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ORIGINAL PAPER

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

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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

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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 suppling 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-Gunneråas 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 susceptibly 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 C_6C_1 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 CINNAMATE-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 (*AtCesA7*, AT5G17420, 1127 bp) and lignin synthase gene (*AtC4H*, 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′-ATGTCTGTACTTACTCGTGTCCAAAGC-3′; reverse, 5′-TTACTCGATAGTCAAAGGAACTGAATCAGCC-3′), *AtCesA7pro* (forward, 5′-AGTAAAAGATCTTTTAGTTGTTTGC-3′; reverse, 5′-AGGGACGGCCGGAGATTAGCAGCGA-3′), *AtC4Hpro* (forward, 5′-AGCTTAGAGGAGGAACTGAGAAAAT-3′; reverse, 5′-TATAGTTTGTGTATCCGCAATGATA-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 *AtCesA7* and *AtC4H* promoters (Fig. 1a) following the method described previously (Qi et al. 2015). After sequence validation, the

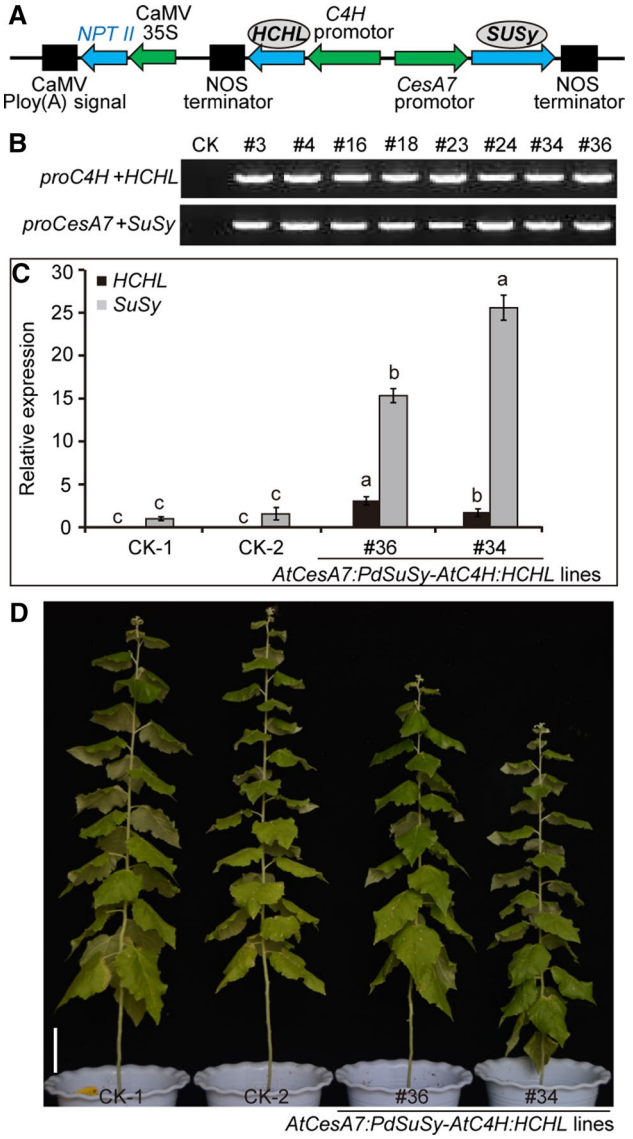

Fig. 1 Phenotypes of transgenic poplars overexpressing *AtCesA7*-driven *PdSuSy* and *AtC4H*-driven *HCHL*. **a** The overexpression construct containing *AtCesA7*-driven *PdSuSy* and *AtC4H*-driven *HCHL*. **b** PCR identification of overexpression lines with the primer pairs (*proC4H*+*HCHL* and *proCesA7*+*PdSuSy*). **c** Relative *HCHL* and *PdSuSy* transcript abundance as determined by RT-qPCR analysis of the stems of 4-month-old vector controls (CK, #1, 2) and representative *AtCesA7:PdSuSy-AtC4H:HCHL* lines (#36, 34). *PdUBQ* was used as the reference gene and the transcript expression of *PdSUSy* in CK was set to 1. Error bars are mean ± SE (n = 5). Duncan's *t* test was used to compare means at *alpha* = 0.05. **d** Phenotypes of 4-month-old CK (#1, 2) and *AtCesA7:PdSuSy-AtC4H:HCHL* (#36, 34) lines. Bar = 10 cm

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: *PdSuSy-PCR* (forward, 5′-ATGTCTGTACTTACTCGTGTCCAAAGC-3′; reverse, 5′-TTACTCGATAGTCAAAGGAACTGAATCAGCC-3′), *HCHL-PCR* (forward, 5′-ATGTCTACATACGAAGGAAGATGGA-3′; reverse, 5′-TTATCTCTTATAAGCTTGAAGACCT-3′), *PdSuSy-qPCR* (forward, 5′-ATCGGGAAGATATTGGCGGG-3′; reverse, 5′-TGACGTACTCCCAGACACCA-3′), *HCHL-qPCR* (forward, 5′-GCGCTGATGAGGCTACATTC-3′; reverse, 5′-TCAGCAGCTTTCTGTCCTCC-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). *PdUBQ* (BU879229, forward, 5′-GTTGATTTTTGCTGGGAAGC′; reverse, 5′-GATCTTGGCCTTCACGTTGT-3′) was used as the reference gene. The relative transcript expression was determined by the $2^{-\Delta\Delta CT}$ 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 trifluroacetic acid (TFA) for 2 h at 120 °C. The

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% H_2SO_4. 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 its 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,

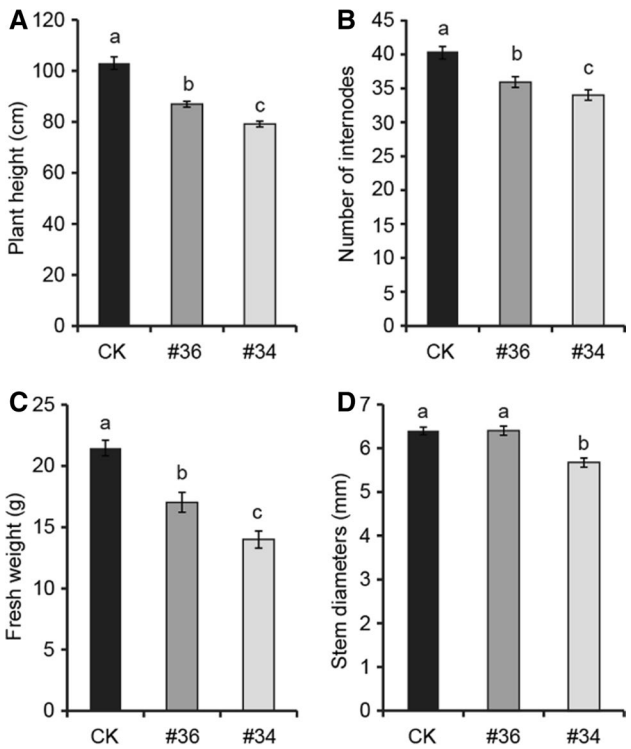

Fig. 2 Growth parameters of 4-month-old *AtCesA7:PdSuSy-AtC4H:HCHL* and control poplars. **a**–**c** Plant height (**a**), number of internodes (**b**) and stem diameter (**d**) were measured from more than 10 plants for each line. **c** Fresh biomass weight of stems was measured from five plants for each line. Duncan's *t*-test, *alpha* = 0.05

basal stem sections of 4-month-old *AtCesA7*:*PdSuSy-AtC4H*:*HCHL* 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 *PdSuSy2* and *HCHL* 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 *HCHL* expression on the cellulosic and non-cellulosic wall polysaccharides, we investigated cell wall compositions of wood from *AtCesA7*:*PdSuSy-AtC4H*:*HCHL* 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*:*HCHL* 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*:*HCHL* lines relative to the controls. These results indicated that overexpression of *AtCesA7*-driven *PdSuSy* and *AtC4H*-driven *HCHL* might promote cellulose and xylan biosynthesis in poplar.

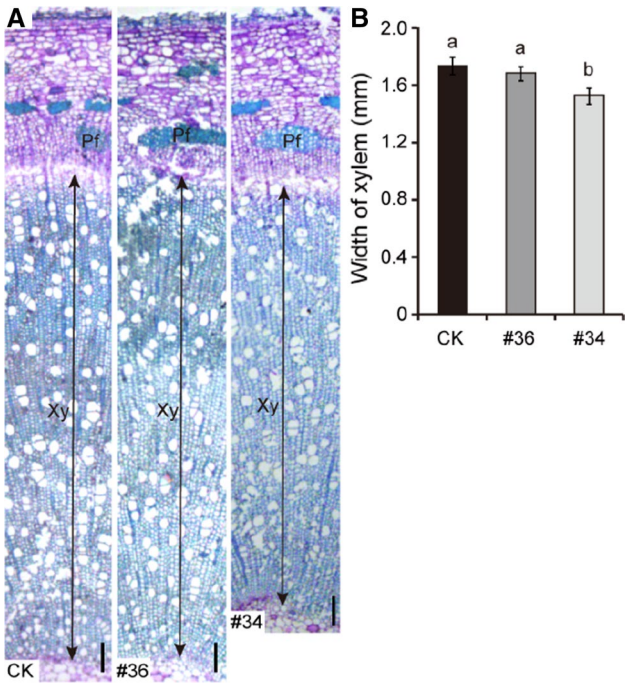

Fig. 3 Microscopic analysis of stem sections from 4-month-old *AtCesA7:PdSuSy- AtC4H*:*HCHL* and control poplars. Statistics are mean ± SE. For each line, at least five plants were used for measurement of xylem radial width. Bars = 500 µm. Duncan's *t*-test at *alpha* = 0.05

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 saccharifiabilty (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*:*HCHL* 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 *HCHL* 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 *HCHL* 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 *HCHL* expression level. Like *IRX15:HCHL Arabidopsis* plants (Eudes et al. 2012), our *AtCesA7:PdSuSy-AtC4H:HCHL* 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:HCHL* 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 *HCHL* expression to the lignifying tissues. Our *AtCesA7:PdSuSy-AtC4H:HCHL* poplars grew slightly shorter than the controls, consistent with the phenotypic alteration of some *IRX15:HCHL Arabidopsis* lines with low *HCHL* expression level (Eudes et al. 2012). In contrast, *IRX15:HCHL Arabidopsis* lines with relatively high expression level of *HCHL* remained

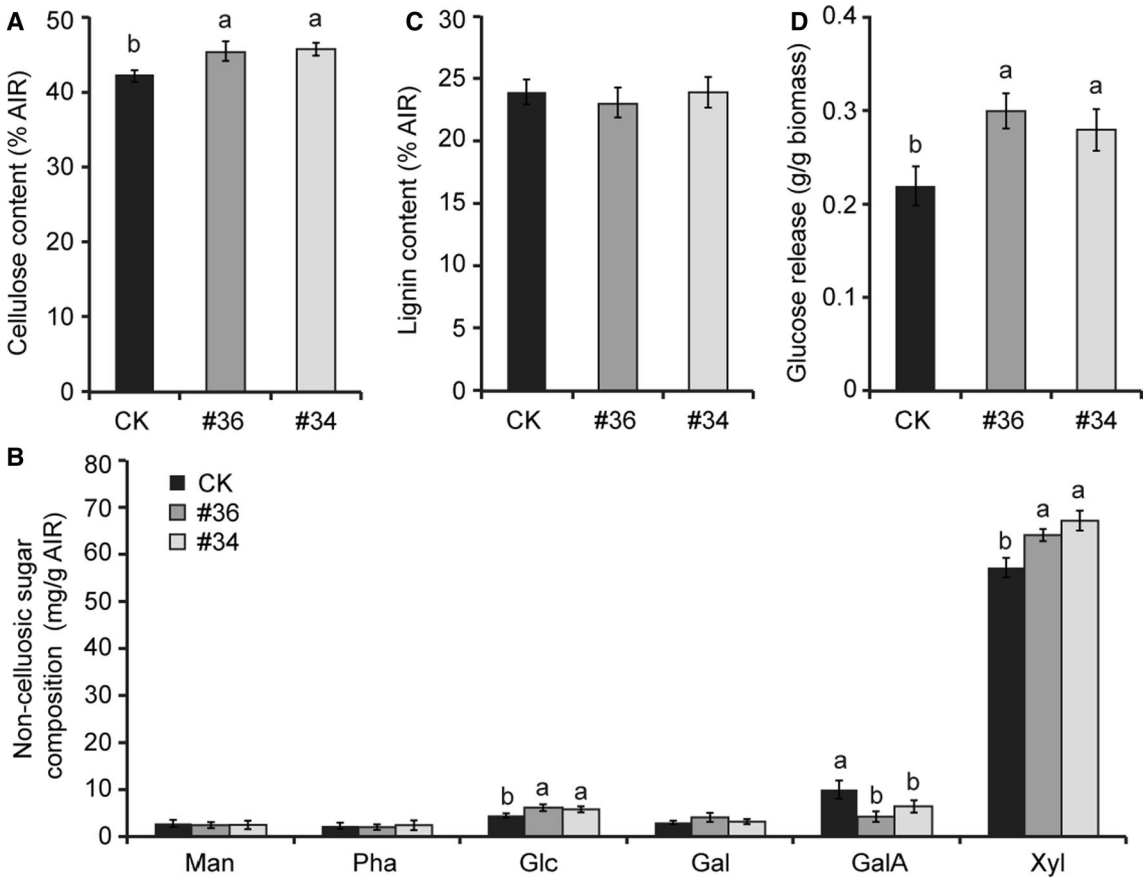

Fig. 4 Cell wall composition and saccharification of mature stems from *AtCesA7:PdSuSy-AtC4H:HCHL* and control poplars. Alcohol-insoluble residues (AIRs) were extracted from the basal stems of 4-month-old poplars. Data are mean ± SE. At least three plants were measured for each line. Statistical significance of the differences among different genotypes was analyzed by Duncan's *t*-test, $P < 0.05$

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 (Wierzbicki 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

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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