AIRs was determined using the method described by Selvendran et al. (1979). Briefly, cell walls were hydrolysed with 2 M trifluoroacetic acid (TFA) for 2 h at 120 °C.
TFA-released materials were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and analyzed on a Thermo ODS-2 C18 column (4.6 × 250 mm) that was connected to a Waters high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA).

Cellulose content was measured following the method of Updegraff (1969). TFA-resistant materials were treated by using the solution (acetic acid/nitric acid/water, 8:1:2) at 100 °C for 30 min. The resulting pellets were completely hydrolysed with 67% H2SO4. The released glucose was detected using a glucose assay kit (Cayman Chemical, Ann Arbor, MI, USA) with a dehydration factor of 0.9.

Total lignin content was determined by using the AcBr method (Fukushima and Hatfield 2001). Three mg of AIRs were solubilized by acetyl bromide solution, and the stop reaction was conducted by adding a solution containing 2 M sodium hydroxide and 0.5 M hydroxylamine hydrochloride. Then absorbance was measured at 280 nm using the UV–visible spectrophotometer model VARIAN Cary 50 (Varian, Inc., Palo Alto, CA, USA).

### Cell wall pretreatment and saccharification

Cell wall residues of the stems from 4-month-old transgenic poplars were sampled for pretreatment and saccharification analyses following the method of Van Acker et al. (2013). Briefly, the biomass was pretreated with 1 ml of 1 M HCl at 80 °C for 2 h. After removing the acid extract, the pretreated material was washed with water and incubated with 1 ml 70% ethanol overnight at 55 °C. The dry material of 30 mg was treated with buffer-enzyme stock (40 µL of 8% Novozymes CTec2 in 1 M sodium citrate buffer, pH 5.0). The diluted saccharified hydrolysate was incubated for 70 h at 50 °C and then analyzed using phenol–sulfuric acid assays.

### Results

#### Transgenic poplar plants overexpressing both PdSuSy and HCHL driven by the secondary cell wall-specific promoters showed slightly inhibited growth

To simultaneously express two wood-forming genes in secondary xylem tissue, we cloned the coding sequence of PdSuSy from *P. deltoides* × *P. euramericana* and the codon-optimized DNA sequence encoding HCHL from *P. fluorescens*. The two genes were inserted into the binary vector pK7m34GW2-8m21GW3, which has two cassettes under control of the promoters of secondary cell wall cellulose synthase gene (*AtCesA7*) and lignin synthase gene (*AtC4H*) (Fig. 1a). The *AtCesA7* and *AtC4H* promoters were applied to restrict *PdSuSy* and *HCHL* expression to lignifying tissues of the *Populus* stem. Forty-two transgenic poplar lines overexpressing both *PdSuSy* and *HCHL* were obtained (Fig. 1b). RT-qPCR showed high transcript abundance for endogenous *PdSuSy* and relatively low transcript abundance for exogenous *HCHL* in these overexpression lines compared with vector controls (CKs) (Fig. 1c). Phenotypically, these overexpression lines displayed inhibited growth characteristics, including significantly reduced plant height (> 16%), reduced number of internodes (> 10%) and reduced fresh weight (> 18%) after growth over 4 months in a greenhouse (Figs. 1d and 2a–c). Further, we recorded dose–response between the phenotypic alterations and *HCHL* transcript level in these transgenic lines (Figs. 1c, d). The overexpression lines (e.g. #34) with lower *HCHL* expression level showed 11% smaller stem radial diameter than other overexpression lines (e.g. #36) and control (Fig. 2d). These results indicated that overexpression of *AtCesA7*-driven *PdSuSy* and *AtC4H*-driven *HCHL* in a hybrid poplar slightly inhibits growth.

#### Transgenic poplars resulted in a decrease of xylem radial width

To examine the effect of *AtCesA7*-driven *PdSuSy* and *AtC4H*-driven *HCHL* expression in secondary tissues,
basal stem sections of 4-month-old AtCesA7:PdSuSy-AtC4H:HCCHL lines (#36 and #34) and control plants were observed by microscopy. There was a significant 12% decrease in the radial width of xylem in stem sections from the overexpression line #34 compared to control, but xylem widths were similar between overexpression line #36 and the control (Fig. 3). These were consistent with the alterations of stem diameters in these transgenic poplars (Fig. 2d). Thus, overexpression of PdSuSy and HCCHL under control of the secondary xylem-specific promoters might inhibit xylem development, thereby slightly decreasing biomass yield in a hybrid poplar.

The contents of cellulose and non-cellulosic wall polysaccharides changed in mature wood tissues of transgenic poplars

To determine the consequence of AtCesA7-driven PdSuSy and AtC4H-driven HCCHL expression on the cellulosic and non-cellulosic wall polysaccharides, we investigated cell wall compositions of wood from AtCesA7:PdSuSy-AtC4H:HCCHL lines (#36 and #34) along with vector controls. Wood cell walls were extracted from the bottom 3 cm of stems of 4-month-old poplars as alcohol insoluble residue (AIR) and were analyzed by HPLC. The contents of cellulose, xylose (Xyl, the dominant hemicellulose in hardwood biomass) and glucose (Glc) were significantly increased in total AIR from AtCesA7:PdSuSy-AtC4H:HCCHL lines compared to controls, i.e., >8% increased cellulose, >12% increased Xyl and >29% increased Glc (Fig. 4a, b). However, a >36% reduction in galacturonic acid (GalA) content was recorded for AtCesA7:PdSuSy-AtC4H:HCCHL lines relative to the controls. These results indicated that overexpression of AtCesA7-driven PdSuSy and AtC4H-driven HCCHL might promote cellulose and xylan biosynthesis in poplar.

Total lignin content is not changed but saccharification is increased in mature wood tissues of transgenic poplars

Lignin confers recalcitrance to the processing of plant cell walls, and negative correlations exist between lignin content and polysaccharide saccharifiability (Chen and Dixon 2007; Taboada et al. 2010). Therefore, we measured lignin content and glucose release in mature wood samples of 4-month-old AtCesA7:PdSuSy-AtC4H:HCCHL and control lines. Two overexpression lines (#36 and #34) showed total lignin content similar to that in the controls (Fig. 4c). However, after a 70-h incubation with cellulase, pretreated biomass of the two overexpression lines released more (>27%) glucose per gram AIR than that of the controls (Fig. 4d), showing improvement of saccharification efficiency.

Discussion

In this study, we investigated how overexpression of PdSuSy and HCCHL driven by the secondary xylem-specific promoters affects tree growth and secondary cell wall chemistry in a hybrid poplar. Transgenic poplar lines with high PdSuSy levels and low HCCHL levels showed inhibited growth characteristics, including reduced plant height, reduced number of internodes and decreased fresh weight, compared to control plants. Further, the growth-inhibitory effects of these overexpression lines were dose–response to HCCHL expression level. Like IRX15:HCCHL Arabidopsis plants (Eudes et al. 2012), our AtCesA7:PdSuSy-AtC4H:HCCHL poplars did not produce undesirable phenotypes, such as senescing and chlorotic leaves, male sterility, stunting and collapsed xylem vessels, which are often observed in 35S:HCCHL transgenic tobacco and sugarcane (McQualter et al. 2005; Merali et al. 2007). It is possible that using the secondary xylem-specific AtC4H promoter effectively restricted HCCHL expression to the lignifying tissues. Our AtCesA7:PdSuSy-AtC4H:HCCHL poplars grew slightly shorter than the controls, consistent with the phenotypic alteration of some IRX15:HCCHL Arabidopsis lines with low HCCHL expression level (Eudes et al. 2012). In contrast, IRX15:HCCHL Arabidopsis lines with relatively high expression level of HCCHL remained...
comparable to wild-type (WT) plants. Combined with the finding that all 35S:GhSuSy and 4CL:GhSuSy Arabidopsis plants exhibit WT-like phenotypes (Coleman et al. 2009), we speculate that AtC4H-driven HCHL expression may contribute to slight dwarfism in AtCesA7:PdSuSy-AtC4H:HCHL poplars. Our work cannot absolutely exclude the possibility that the HCHL and PdSuSy genes have different response in Populus. Further studies by elevated expression of the single gene PdSuSy or HCHL in poplar may resolve this ambiguity.

Analysis of cell wall sugar composition in the AIR of mature wood tissue showed significantly increased cellulose, Glc, and Xyl contents but reduced GalA content in AtCesA7:PdSuSy-AtC4H:HCHL poplar lines compared to controls. More importantly, saccharification efficiency was promoted by at least 27% without affecting lignin content in these overexpression poplars. SuSy catalyzes the formation of fructose and UDP-glucose (Amor et al. 1995; Coleman et al. 2009). The latter serves as a precursor for Xyl biosynthesis (Bar-Peled and O’Neill 2011). Further, cellulose and xylan biosynthesis are co-regulated transcriptionally to balance UDP-Glc usage for cellulose and Xyl via UDP-Xyl (Wierzbitcki et al. 2019). Therefore, it is possible that in AtCesA7:PdSuSy-AtC4H:HCHL poplars PdSuSy overexpression promotes the accumulation of UDP-glucose, which provides the material for the biosynthesis of both cellulose and Xyl. Recently, the HG-containing polymers derived from the sole sugar residue GalA are shown to interact with other cell wall polymers, for instance, the hemicellulose (e.g., xylan) moieties in HG-containing proteoglycans (e.g., APAP1) and cellulose microfibrils using solid-state NMR analysis (Wang and Hong 2016). This finding is subsequently confirmed in planta. Reduced expression of Guronosyltransferase 4 (GAUT4), a pectin biosynthesis gene, in switchgrass and poplar decreases GalA content and increases Xyl content in total AIR, affecting cell wall structure and thereby enhancing saccharification (Biswal et al. 2018). Thus, the changes of Xyl and GalA levels in the wood tissue of AtCesA7:PdSuSy-AtC4H:HCHL poplars may contribute to enhancing saccharification. A more plausible explanation for enhancing saccharification in AtCesA7:PdSuSy-AtC4H:HCHL poplars is that AtC4H-driven HCHL expression may decrease lignin polymerization degree and in turn repress cell wall

![Fig. 4](Image)

Fig. 4 Cell wall composition and saccharification of mature stems from AtCesA7:PdSuSy-AtC4H:HCHL and control poplars. Alcohol-insoluble residues (AIRs) were extracted from the basal stems of 4-month-old poplars. Data are mean±SE. At least three plants were measured for each line. Statistical significance of the differences among different genotypes was analyzed by Duncan’s t-test, P < 0.05.
recalcitrance to enzymatic hydrolysis. Further experimental confirmation is needed to validate this hypothesis.

In this study, we successfully used a secondary xylem-specific system to express both PdSuSy and HCHL in a hybrid poplar. Our AtCesA7::PdSuSy-AtC4H::HCHL poplars showed increased holocellulose (cellulose + hemicellulose) content and enhanced saccharification efficiency, without affecting lignin content. Further, the levels of other cell wall compositions such as glucose and galacturonic acid were changed in the stem cell walls of these transgenic poplars. Unexpectedly, these overexpression poplars exhibited slightly inhibited phenotypes. This work provides fundamental information for understanding the structural characteristics of the cell wall polymers in woody species.

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