Downregulation of RWA genes in hybrid aspen affects xylan acetylation and wood saccharification

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Summary

- High acetylation of angiosperm wood hinders its conversion to sugars by glycoside hydrolases, subsequent ethanol fermentation and (hence) its use for biofuel production.
- We studied the *REDUCED WALL ACETYLATION* (*RWA*) gene family of the hardwood model *Populus* to evaluate its potential for improving saccharification.
- The family has two clades, *AB* and *CD*, containing two genes each. All four genes are expressed in developing wood but only *RWA-A* and *-B* are activated by master switches of the secondary cell wall *Pt*NST1 and *Pt*MYB21. Histochemical analysis of promoter::GUS lines in hybrid aspen (*Populus tremula* × *tremuloides*) showed activation of *RWA-A* and *-B* promoters in the secondary wall formation zone, while *RWA-C* and *-D* promoter activity was diffuse. Ectopic downregulation of either clade reduced wood xylan and xyloglucan acetylation. Suppressing both clades simultaneously using the wood-specific promoter reduced wood acetylation by 25% and decreased acetylation at position 2 of Xyl*p* in the dimethyl sulfoxide-extracted xylan. This did not affect plant growth but decreased xylose and increased glucose contents in the noncellulosic monosaccharide fraction, and increased glucose and xylose yields of wood enzymatic hydrolysis without pretreatment.
- Both RWA clades regulate wood xylan acetylation in aspen and are promising targets to improve wood saccharification.

Introduction

Wood is the most abundant terrestrial form of biomass, and increasing its utilization is considered a desirable strategy to mitigate anthropogenic increases in atmospheric CO_2 concentrations. Wood is composed of dead cells with thick secondary wall layers, surrounded by a thin primary cell wall layer and a middle lamella jointly called the compound middle lamella. Different polymers are deposited in the compound middle lamella and secondary cell wall layers of hardwoods (Mellerowicz *et al.*, 2001; Plomion *et al.*, 2001; Mellerowicz & Gorshkova, 2012). In *Populus*, a model hardwood tree genus, the compound middle lamella is mostly composed of a pectin matrix of homogalacturonan (HG) and rhamnogalacturonan I (RGI), and cellulose microfibrils coated with xyloglucan (XG), primary wall xylan and mannan. By contrast, the secondary wall layers are enriched in cellulose micro- and macrofibrils forming a honeycomb-like network and coated with acetylated glucuronoxylan (AcGX). Small amounts of glucomannan and type II arabinogalactan are also present in these wall layers. The polysaccharides in mature wood cells are surrounded and covalently linked to lignin – a polymer composed primarily of syringyl (S) and guaiacyl (G) phenylpropanoid units with traces of *p*-hydroxylphenyl (H) units.

In addition to use as a raw material for constructing diverse artifacts, a major application of wood is as a source of energy, either through direct combustion or following conversion to sugars (saccharification) and subsequently ethanol. The ease of this conversion is strongly influenced, *inter alia*, by the wood's lignin content and composition (Chang & Holtzapple, 2000; Sannigrahi *et al.*, 2010; Studer *et al.*, 2011), and its covalent links to AcGX and other carbohydrates (Yuan *et al.*, 2011; Min *et al.*, 2014a,b). Another factor that may (negatively) affect wood conversion to sugars is its acetyl content (Pawar *et al.*, 2013). The acetyl content of hardwoods varies between 3.5 and 4.5% of their dry weight, and is mostly associated with GX residing in secondary wall layers, where *c.* 60% of xylopyranosyl residues (Xyl*p*) are mono- or di-acetylated

(Teleman *et al.*, 2000, 2002). Recent studies have revealed that most acetyl groups are present on alternate Xyl*p* units at position 2 and/or 3, or at position 3 of (Me)GlcA-Xyl*p* in Arabidopsis (Busse-Wicher *et al.*, 2014; Chong *et al.*, 2014). In the compound middle lamella, acetyl groups are mostly associated with RGI, HG or XG (Pawar *et al.*, 2013). Acetylation of polysaccharides is known to affect their solubility in water, interactions with cellulose and many other physicochemical properties (Grondahl *et al.*, 2003; Fundador *et al.*, 2012; Busse-Wicher *et al.*, 2014). Thus, acetylation patterns are presumably involved in regulation of cell wall and lignocellulose properties. Acetylation also protects polymers from degradation by restricting accessibility of glycanases, making cell walls durable and resistant to microbial attack (Rowell, 2009). This is desirable for solid wood products, but not for saccharification of woody biomass. It is also problematic for subsequent fermentation because acetic acid is a potent inhibitor of microbial activity (Helle *et al.*, 2003; Jönsson *et al.*, 2013). Therefore, reduction of acetylation in plants cultivated to provide wood for saccharification is thought to be beneficial for both sugar and ethanol yields.

The polysaccharides are acetylated during biosynthesis in the Golgi using acetyl-coenzyme A (CoA) as a donor substrate (Pauly & Scheller, 2000). Acetyl-CoA is thought to be transported from cytoplasmic pools to the Golgi by REDUCED WALL ACETYLATION (RWA) proteins. Four RWA proteins have been found in Arabidopsis, designated RWA1–4. Reductions in acetylation of 20%, relative to wild-type (WT) levels, have been observed in knock-out *rwa2* mutants (Manabe *et al.*, 2011), and up to 60% in *rwa1/2/3/4* mutants, suggesting the existence of an RWA-independent route for cell wall acetylation (Manabe *et al.*, 2013). Mutation of several TRICHOME BIREFRINGENCE-LIKE (TBL) proteins also reportedly induces deficiency of acetylation in specific polymers in Arabidopsis, and the TBL29 protein was recently biochemically characterized as a XYLAN *O*-ACETYLTRANSFERASE 1 (XOAT1) (Urbanowicz *et al.*, 2014). Deficiencies in XG acetylation have been observed in the vegetative plant body and seeds of *axy4/tbl27* and *axy4L/tbl22* mutants, respectively (Gille *et al.*, 2011), and reductions in xylan acetylation in *eskimo1/tbl29, tbl3*, *tbl31*, *tbl32xtbl33* and *tbl34xtbl35* mutants (Xiong *et al.*, 2013; Yuan *et al.*, 2013, 2016a–c). Regiospecificity of xylan acetyl transferases is supported by the dynamics of the acetylation pattern observed during biosynthesis *in vitro* (Urbanowicz *et al.*, 2014), and by defects observed specifically at positions 2 or 3 of monoacetylated Xyl*p* in *tbl29* mutants (Xiong *et al.*, 2013; Yuan *et al.*, 2013), at position 3 of mono- and di-acetylated Xyl*p* in *tbl3*, *tbl31* and *tbl34 tbl35* mutants (Yuan *et al.*, 2016b,c), and at position 3 in mono- and di-acetylated Xyl*p* and (Me)GlcA-Xyl*p* in *tbl32 tbl33* mutants (Yuan *et al.*, 2016a). However, the interpretation of these data is not straightforward because the acetyl group was observed to spontaneously migrate between positions 2 and 3 of Xyl*p* (Mastihubova & Biely, 2004). Large (80%) reductions in xylan acetylation have also been detected in an Arabidopsis mutant with a lesion in a member of a different plant-specific gene family, *axy 9-1*, and the AXY9 protein was localized to Golgi, but its function is not clear (Schultink *et al.*, 2015). Thus, many aspects of the acetylation biosynthesis pathway in Arabidopsis remain unknown, and very little is known about *O*-acetylation mechanisms in other species. Moreover, no improvements in sugar yields have been obtained in saccharification tests of the acetylation mutants described earlier, and severe deacetylation causes xylem cell collapse and dwarfism (Lee *et al.*, 2011; Manabe *et al.*, 2013; Xiong *et al.*, 2013; Yuan *et al.*, 2013). This raises questions about the feasibility of improving this trait by biosynthetic reduction of acetylation.

Populus is a good model species to address these questions, as it offers large quantities of pure xylem tissue for analysis. Thus, as reported here, we have analyzed the promoter activity, transactivation and effects of *in planta* suppression of the *RWA* genes in *Populus*, and probed the (overlapping) functions of RWA proteins in xylan acetylation in the xylem. We demonstrate that suppression of *RWA* genes alters xylem sugar composition and improves saccharification without affecting plant growth.

Materials and Methods

Construction of promoter:GUS vectors and transactivation

RWA genes in *Populus trichocarpa* (with IDs presented in Supporting Information Table S1) were identified from Phytozome (http://www.phytozome.net/). Their promoters were then cloned from *Populus tremula* genomic DNA, using primers based on sequences *c.* 1 kb upstream of the translation sites (Table S2), and transferred into the pCF201 vector. *Pt*MYB21 (*Potri009G053900*) and *Pt*NST1 (*Potri002G178700*) effector constructs were cloned following Ratke *et al.* (2015). The plasmids were introduced into *Agrobacterium* strain C58C1-RS (pCH32).

Leaves of 6- to 8-wk-old *Nicotiana benthamiana* plants were infiltrated with the transformed *Agrobacterium* carrying these constructs, and β-glucuronidase (GUS) activity of their proteins was determined after growth for a further 5–6 d following Guerriero *et al.* (2009). Briefly, protein extracts were incubated with *p*-nitrophenyl β-D-glucuronide (PNPG) substrate at 37°C and absorbance of the product, *p*-nitrophenol (PNP), was measured at 410 nm. Leaves infiltrated with empty effector plasmids were used as controls. To determine specific GUS activity in extracts, their total protein contents were determined using the Bradford assay.

Histochemical GUS analysis in transgenic aspen lines

Sets of 12 transgenic lines expressing GUS via each of the RWA promoters were generated using *Agrobacterium* (GV3101) as described by Gray-Mitsumune *et al.* (2004). Whole mounts of stem, roots and leaves of the *in vitro* grown transgenic plants were fixed in ice-cold acetone, washed with 50 mM phosphate buffer and incubated in solution containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Glc), 50 mM phosphate buffer, 1 mM potassium ferricyanide ($K_3Fe(CN)_6$) and 1 mM potassium ferrocyanide ($K_4Fe(CN)_6$) for 36 h at 37°C in the dark. The tissues were further fixed in FAA solution (50% ethanol, 5% formaldehyde, 10% acetic acid), and rehydrated by passage through an ethanol series. Vibratome-obtained stem sections and whole mounts of roots and leaves were observed under a Zeiss Axioplan 2 microscope (Ratke *et al.*, 2015).

Generation of RWA::RNAi hybrid aspen (*P. tremula* L. × *tremuloides* Michx.) transgenic trees

Sequences common to RWA-A and -B, RWA-C and -D, and RWA-A, -B, -C and -D were amplified from cDNA of hybrid aspen developing wood, using the primers listed in Table S2. The amplified fragments, 253, 249 and 243 nt long, respectively, for the three RNAi constructs were cloned into Gateway vectors pK2GW7 and its derivative pK-pGT43B-GW7 (Karimi *et al.*, 2002; Ratke *et al.*, 2015) as described by Ratke *et al.* (2015). The constructs were named *35S::RWA-AB*, *35S::RWA-CD* and *pGT43B::RWA-ABCD*, respectively.

Hybrid aspen was transformed using *Agrobacterium* (GV3101) as described by Gray-Mitsumune *et al.* (2004). Twenty transgenic lines carrying each construct were screened *in vitro* by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and the five of each set that suppressed expression of the target genes most strongly were selected for the glasshouse experiment. These lines were grown for 2 months in the glasshouse, then harvested and samples were stored for expression and cell wall analyses following Gandla *et al.* (2015). The two lines carrying each construct that suppressed target gene expression most strongly, according to quantitative RT-PCR (RT-qPCR) assays, were analyzed in detail as described in the section below.

RT-qPCR

Total RNA was isolated from developing xylem by the cetyl trimethyl ammonium bromide (CTAB) method (Chang *et al.*, 1993). It was treated with DNAse (Ambion) and cDNA was prepared using iScript (Bio-Rad). Relative expression levels of the target RWA genes were determined using a PCR Light Cycler 480 II (Roche) and SYBR green with a premixed PCR mixture (Bio-Rad) following standard protocols (Pfaffl, 2001; Hellemans *et al.*, 2007), and normalization with respect to expression levels of two reference genes selected using geNorm (Vandesompele *et al.*, 2002). Primers and their efficiencies are listed in Table S1.

Preparation of wood powder

To prepare wood powder, bark and pith of hybrid aspen stem were removed and the wood was freeze-dried overnight. Dried stem segments were cut into small pieces and ground into rough powder and fine powder as previously described (Gandla *et al.*, 2015).

Acetyl content analysis

Fine wood powder was saponified by incubation in 0.5 M NaOH at room temperature for 1 h. The resulting solution was neutralized with 1 M HCl and its acetic acid content was determined using a Megazyme kit (Megazyme, Wicklow, Ireland).

Xylan and xyloglucan acetylation analysis by OLIMP

Fine wood powder was sequentially washed with 70% ethanol, methanol : chloroform 1 : 1 (v/v) and acetone, and the resulting alcohol insoluble residue (AIR) was then digested with GH10 endoxylanase as previously described (Chong *et al.*, 2011). The xylan oligosaccharides (XOS) released by digestion were separated into acidic and neutral fractions, and the acidic fraction was analyzed by atmospheric pressure-matrix-assisted laser desorption ionization-ion trap mass spectrometry (AP-MALDI-ITMS). Relative signal intensities for all detected oligosaccharides in the samples are presented (see Figs 4, 5 in the Results).

Cambium/phloem tissues were freeze-dried and ground using a bead mill. Dry powder was ground in 96% ethanol using ceramic beads, left for 10 min at 80°C, and the pellet was washed with ethanol and dried overnight at room temperature. Approximately 100 mg of the pellet was digested with an endoglucanase and the released oligosaccharides were analyzed using MALDI-time-of-flight-MS as described by Lerouxel *et al.* (2002).

Nuclear magnetic resonance analysis of xylan

AIR, prepared as described in the previous section, was incubated with peracetic acid solution (Sigma-Aldrich) at 85°C for 20 min to remove lignin. The pellet was collected by centrifugation and treated with dimethyl sulfoxide (DMSO) at 60°C for 24 h. The soluble part was collected by centrifugation and the pellet was again treated with DMSO. The two DMSO-soluble fractions were pooled and treated with an ethanol : methanol : water mixture (70 : 20 : 10, v/v, with pH adjusted to 3 by adding formic acid) for 48 h at 4°C. Precipitated material was collected by centrifugation, dissolved in 20 µl of water and freeze-dried. Five milligrams of freeze-dried powder was dissolved in 5 ml of D_2O and transferred to NMR tubes for two-dimensional (2D) NMR heteronuclear single quantum coherence (HSQC) analysis (Chong *et al.*, 2014), using a Bruker Avance III HD 600 MHz spectrometer equipped with a TCI HCP cryoprobe operating at 150.91 MHz ^{13}C frequency. A Bruker SampleXpress sample changer and ICON-NMR software (Bruker Biospin, Rheinstetten, Germany) were used to record all spectra automatically. 2D ^{1}H-^{13}C HSQC spectra with multiplicity editing (hsqcedetgpsisp2.3) were obtained at 300K, with 256 increments, 24 transients, 2 s relaxation delay, sweep widths of 10 and 165 p.p.m., and optimized for a direct coupling constant of 145 Hz. The total acquisition time of each experiment was 226 min. TOPSPIN 3.2 (Bruker Biospin) was used for processing and analysis.

Wood sequential extraction

Wood samples were sequentially extracted from 150 mg samples of fine wood powder using a protocol presented by Ona *et al.* (1995), with modifications, as follows. Extractives were removed by sequential Soxhlet extraction using 95% ethanol : toluene (1 : 2, v/v, 6 h), 95% ethanol (4 h) and distilled water (2 h). The extractive-free wood was dried overnight and weighed to calculate amounts of extractives, and a 75 mg subsample was delignified by treatment with 2 ml of 20% sodium chlorite at 100°C for 2 h. The resulting mixture was centrifuged and the pellet (holocellulose) was then dried overnight and weighed. Twenty milligrams of the holocellulose was treated with 17.5% of NaOH at room

temperature for 5 min. It was then mixed with 4 ml of NaOH and incubated for 1 h. This solution was diluted with 4 ml of water, re-centrifuged and the pellet was dried and weighed.

Cell wall analysis

The extractive-free wood powder was used to determine Updegraff cellulose and Klason lignin contents. The monosaccharide composition of delignified wood samples was analyzed following methanolysis and conversion to TMS derivatives as described by Gandla *et al.* (2015). The relative carbohydrate and lignin contents were determined by pyrolysis GC-MS analysis according to Gerber *et al.* (2012). Cellulose crystallinity was determined by NMR as described in Methods S1.

Wood digestibility assay with and without acid pretreatment

The saccharification procedures have been described in detail by Gandla *et al.* (2015). Briefly, wood powder was sieved to particle sizes between 0.1 and 0.5 mm, then 50 mg of the sieved dry wood powder was subjected to enzymatic hydrolysis both with and without acid pretreatment. The pretreatment involved incubating wood powder with 1% sulfuric acid for 10 min at 165°C, then centrifuging, collecting the supernatant (the pretreatment liquid) for analysis and digesting the pellet enzymatically. The wood and the pellet from the pretreated wood was digested with 50 mg of a 1 : 1 (w/w) mixture of Celluclast 1.5L (a cellulase-rich liquid enzyme preparation from *Trichoderma reesei* ATCC 26921 with a measured CMCase activity of 480 units per gram of liquid enzyme preparation, as defined in Ghose, 1987) and Novozyme 188 (a β-glucosidase-rich liquid enzyme preparation from *Aspergillus niger* with a measured β-glucosidase activity of 15 units g^{-1} liquid enzyme preparation, based on an assay with 5 mM *p*-nitrophenyl glucopyranoside as the substrate, as defined in Mielenz, 2009). Enzyme preparations were obtained from Sigma-Aldrich. Sodium citrate buffer (50 mM, pH 5.2) was added so that the total weight of the reaction mixture was 1000 mg. The monosaccharide composition of the enzymatic hydrolysates and the pretreatment liquids was analyzed by using high-performance anion-exchange chromatography (HPAEC).

Results

The RWA gene family in *Populus*

P. trichocarpa genome assembly (v.3.0; http://www.phytozome.net/poplar.php) contains four RWA genes that we have named RWA-A–D. Alignment of amino acid sequences of *Populus* and Arabidopsis RWA members (Fig. S1) showed strong conservation in the Cas1p domain that is characteristic of this gene family (Lee *et al.*, 2011; Manabe *et al.*, 2011). This domain, comprising several transmembrane helices, was first identified in the *Cryptococus neoformans* CAS1P protein involved in *O*-acetylation of capsular polysaccharides (Janbon *et al.*, 2001). According to the topology prediction program TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) *Populus* RWA proteins have 10–12 transmembrane domains (Fig. S2a), similar to those in Arabidopsis (Lee *et al.*, 2011).

As shown in Fig. 1, phylogenetic analysis of RWA sequences in selected embryophtes, following Dereeper *et al.* (2008) indicated that the RWA family has two major clades in these taxa: AB (including *Populus* RWA-A and -B) and CD (including *Populus* RWA-C and -D). Pairs of *Populus* proteins within each clade are over 90% identical, and pairs of different clades are *c.* 70% identical (Fig. S2b). Clade AB includes Arabidopsis RWA1, RWA3 and RWA4, whereas clade CD includes Arabidopsis RWA2. Proteins that can be clearly assigned to the AB and CD clades are also apparently present in other advanced dicotyledons, including eucalyptus, tomato and grape vine, but not in the basal flowering plant species *Amborella trichopoda* or the moss *Physcomitrella patens*, which only apparently have CD clade

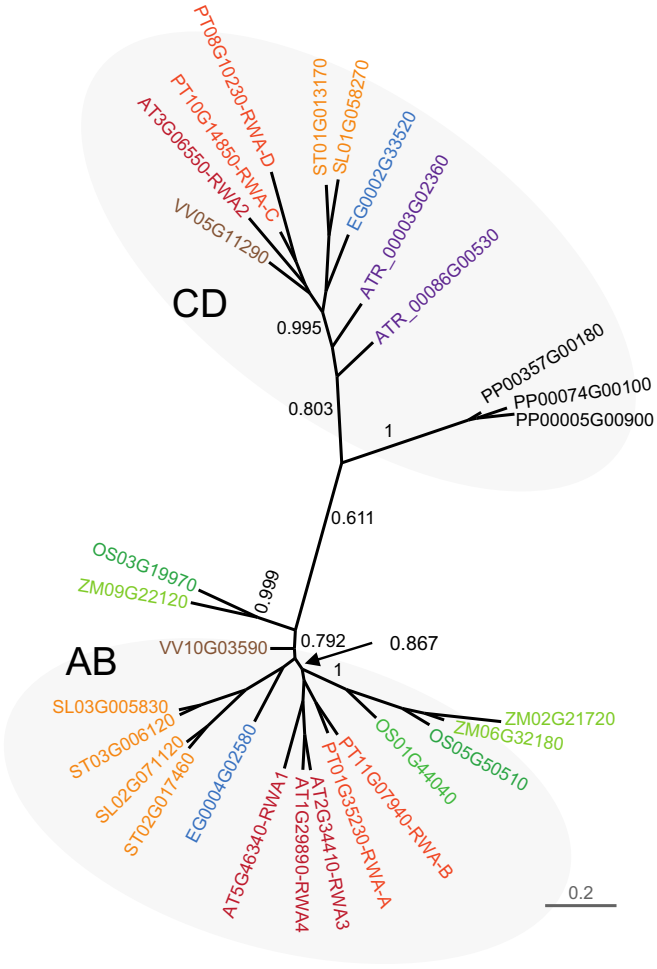

Fig. 1 Phylogenetic tree of the REDUCED WALL ACETYLATION (RWA) protein family. The tree was generated using PhyML at http://phylogeny.lirmm.fr, based on protein sequences retrieved from the Plaza v.3.0 database (http://http://bioinformatics.psb.ugent.be/plaza/). ATH, *Arabidopsis thaliana*; ATR, *Amborella trichopoda*; EG, *Eucalyptus grandis*; PP, *Physcomitrella patens*; PT, *Populus trichocarpa*; OS, *Oryza sativa*; SL, *Solanum lycopersicum*; ST, *Solanum tuberosum*; VV, *Vitis vinifera*; ZM, *Zea mays*. The numbers indicate branch support values based on the maximum-likelihood method.

representatives (Fig. 1). Monocot RWA proteins form two well-defined clades, one of which clearly groups with the AB clade, while the other forms a separate branch that is closer to the AB than to the CD clade, according to our analysis.

Expression and transactivation analyses reveal differences in expression of RWA genes

Publicly available *Populus* expression data (http://popgenie.org/) indicate that genes of both *RWA* clades are expressed simultaneously in various organs of *P. tremula* plants grown under diverse physiological conditions, and that *RWA-A* is always expressed more strongly than *RWA-B* (Fig. S3). Previously reported semi-quantitative RT-PCR analysis in hybrid aspen (*P. tremula* × *tremuloides*) has confirmed the expression of all four *RWA* genes in developing wood (Ratke *et al.*, 2015).

To investigate if the expression of any of the *RWA* genes is induced as part of the secondary wall program, we tested their promoter activation by the transcription factors *Pt*NST1 (PNAC085) and *Pt*MYB21. These *Populus* transcription factors are homologs of Arabidopsis NST1 (Hu *et al.*, 2010) and MYB46 (Zhong *et al.*, 2013), respectively, the master switches inducing the secondary wall formation program. *RWA-A* and *RWA-B* promoters were found to be strongly activated by *Pt*MYB21 and *Pt*NST1, but not *RWA-C* or *RWA-D* promoters (Fig. 2).

The same promoter-GUS constructs were transferred to hybrid aspen to study the tissue specificity of *RWA* promoters' activities in wood-forming tissues (Fig. 2c). The results indicate that *RWA-A* and -B promoters are both specifically activated in developing wood, but *RWA-A* much more strongly than *RWA-B*, while *RWA-C* and *-D* promoters apparently generated diffuse signals in all living cells of the xylem and other cell types in the stem. These patterns correspond well to data on *Pt*NST1 and *Pt*MYB21 activation (Fig. 2a,b) and to RT-PCR expression data (Ratke *et al.*, 2015).

Suppression of RWA AB and CD clades in hybrid aspen does not affect growth

To elucidate the biological function of *Populus RWA* genes in xylem tissues, hybrid aspen was transformed with RNAi

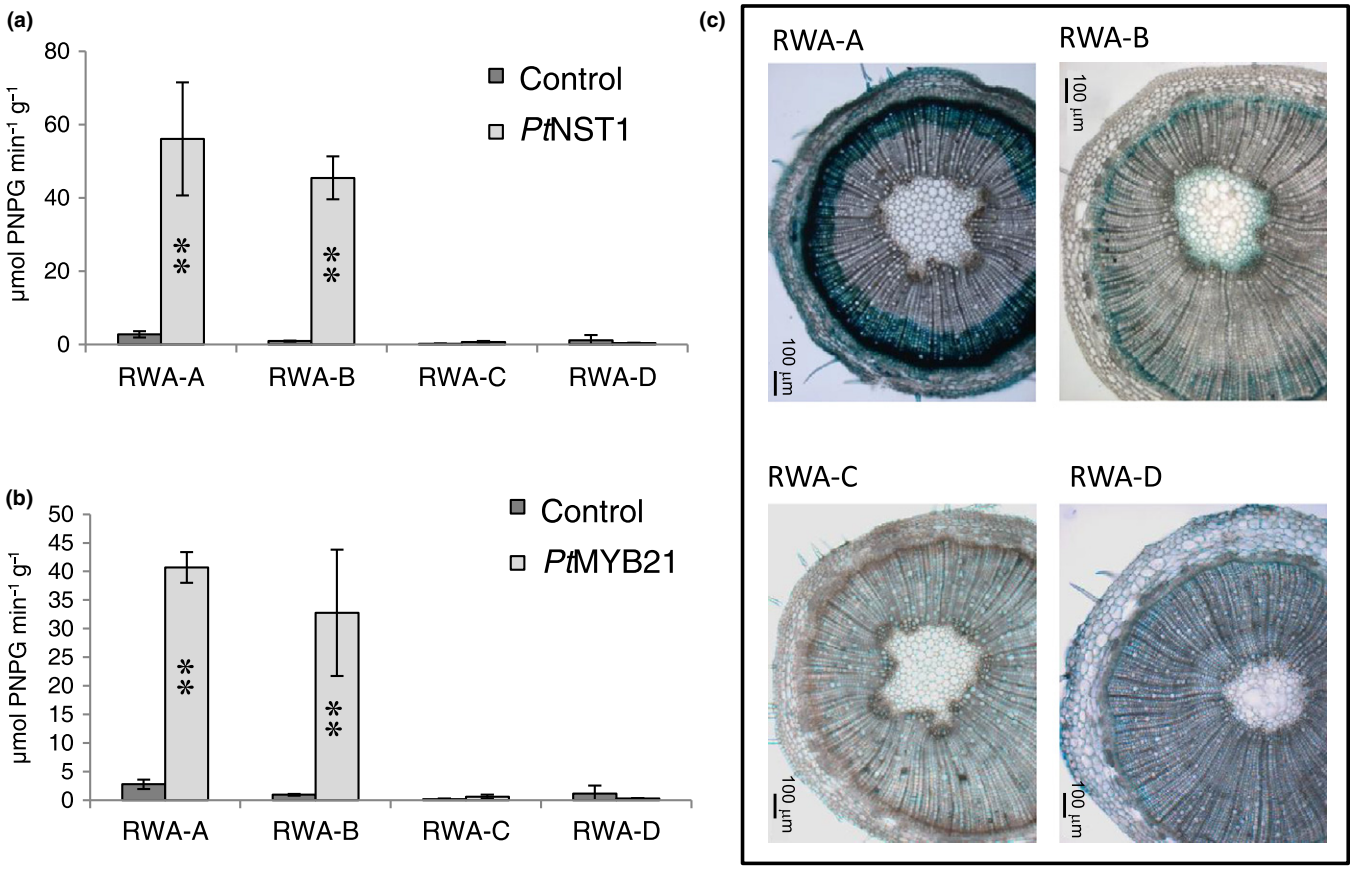

Fig. 2 Promoter activity of *Populus REDUCED WALL ACETYLATION* (*RWA*) genes. Activation of RWA promoters by (a) *Pt*NST1 and (b) *Pt*MYB21 transcription factors, according to β-glucuronidase (GUS) activities with *p*-nitrophenyl β-D-glucuronide (PNPG) substrate in extracts of tobacco leaves transfected with a reporter construct alone (control) or with reporter and effector constructs. The reporter constructs contained RWA promoters fused with GUS, while effector constructs contained the 35S promoter and either *Pt*NST1 or *Pt*MYB21. Data are means ± SD of $n = 3$ biological replicates. Asterisks indicate means significantly different from control: **, $P \leq 0.01$ (Student's *t*-test). (c) Results of histochemical analysis of GUS activities in stem internode 7 of pRWA::GUS transgenic hybrid aspen lines (representative patterns observed in the strongest GUS expressers of 10–15 transgenic lines screened for each promoter::GUS construct).

constructs designed to silence the AB or CD clade, under control of the *35S* constitutive promoter (Fig. 3). Two lines, selected from 20 independently generated using each construct, and WT plants, were grown for 2 months in the glasshouse, then expression of all *RWA* genes in their developing xylem tissues was analyzed (Fig. 3b). In lines carrying the *35S::AB-RWA* RNAi construct, transcript levels of the *RWA-A* target gene were approximately two-fold lower than in WT plants, but those of the *RWA-B* target were not significantly affected. In transgenic lines carrying the *35S::CD-RWA* RNAi construct, transcript levels of both target genes, *RWA-C* and *-D*, were approximately two-fold lower than WT levels (Fig. 3b).

Suppression of either clade did not visibly affect plant development (Fig. S4), it caused no consistent among-line effects on height, and no significant effects on stem diameter in any transgenic line (Table S3).

Both RWA clades participate in wood acetylation

To determine if the acetate content of the wood was affected by RWA gene suppression we quantified acetic acid released during saponification of transgenic and WT wood tissues. Approximately 15 and 20% less was released from samples of lines carrying the *35S::AB-RWA* and *35S::CD-RWA* RNAi constructs, respectively, than from WT samples (Fig. 4), strongly indicating that both clades contribute to wood acetylation.

To test whether the reduction in wood acetylation in transgenic RNAi lines is due to a decrease in xylan acetylation, we applied xylan oligosaccharide mass profiling (OLIMP) analysis (Chong *et al.*, 2011). Acidic XOS released by endoxylanases had degree of polymerization (DP) values ranging from 3 to 7 and were substituted with 0–7 acetyl groups (Fig. 4b). Detailed MS analysis and multivariate analysis of the data (Fig. S5) indicated that the most significant changes in the proportions of XOS in the transgenic lines were the shifts towards lower DP values and lower degrees of acetylation. For example, signals from MeGlc-Xyl6-Ac6, which was the most abundant XOS in the WT, were lower in transgenic lines, while signals corresponding to shorter and less acetylated XOS, such as MeGlcA-Xyl5-Ac3, MeGlcA-Xyl5-Ac2 and MeGlcA-Xyl3-Ac1, were higher. The overall DP decreased, as shown in the insets in Fig. 4(b). Similar shifts in XOS distributions have been observed in several Arabidopsis *rwa* mutants (Manabe *et al.*, 2013). Thus, the OLIMP analysis (Fig. 4b) revealed that xylan acetylation is reduced in both *AB* and *CD* RNAi lines, corroborating the hypothesis that both *Populus RWA* clades contribute to xylan acetylation in the wood.

Arabidopsis RWA proteins were shown to affect the acetylation in other cell wall polysaccharides beside xylan (Manabe *et al.*, 2011, 2013). To investigate if this is similar for aspen, we analyzed acetyl content of xyloglucan in the *AB* and *CD* RNAi lines. Xyloglucan is an abundant polymer of developing primary walled xylem (Mellerowicz *et al.*, 2001), and therefore we used the primary walled xylem tissues for its extraction. Xyloglucan acetylation was analyzed by OLIMP according to published protocols (Lerouxel *et al.*, 2002; Gille *et al.*, 2011). We observed small but statistically significant increases in the non-acetylated xylogluco-oligosaccharides (XGOs) and decreases in the sum of acetylated XGOs in the transgenic lines without apparent difference among the lines (Fig. 4c), indicating that the RWA proteins mediate also the acetylation of xyloglucan in aspen xylem.

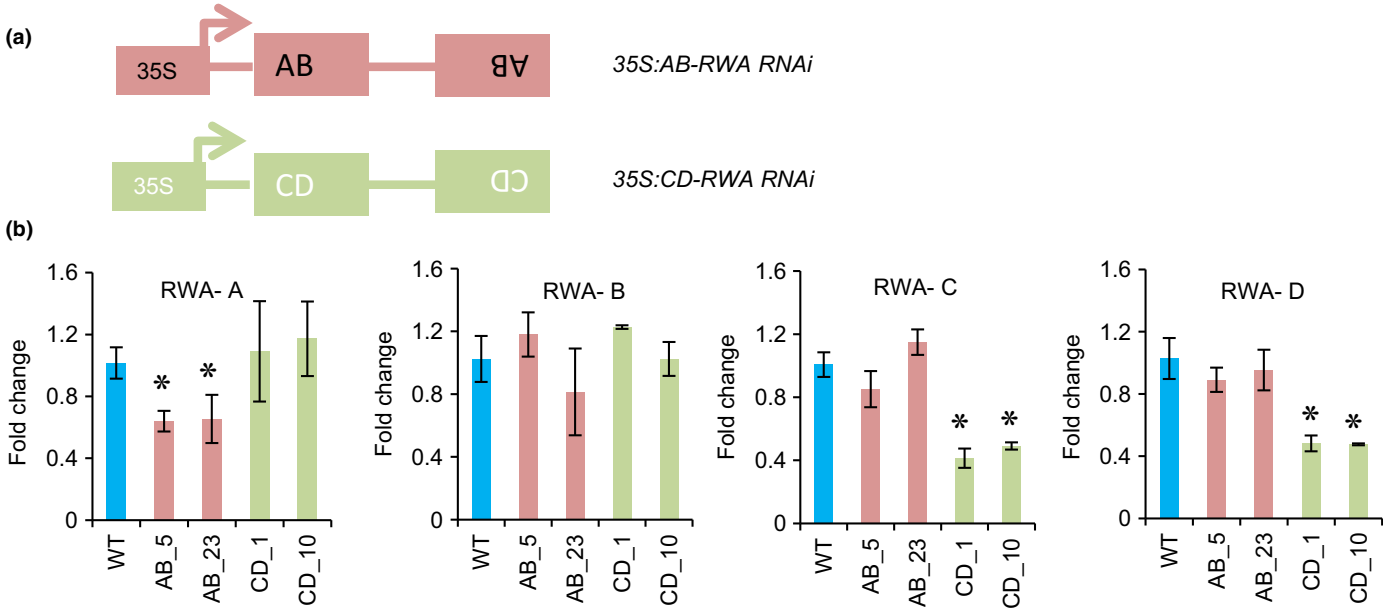

Fig. 3 Suppression of *Populus REDUCED WALL ACETYLATION* (*RWA*) genes in hybrid aspen by RNAi. (a) Schematic representation of RNAi vectors designed to suppress either *RWA-A* and *RWA-B* (*AB*) or *RWA-C* and *RWA-D* (*CD*). (b) Expression of RWA genes in developing xylem in the transgenic lines: transcript levels detected by RT-qPCR assays of independent transgenic lines carrying constructs *35S::AB-RWA RNAi* (AB_5, AB_23) or *35S::CD-RWA RNAi* (CD_1 and CD_10). Data are mean ± SE of $n = 3$–4 biological replicates. Asterisks indicate means significantly different from wild-type (WT): *, $P \leq 0.05$ (Student's *t*-test).

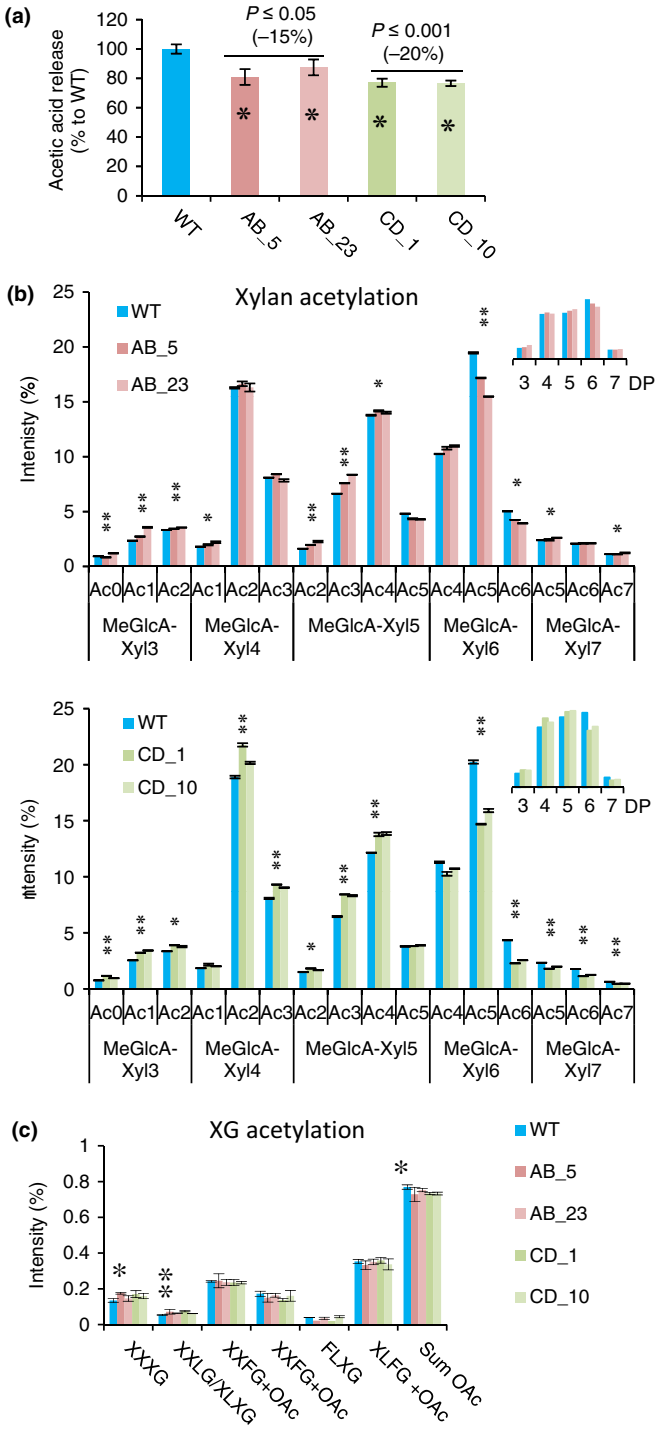

Fig. 4 Changes in acetylation of cell wall of transgenic lines with suppressed *REDUCED WALL ACETYLATION* (*RWA*) gene expression relative to wild-type (WT) of hybrid aspen. (a) Amounts of acetic acid released during saponification of ground wood powder. Data are means ± SE of $n = 3$–5 biological replicates. Asterisks indicate means significantly different from WT (Student's *t*-test). *P*-values above bars indicate the significance of differences between the transgenic lines and WT plants according to post-ANOVA contrast analysis. (b, c) Results of oligosaccharide mass profiling (OLIMP) analysis of (b) acidic xylo-oligosaccharides (XOS) in xylan revealing reductions in xylan acetylation in the wood or (c) xyloglucan-oligosaccharides (XGOS) revealing reductions in xyloglucan (XG) acetylation in the developing primary-walled xylem tissues of AB-RWA RNAi and CD-RWA RNAi transgenic trees. Relative intensities (%) of signals from indicated XOS and XGOS species released by xylanase and xyloglucanase, respectively, in powdered samples. In (b), the acidic XOS had degree of polymerization (DP) values from 3 to 7 (MeGlcA-Xyl3 to MeGlcA-Xyl7) with 0–7 acetyl groups (Ac0 to Ac7). The summed signals for each DP are shown in the insets to demonstrate overall reduction in the length of XOS in the transgenic lines. Data are means ± SE of $n = 2$ technical replicates representing pooled samples from five trees per transgenic line (b) or $n = 2$–3 biological replicates representing four trees (c). The asterisks above the WT bar indicate the significance of differences between WT and transgenic lines according to post-ANOVA contrast analysis:
**, $P \leq 0.01$; *, $P \leq 0.05$.

Reduction of xylan acetylation in the wood by suppressing all RWA genes under control of wood-specific promoters

Since both *RWA* clades were found to affect xylan acetylation in the wood, we designed a strategy to downregulate all four *RWA* genes in this tissue, involving use of a common gene fragment in an RNAi construct (Fig. 5a) driven by the wood-specific promoter of *PtGT43B* (Ratke *et al.*, 2015). Two transgenic lines (ABCD_11 and ABCD_15) were selected from 20 originating from independent transformation events according to gene expression analysis. RT-qPCR analysis confirmed that all of the target genes were expressed more weakly in the developing wood tissues in the transgenic lines than in WT plants (Fig. 5b). *RWA-A*, which is by far the most highly expressed *RWA* gene in developing wood, was most strongly affected, being expressed in the transgenic lines at *c.* 30% of WT levels.

The transgenic plants grew similarly to WT plants (Fig. S4). Quantitative analysis of morphological parameters detected no significant differences between them in either height or stem diameter (Table S4). However, wood acetyl contents in both *pGT43B::RWA-ABCD* RNAi lines were *c.* 25% lower than in WT plants (Fig. 5c). Xylan OLIMP analysis revealed changes in distribution patterns of acidic XOS, indicating that xylan acetylation was reduced (Fig. 5e). This is clearly shown by increases in the prominence of lightly acetylated XOS at the expense of highly acetylated XOS (e.g. in XOS with DP values of 6, 5 and 3) and similar shifts towards lower DP values, for example in MeGlcA-Xyl6-Ac5 and MeGlcA-Xyl 3-Ac1 contents.

NMR-detected differences in Xyl*p* substitution patterns between *pGT43B::RWA-ABCD* RNAi lines and WT

Acetyl esters are found on positions 2 and/or 3 of Xyl*p* and at position 3 of (Me)GlcA-substituted Xyl*p* of hardwood xylan (Teleman *et al.*, 2000, 2002). To determine if downregulation of RWA genes affected xylan structure, DMSO-extracted xylan was analyzed by 2D HSQC NMR following Chong *et al.* (2014). Signals from anomeric carbon and hydrogen corresponding to five types of Xyl*p* residues – nonsubstituted (X), mono-acetylated at position 2 (X2), mono-acetylated at position 3 (X3), di-acetylated (X23), and mono-acetylated at position 3 and substituted with (Me)GlcA at position 2 (X3G2) – were integrated (Fig. 5d). As there were overlaps in anomeric signals between X and X3, and between X2 and X23, the corresponding signals were back-calculated from either

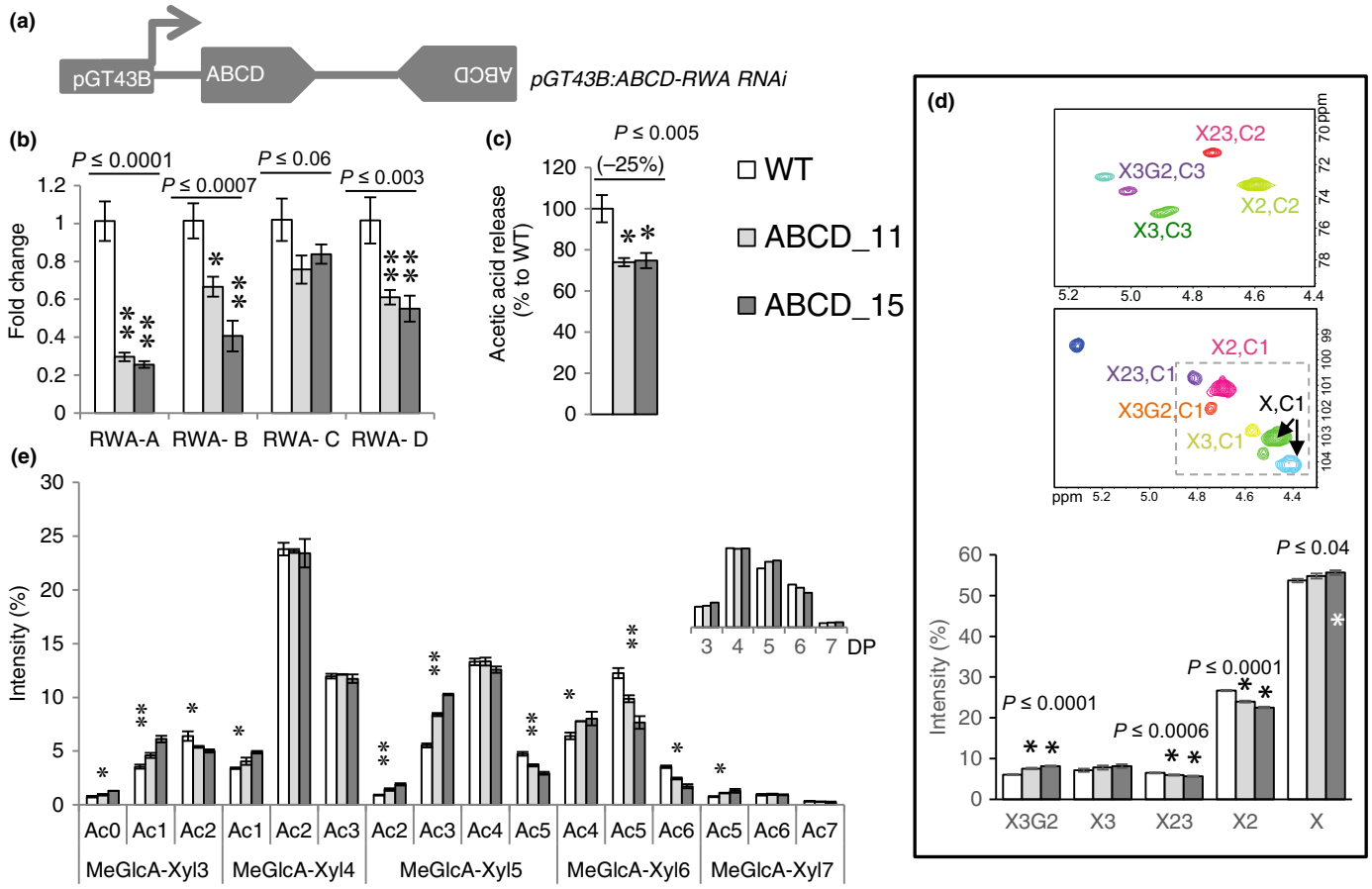

Fig. 5 Suppressing all *REDUCED WALL ACETYLATION* (*RWA*) genes by a construct with a wood-specific promoter reduces xylan acetylation in hybrid aspen wood. (a) Schematic representation of the construct. (b) Transcript levels of *RWA-A*, *-B*, *-C* and *-D* genes in the developing wood of the indicated transgenic lines determined by RT-qPCR; $n = 3$ biological replicates. (c) Amounts of acetic acid released from mature xylem tissues during saponification; $n = 3$–5 biological replicates. (d) Xylan structure according to 2D HSQC NMR analysis. X and G refer to Xyl and (Me)-glucuronosyl substituents, respectively, the number beside an X indicates the acetyl position in the xylopyranose ring, and the number after a G indicates its position on Xyl. The inset shows an example of the signals quantified in the bar graph (summed intensities of H1/C1 signals within the marked box were considered 100%); $n = 4$–6 biological replicates. (e) Results of oligosaccharide mass profiling (OLIMP) analysis of acidic xylo-oligosaccharides (XOS) in xylan. Labels are as in Fig. 3(b,c). The inset shows summed intensities for each XOS length to show changes in degree of polymerization (DP) of XOS released by xylanase in the transgenic lines; $n = 2$ technical replicates representing pooled samples from five trees. The asterisks above the wild-type (WT) bar indicate the significance of differences between WT and two transgenic lines according to post-ANOVA contrast analysis. Data in (b)–(e) are means ± SE. In (b), (c) and (d), asterisks indicate means significantly different from WT (Student's *t*-test) and *P*-values above bars indicate the significance of differences between WT and the two transgenic lines according to post-ANOVA contrast analysis. For all statistical tests: **, $P \leq 0.01$; *, $P \leq 0.05$.

C2/H2 or C3/H3 signals that were fully resolved with no overlap (inset in Fig. 5d), as in the cited study (Chong *et al.*, 2014). Reductions in relative abundances of X2, X3G2 and X23 units (but not X3) were detected in samples from the transgenic lines, compared with those in WT samples. These results indicate that acetylation of DMSO-extractable xylan of transgenic lines is specifically affected at position 2.

Change in cell wall composition in pGT43B::RWA-ABCD RNAi transgenic trees

To explore effects of xylan deacetylation on overall cell wall composition, we subjected samples of powdered wood to pyrolytic GC-MS analysis. No differences between the WT and transgenic lines were detected in total lignin contents, total carbohydrate contents or lignin monomeric composition (Table S5). The syringyl : guaiacyl ratio was slightly, but nonsignificantly, lower in transgenic lines. To further test effects of xylan deacetylation on wood composition, wood samples were sequentially extracted and their contents of extractives, lignin, hemicellulose and cellulose were determined (Ona *et al.*, 1995). Proportions (by weight) of extractives and lignin were unaffected, but alkali-extractable hemicellulose proportions were reduced and those of cellulose slightly increased, in samples from the transgenic lines relative to WT samples (Table S6). However, no significant differences in acid-resistant (Updegraff) cellulose contents of their extractive-free wood were detected (Table 1), suggesting that the increase in weight of the cellulose fraction was due to lower extractability of hemicelluloses. Similarly, there were no differences in Klason lignin contents of the extractive-free wood, but monosaccharide analysis of noncrystalline cell wall polysaccharides following acid methanolysis revealed a small decrease in Xyl together with

Table 1 Monosaccharide composition (mol%), cellulose content (%) and Klason lignin content (%) of extractive-free wood from transgenic and wild-type (WT) hybrid aspen lines

Line	Ara	Rha	Fuc	**Xyl***	Man	**MeGlcA***	Gal	GalA	**Glc***	GlcA	Cellulose	Klason lignin
WT	3.08 ± 0.08	4.69 ± 0.15	0.71 ± 0.01	60.89 ± 0.81	3.23 ± 0.14	5.73 ± 0.19	1.90 ± 0.13	5.32 ± 0.13	13.08 ± 0.53	1.37 ± 0.03	42.4 ± 1.17	17.4 ± 1.26
ABCD_11	3.09 ± 0.21	4.21 ± 0.17	0.76 ± 0.03	59.08 ± 0.90	3.38 ± 0.11	6.28 ± 0.14	1.73 ± 0.09	5.24 ± 0.06	14.85 ± 0.90	1.42 ± 0.08	42.0 ± 1.78	16.7 ± 0.51
ABCD_15	3.23 ± 0.11	4.41 ± 0.21	0.76 ± 0.04	56.70 ± 0.40*	3.40 ± 0.07	6.40 ± 0.13*	2.08 ± 0.07	5.38 ± 0.15	16.05 ± 0.34*	1.59 ± 0.05*	43.3 ± 1.92	16.9 ± 0.10

Data are means ± SE of $n = 3$ biological replicates. Asterisks after numbers indicate means significantly different from WT (Student's *t*-test). Bold type indicates significant differences between the two transgenic lines and WT plants according to post-ANOVA contrast analysis. *, $P \leq 0.05$.

increases in Glc and MeGlcA in transgenic lines (Table 1). Furthermore, proton spin relaxation editing (PSRE) analysis (Newman & Hemingson, 1990) detected no major differences in cellulose crystallinity between ground wood samples from transgenic and WT plants (Table S7).

RWA-ABCD suppression improved saccharification efficiency

Reductions in xylan acetylation generally increase its accessibility to hydrolytic enzymes (Poutanen *et al.*, 1990; Kong *et al.*, 1992; Selig *et al.*, 2009; Zhang *et al.*, 2011). To test the consequent possibility that the reduced xylan acetylation in the transgenic lines improved their cell walls' digestibility, their woody biomass was subjected to enzymatic hydrolysis by a commercial saccharification enzyme mixture, with and without acid pretreatment.

Enzymatic hydrolysis without pretreatment yielded more sugars (Ara, Gal, Glc, Xyl and Man) from the wood of transgenic lines than from the wood of WT plants (Fig. 6): 20% more per unit weight of wood, on average, including *c.* 30 and 20% more Xyl and Glc, respectively. Since wood from the transgenic lines had *c.* 20% higher noncrystalline Glc contents (Table 1), and 4% more alpha cellulose (Table S6), but the same crystalline cellulose contents, as wood from WT plants (Table 1), the higher Glc yield could be due to compositional changes and/or higher digestibility. Comparison of Glc contents of Updegraff cellulose and the acid-labile sugar fraction to the Glc yields indicates that the enzymes converted *c.* 14% more of the Glc in transgenic lines (Fig. 6c), and Xyl conversion rates were even more strongly increased (by 40%). These findings suggest that transgenic plants contained more digestible xylan and cellulose than WT plants and (hence) yields of all sugars from them were higher.

When woody biomass was pretreated with 1% sulfuric acid and the residue was subjected to enzymatic hydrolysis, the combined acid and enzymatic hydrolysis yields from samples of the transgenic lines were higher than WT yields for Glc, but lower for some minor sugars such as Man and Ara (Fig. 6b). Pretreatment-extracts of transgenic samples contained less Ara and Man, but more Glc, than WT samples (Table 2). Glc yields from enzymatic hydrolysis of these samples after pretreatment were also slightly higher than those of WT samples (inset in Fig. 6b). Conversion rates of Glc and Xyl in transgenic lines, based on combined yields from acid and enzymatic hydrolyses, were not significantly different from those obtained for WT samples. These data indicate that deacetylation of xylan has a small positive effect on Glc yields from saccharification with acid pretreatment, due to increases in yields from acid and enzymatic hydrolysis, but a small negative effect on yields of the minor hemicellulose sugars (Ara and Man).

Discussion

Both clades of *Populus* RWA genes participate in xylan acetylation in the wood

Our phylogenetic analysis showed that *Populus RWA* genes are members of two clades identified in embryophytes, *AB* and *CD*,

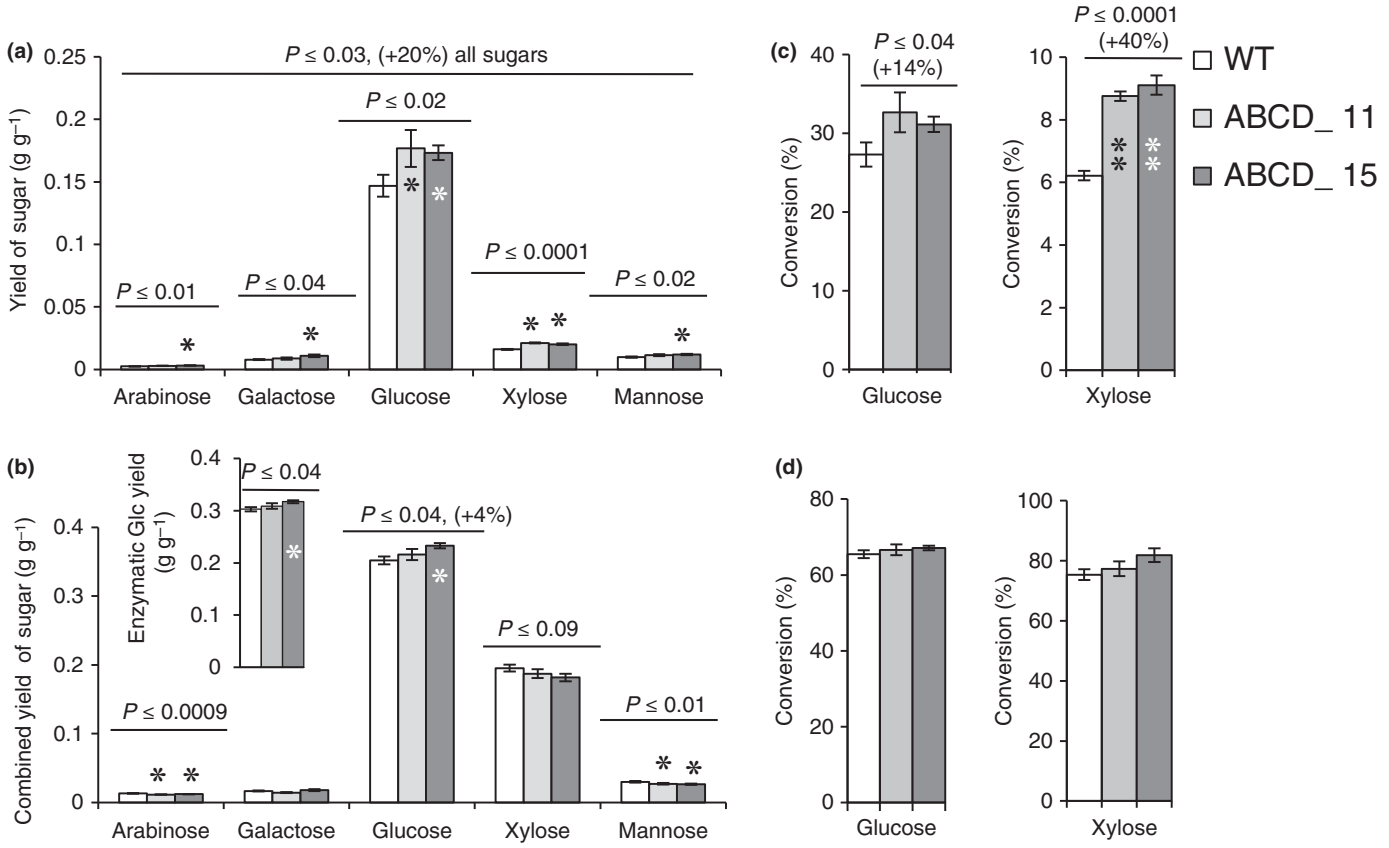

Fig. 6 Effects of suppressing *AB* and *CD REDUCED WALL ACETYLATION* (*RWA*) genes by a construct with a wood-specific promoter on wood saccharification in hybrid aspen. (a, b) Sugar yields (g g^{-1} dry wood) from enzymatic hydrolysis without pretreatment and combined yields from acid pretreatment and enzymatic hydrolysis, respectively. The inset shows the Glc yield from enzymatic saccharification. (c) Glc and Xyl conversion rates (%) in saccharification without pretreatment. (d) Glc and Xyl conversion rates of acid pretreament and enzymatic hydrolysis. Water weights were considered when calculating theoretical Glc and Xyl contents (weights) of wood samples. Data are means ± SE of $n = 8$–10 biological replicates. Asterisks indicate means significantly different from wild-type (WT) (Student's *t*-test). *P*-values indicate the significance of differences between samples from the two transgenic lines and WT plants according to post-ANOVA contrast analysis. For all statistical tests: *, $P \leq 0.05$; **, $P \leq 0.01$.

Table 2 Relative proportions (%, w/w) of the indicated sugars in acid pretreatment extracts of transgenic and wild-type (WT) hybrid aspen samples

Line	**Ara***	Gal	**Glc***	Xyl	**Man***
WT	4.27 ± 0.12	5.04 ± 0.19	16.75 ± 0.48	64.67 ± 0.61	9.28 ± 0.15
ABCD_11	4.00 ± 0.07*	4.45 ± 0.20	18.58 ± 0.95	64.27 ± 1.13	8.70 ± 0.17*
ABCD_15	4.02 ± 0.08	5.57 ± 0.35	19.59 ± 1.07*	62.39 ± 0.86	8.43 ± 0.19*

Data are means ± SE of $n = 8$–10 biological replicates. Asterisks next to numbers indicate means significantly different from WT (Student's *t*-test). Bold type indicates significant differences between the two transgenic lines and WT plants according to post-ANOVA contrast analysis. *, $P \leq 0.05$.

and orthologous sequences are present in other dicotyledonous species including Arabidopsis, tomato, grape vine and eucalyptus (Fig. 1). Since the basal flowering species *A. trichopoda* and the moss *P. patens* only have members of the *CD* clade, this clade appears to be basal and the *AB* clade presumably evolved from it after the emergence of flowering plants. By contrast, analyzed grasses (rice and maize) have clear *AB* orthologs, but apparently lack *CD* orthologs, suggesting that grasses may have lost *CD* genes and instead have a separate clade that is not present in other angiosperms but is more similar to the *AB* than to the *CD* clade, suggesting that it may have evolved from *AB* during evolution of the grass lineage.

Arabidopsis has one member of the *CD* clade (*RWA2*), which appears to be primarily responsible for acetylation of primary wall matrix components, XG and pectins, while members of the *AB* clade (*RWA1*, *RWA3* and *RWA4*) play more prominent roles in xylan acetylation (Lee *et al.*, 2011; Manabe *et al.*, 2011, 2013). This may be due to *RWA2* being more strongly expressed in primary walled tissues, and less strongly responsive to SND1, than genes of the *AB* clade (Lee *et al.*, 2011). Similarly, in *Populus*, both clade *AB* genes (but not *CD* genes) were clearly transactivated by the secondary wall master switches *Pt*NST1 and *Pt*MYB21, and specifically upregulated in the wood-forming tissues (Fig. 2). Thus, the two clades seemed to diverge in their

expression patterns, with the *AB* clade acquiring the expression within the secondary wall biosynthesis program. However, biochemical activities of the clades may be similar or partially overlapping. This notion is supported by the reported ability of *RWA2* alone to complement the dwarfism and acetyl content deficiency of *rwa* quadruple mutants (Manabe *et al.*, 2013). By contrast, some combinations of triple mutations reportedly result in different perturbations of acetylation in XG relative to other polymers (Manabe *et al.*, 2013). These variations in defects could result from reductions in dosage of RWA activities in particular tissues, or subtle functional differences among the RWA proteins, such as their interactions with specific TBL proteins conferring specificities to different polymers and sites on these polymers (Gille *et al.*, 2011; Xiong *et al.*, 2013; Yuan *et al.*, 2013, 2016a, b). Interestingly, in Arabidopsis the *AB* clade alone is sufficient for maintaining cell wall acetylation in the stem tissues (Manabe *et al.*, 2011), but not in hybrid aspen, where both clades (*AB* and *CD*) seem to be important for xylan acetylation in the wood and for wood acetylation (Fig. 4). Whether this is due to dosage effects or functional specificities remains to be established.

Effects of reducing RWA activity on secondary walls

The downregulation of RWA is expected to decrease acetyl-CoA pools in the Golgi, thereby limiting activities of acetyl transferases (Gille & Pauly, 2012). Accordingly, we found that specific downregulation of both *RWA* clades in developing *Populus* wood during the secondary wall formation stage resulted in reductions in wood acetyl contents and xylan acetylation (Figs 4, 5). Furthermore, xylan with unsubstituted position C-2 can be used as a substrate for the Golgi-residing glucuronyl transferases that apparently compete with acetyl transferases (Yuan *et al.*, 2013; Chong *et al.*, 2014; Lee *et al.*, 2014; Xiong *et al.*, 2015). We observed corresponding increases in MeGlcA contents of noncellulosic sugars in plants with reduced *RWA* expression (Table 1), and increased proportions of acetylated (Me)GlcA-substituted Xyl*p* residues in DMSO extracts of their xylan (Fig. 5d).

RWA suppression in hybrid aspen resulted in preferential deacetylation at position C-2 in DMSO-extracted xylan, as shown by reductions in X2 and X23 signals in 2D HSQC NMR experiments (Fig. 5). Similarly, the acetylation in *rwa1/2/3/4* quadruple mutants is most affected at C-2, according to Lee *et al.* (2014). As RWA proteins are not directly involved in acetylation, this result probably reflects an indirect effect. For example, RWA proteins could specifically interact with C-2-specific acetyl transferases such as TBL-29 (Urbanowicz *et al.*, 2014). Moreover, the spontaneous migration of acetyl groups is affected by the acetyl content (Mastihubova & Biely, 2004) and thus it might differ between the RWA-suppressed lines and WT.

Our results, and previous studies of mutants affected in xylan acetylation biosynthesis, such as *rwa1rwa2rwa3rwa4* (Lee *et al.*, 2011; Manabe *et al.*, 2013), *axy9* (Schultink *et al.*, 2015), *tbl29* (*esk1*) (Xiong *et al.*, 2013; Yuan *et al.*, 2013), and the double and triple mutants in *tbl* genes from the *ESK1* clade combined with *esk1* (Yuan *et al.*, 2016a–c) indicate that at least 70% of WT xylan acetylation, which roughly corresponds to 70% of WT cell wall acetyl content in the stem tissues, is required for proper biosynthesis of secondary cell wall, whereas reductions by 30% or larger lead to growth penalty and aberrant secondary wall formation (cf. compiled summary in Table S8). Interestingly, larger reductions of cell wall acetyl content (by 32–50%) are possible, without any adverse effects on plants, either when the acetyl groups are removed in the cell wall by wall-resident esterases (Pogorelko *et al.*, 2013; Pawar *et al.*, 2016), or when the reductions are compensated by (Me)GlcA side chains added in lieu of acetyl groups to the xylan backbone during its biosynthesis in the Golgi (Xiong *et al.*, 2015). These observations are consistent with the view that precipitation of highly deacetylated and de-branched xylan (Grondahl *et al.*, 2003) may affect its biosynthesis in the Golgi and/or delivery to the cell wall. Moderately deacetylated xylan can probably be normally secreted to cell walls, but it is expected to have more free hydroxyl groups available for contacts with hydrophilic surfaces of cellulose microfibrils (Busse-Wicher *et al.*, 2014), which would probably affect the self-assembly of cellulose–hemicellulose networks. We have not observed any major perturbations of cellulose structure in the transgenic lines as characterized by NMR spectroscopy, but the higher Glc yields from acid methanolysis of their noncrystalline polysaccharides and increased alpha-cellulose contents may reflect such subtle changes in cell wall cellulose assembly.

The presence of partially deacetylated xylan in cell walls should hypothetically affect *in muro* xylan metabolism, as it should theoretically be more susceptible to wall-residing GH10 enzymes such as *Ptxt*Xyn10A, which has been found in aspen wood (Derba-Maceluch, *et al.*, 2015). This hypothesis is consistent with the reductions in Xyl and hemicellulose contents in the wood of transgenic lines (Tables 1, S6). GH10 enzymes cannot bind to the xylan backbone if Xyl*p* residues interacting at subsites −1 or +2 have acetyl group substituents, whereas they can probably tolerate monoacetylation at position 3 at the subsite −2 (Pell *et al.*, 2004; Busse-Wicher *et al.*, 2014; Chong *et al.*, 2014), so acetylation, which occasionally occurs on adjacent Xyl*p* residues in the xylan chain (Busse-Wicher *et al.*, 2014; Chong *et al.*, 2014), may protect these regions from degradation by these enzymes. However, deacetylated xylan can be cleaved and then metabolized or presumably removed with the transpiration flow. Reductions in Xyl contents have also been reported in *tbl29* mutants complemented with GUX1, which have highly deacetylated xylan (Xiong *et al.*, 2015). Conversely, *gux1gux2* double mutants, in which monoacetylation is reportedly increased, have increased cell wall Xyl contents indicative of xylan (Chong *et al.*, 2014, 2015), consistent with the protection of xylan in cell walls by acetylation.

Reducing wood acetylation by suppressing RWA genes improves saccharification

Suppressing RWA using an RNAi construct with a wood-specific promoter clearly facilitated saccharification of the resulting wood (Fig. 6). This particularly applied to enzymatic saccharification

without pretreatment, where it enhanced yields of all sugars per unit wood dry weight by *c.* 20%, and yields of matrix polysaccharide sugars even more, for example Xyl and Gal yields by 30 and 25%, respectively. Glc and Xyl conversion degrees were also substantially increased, by 14 and 40%, respectively. Effects were smaller with acid pretreatment. In this case, the Glc yields were slightly increased (by 4%), due to both extraction of more Glc during acid pretreatment and higher enzymatic hydrolysis after pretreatment (Fig. 6b; Table 2). However, the yields of matrix polysaccharide sugars per unit wood dry weight were slightly decreased. The lower Xyl yield per unit wood dry weight was due to its reduced content in cell walls (Table 1), as Xyl conversion in samples from the transgenic lines remained high (Fig. 6d). These data and results of our previous Arabidopsis analyses (Pawar *et al.*, 2016) demonstrate a clear link between reductions in cell wall acetylation and improvements in saccharification yields and conversion degrees. Previously, we used the postsynthetic deacetylation approach to remove acetyl groups from the xylan already secreted to cell walls (Pawar *et al.*, 2016). Here we used a different strategy, suppressing acetylation during biosynthesis in the Golgi, and in both cases positive effects on saccharification were detected. The reduced acetylation was demonstrated here in xylan and xyloglucan, the main hemicelluloses in aspen mature and developing wood, respectively, but if RWA genes are responsible for maintenance of acetyl-CoA pools in the Golgi, similar reductions in acetylation of several other polymers are likely, as previously observed in Arabidopsis *rwa* mutants (Lee *et al.*, 2011; Manabe *et al.*, 2011, 2013). In particular, reduced acetylation of glucomannan could be relevant for the saccharification due to its anticipated tight interaction with cellulose (Åkerholm & Salmén, 2001). Reductions in matrix polysaccharide acetylation could enhance saccharification yields and/or conversion rates by allowing the binding of hydrolytic enzymes to the polymers, as previously demonstrated (Biely, 2012; Pawar *et al.*, 2013). However, the observation that lignocellulose of Arabidopsis plants expressing fungal acetyl xylan esterase exhibited higher sugar production rates even after alkali pretreatment that hydrolyzes acetyl esters (Pawar *et al.*, 2016) suggests strongly that benefits of deacetylation may partially lie in changes in cell wall architecture occurring when acetyl groups are removed. Xylan deacetylation could affect its interactions with other polymers, especially cellulose (Busse-Wicher *et al.*, 2014), as discussed earlier, and lignin (Reis & Vian, 2004; Giummarella & Lawoko, 2016), leading to enhanced cell wall saccharification.

Together, the current and previous (Pawar *et al.*, 2016) results suggest that reducing cell wall acetylation by 15–25% has positive overall effects on the saccharification potential of woody biomass, and that plants can withstand such manipulation without detrimental effects on their growth and development. However, higher degrees of deacetylation may be detrimental for cell wall biosynthesis, as discussed earlier, leading to irregular xylem phenotypes and dwarfism. Secondary effects of such severe deacetylation on plant development are likely to be causes of the lack of improvement in saccharification reported by other studies (Lee *et al.*, 2011; Xiong *et al.*, 2013). In addition, the modification in the present study was targeted to a specific tissue to limit potential unintended effects. A different strategy for reducing cell wall acetylation was recently tested in Arabidopsis, involving prevention of negative effects of deacetylation on cell wall formation by introducing GlcA substitutions on the xylan backbone, which rescued the dwarf phenotype caused by deacetylation (Xiong *et al.*, 2015). However, this strategy is not suitable for lignified tissues such as wood, as it could lead to more lignin–carbohydrate bonds via (Me)GlcA, which are known to inhibit saccharification (Min *et al.*, 2014a,b), nullifying a positive effect of deacetylation.

In conclusion, we have shown that two clades of the *RWA* gene family contribute to xylan acetylation in hybrid aspen wood: one comprising *RWA-A* and *RWA-B* genes (parts of the secondary wall biosynthetic network) and another comprising *RWA-C* and *RWA-D*, which are ubiquitously expressed. Suppression of *RWA* genes during secondary wall formation slightly alters xylem sugar composition without affecting lignin content or composition, and improves saccharification without pretreatment. The growth of such plants is not affected. Thus, reducing secondary cell wall acetylation is a viable strategy for increasing the saccharification potential of plants' biomass.

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Author contributions

P.M-A.P. performed most of the work and wrote the paper with contributions from all authors; C.R. made the transgenic lines; V.K.B. performed gene expression analyses; S-L.C. and M.T. were responsible for xylan fingerprinting analyses; M.L.G and L.J.J. carried out saccharification assays; M.A. and I.E. performed the transactivation assays; T.S. and M.H. performed NMR analyses; K.S. and M.D-M. performed GUS histochemical analyses; G.M. assisted by C.G. performed xyloglucan OLIMP analysis; E.J.M. conceived and supervised the project, and finalized the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Amino acid sequence alignment of RWA proteins from *Arabidopsis thaliana* and *Populus trichocarpa*.

Fig. S2 Bioinformatic analysis of RWA proteins in *Populus trichocarpa*.

Fig. S3 *Populus tremula* RWA gene expression in different organs and physiological conditions based on publically available RNA seq data visualized at www.popgenie.org.

Fig. S4 Representative picture of 8-wk-old transgenic aspen trees grown in the glasshouse.

Fig. S5 Multivariate analysis of the OLIMP data presented in Figs 4(b) and 5(e) using orthogonal projections to latent squares (OPLS) modeling.

Table S1 Primers for qPCR

Table S2 Primers used for cloning

Table S3 Morphological parameters of 8-wk-old transgenic trees carrying 35S::AB-RWA RNAi and 35S::CD-RWA RNAi constructs

Table S4 Morphological parameters of 8-wk-old transgenic trees carrying PtGT43B::ABCD-RWA RNAi construct

Table S5 Cell wall composition analyzed by Pyrolysis GC-MS of *PtGT43B::ABCD-RWA* RNAi and WT trees

Table S6 Wood composition of *PtGT43B::ABCD-RWA* RNAi and WT trees

Table S7 Cellulose crystallinity in the ground wood of transgenic lines and WT analyzed by NMR employing the PSRE method

Table S8 Summary of previously reported effects of altered cell wall acetylation on cell walls, plant morphology and saccharification

Method S1 Method of cellulose crystallinity analysis by NMR.

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