Suppression of xylan endotransglycosylase PtxtXyn10A affects cellulose microfibril angle in secondary wall in aspen wood

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Suppression of xylan endotransglycosylase \textit{PtxtXyn10A} affects cellulose microfibril angle in secondary wall in aspen wood

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Supporting Information: Fig. S1 – S6, Method S1, Tables S1 – S6.

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Summary

- Certain xylanases from family GH10 are highly expressed during secondary wall deposition, but their function is unknown. We carried out functional analyses of the secondary-wall specific PtxtXyn10A in hybrid aspen (*Populus tremula* L. × *tremuloides* Michx.).
- *PtxtXyn10A* function was analysed by expression studies, overexpression in Arabidopsis protoplasts and by downregulation in aspen.
- *PtxtXyn10A* overexpression in Arabidopsis protoplasts resulted in increased xylan endotransglycosylation rather than hydrolysis. In aspen, the enzyme was found to be proteolytically processed to a 68 kDa peptide and residing in cell wall. Its downregulation resulted in a corresponding decrease in xylan endotransglycosylase activity and no change in xylanase activity. This did not alter xylan molecular weight or its branching pattern but affected the cellulose-microfibril angle in wood fibres, increased primary growth (stem elongation, leaf formation and enlargement) and reduced the tendency to form tension wood. Transcriptomes of transgenic plants showed downregulation of tension wood related genes and changes in stress-responsive genes.
- The data indicate that *PtxtXyn10A* acts as a xylan endotransglycosylase and its main function is to release tensional stresses arising during secondary wall deposition. Furthermore, they suggest that regulation of stresses in secondary walls plays a vital role in plant development.

**Key words:** *Populus*, hybrid aspen, secondary cell wall, wood formation, xylanase, xylan endotransglycosylase, cellulose microfibril angle, growth stresses
Introduction

Xylans are among most abundant polysaccharides found in nature (Ebringerová & Heinze, 2000; Scheller & Ulvskov, 2010). They are polymers with a $\beta$-1,4-D-xylopyranose backbone and include homoxylans and heteroxylans, such as arabinoxylans, glucuronoxylans and glucuronoarabinoxylans. Glucuronoxylans are abundant in the secondary walls of dicotyledonous species, where they are the main hemicellulose, comprising roughly one fourth of wood biomass, whereas arabinoxylans and glucuronoarabinoxylans are found in type II primary cell walls of grasses and secondary walls of conifers, respectively. Small amounts of glucuronoarabinoxylans are also present in the primary cell walls of eudicots and lower vascular plants (Darvill et al., 1980; McCartney et al., 2006; Brummell & Schröder, 2009). The importance of understanding the biosynthesis and modification of xylans in plants is emphasised by the increasing significance of plant biomass as a potential source of renewable energy and use of hemicelluloses as food additives and pharmacologically active ingredients (Bevan & Franssen, 2006).

In *Populus* wood, xylans have a backbone of approximately 100 units long with side chains of 4-\(O\)-methyl-\(\alpha\)-D-glucuronic acid (Me-\(\text{GlcUA}\)) at \(O\)-2 in approximately every tenth xylose residue (Timell, 1967; Teleman et al., 2000). In addition, approximately 50% of the xylose residues are \(O\)-acetylated at the C-2, C-3 or both positions (Naran et al., 2009). An oligosaccharide containing $\beta$-D-Xyl-(1,4)-$\beta$-D-Xyl-(1,3)-$\alpha$-L-Rha-(1,2)-$\alpha$-D-GalUA-(1,4)-D-Xyl resides at the reducing end of the *Populus* xylan, similar to found in other eudicots and conifers (Lee et al., 2011). In secondary walls, glucuronoxylans are thought to interact with lignin via ester bonding to GlcUA and Me-GlcUA (Imamura et al., 1994; Spániková & Biely, 2006; Spániková et al., 2007; Li et al., 2007).

The biosynthesis of xylan involves several different classes of glycosyltransferases (GTs) that make up the backbone, the reducing end sequence and different side chains (recently reviewed by Rennie & Scheller, 2014). These enzymes reside in the Golgi apparatus, where they probably form synthesising complexes along with other enzymes involved in the methylation of glucuronate side chains and acetylation of the backbone. The
preformed xylan is deposited in the cell wall, where it associates with cellulose microfibrils by hydrogen bonding (Kabel et al., 2007; Busse-Wicher et al., 2014) and may traverse several wall layers or be modified in muro, resulting in xylan epitope accumulation in the outer wall layers (Awano et al., 2002). Different types of xylan-acting enzymes are known to reside in plant cell walls: endo-1,4-β-xylanase (EC 3.2.1.8), xylan endotransglycosylase (also known as trans-β-xylanase), 1,4-β-xylosidase (EC 3.2.1.37) and bifunctional α-arabinofuranosidase/β-xylosidase (Goujon et al., 2003; Fry, 2004; Minic & Jouanin, 2006; Ichinose et al., 2010; Franková & Fry, 2011; 2013; Johnston et al., 2013).

Plant endo-1,4-β-xylanases belong to the glycoside hydrolase family 10 (GH10) and appear to be involved in xylan modification in primary and secondary walls, but their function is only understood for tissues undergoing decomposition involving digestion of cell wall xylan (Paull & Chen, 1983; Benjavongkulchai & Spencer, 1986; Slade et al., 1989; Banik et al., 1996; Cleemput et al., 1997a, b; Bih et al., 1999; Wu et al., 2002; Simpson et al., 2003; Chen & Paull, 2003; Suen & Huang, 2006). GH10 enzymes are also known to be highly expressed in xylem, but their function in this tissue is not yet clear (Mellerowicz et al., 2001; Suzuki et al., 2002; Geisler-Lee et al., 2006). One of these genes, PtxtXyn10A, which shows high similarity to AtXyn1 (Suzuki et al., 2002), has been found to be upregulated during xylem secondary cell wall formation in hybrid aspen (Populus tremula L. x tremuloides Michx.) (Hertzberg et al., 2001; Aspeborg et al., 2005). Therefore, to investigate its function during xylogenesis, we analysed its activity, expression and effects of its suppression in hybrid aspen. We found that PtxtXyn10A acts mainly as a xylan endotransglycosylase and affects the cellulose microfibril angle (MFA) and other aspects of plant development. Based on our data, we propose that the main function of Xyn10A in secondary walls is to release mechanical stress arising during cell wall deposition.
Materials and Methods

Cloning of full length PttxXyn10A

A partial clone of PttxXyn10A was identified among the EST clones from a cambial region cDNA library of hybrid aspen (Sterky et al., 2004). 5’ RACE was carried out and full-length clones were obtained, cloned into the pGEM-T Easy vector (Promega, USA) and sequenced. The cDNA GenBank accession number is AY935501.

Plant material and growth conditions

Hybrid aspen, *Populus tremula* L. x *tremuloides* Michx., trees (clone T89) were grown in a greenhouse with a long photoperiod as described previously (Gray-Mitsumune et al., 2008) until they reached approximately 2 m in height.

RT-qPCR analysis

One µg of total DNA-free RNA isolated from the primary- and secondary-walled developing xylem or transformed *A. thaliana* protoplasts was used for reverse transcription using an iScriptTM cDNA biosynthesis kit (Bio-Rad). Primers (Table S5) were designed using QuantPrime (http://www.quantprime.de) and Primer3Web 0.4.0 (http://primer3.sourceforge.net). The best reference gene (CYP in aspen and EF1a and UBQ5 in *Arabidopsis*) was selected using GeNorm (http://www.bigazelle.com; Vandesompele et al., 2002) among ADF6, actin, UBQ CYP, Eif1a, clatrin and APT. An iQ™ SYBR Green Supremix (BioRad) kit was used and Cq values were acquired using a Light Cycler 480 1.5.0.sp3 (Roche). Relative expression was calculated as $E_T^{(Cq1-Cq2)/E_R}$ in aspen (where $E_T$ and $E_R$ are the efficiencies of the target and reference genes, respectively, and Cq1 and Cq2 are the Cq levels for the sample and control, respectively) or as $2^{-\Delta\Delta Cq}$ (Livak et al., 2001) in Arabidopsis.

Immunoblotting

For the production of polyclonal antibody Kamisa detecting PttxXyn10A, a cDNA fragment from the clone A020P21 (accession number AI162606) encoding a C-terminal PttxXyn10A fragment was cloned into pAFF8c-3c (Larsson et al., 2000). The recombinant protein was produced in *E. coli* and purified using TALON® protein
purification columns (Clontech, USA). The purified 66 kDa soluble recombinant protein was used as antigen. For the production of antibody Abbe against the PtxtCel9B3 protein, a protein based on the full-length cDNA clone (accession number AY660968) was used in a similar fashion. The polyclonal antibodies were produced in rabbits by Agrisera AB, Sweden.

Soluble proteins were extracted according to Biswal et al. (2014), and cell wall bound proteins were extracted from the remaining pellet by incubation in Laemmli buffer (10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (v/v) SDS, 62.5 mM Tris-HCl, pH 6.8) at 100°C for 10 min. The suspension was cooled to room temperature, subjected to centrifugation at 15000 g for 20 min, then 30 µg of each protein sample was loaded onto a NuPage® Novex Bis-Tris gel (Invitrogen, USA) and blotted onto a nitrocellulose membrane. The membrane was probed overnight at 4°C with a 1:1000 dilution of Kamisa antibody. Signals were revealed using the Amersham ECL System. The same membrane was subsequently probed with the antibody Abbe raised against a cell wall bound carbohydrate PtxtCel9B3 (Takahashi et al., 2009).

**Intracellular localization of PtxtXyn10A**

PtxtXyn10A cDNA was subcloned into the binary vector pEarleyGate103 using the primers listed in Table S6. Arabidopsis was transformed using the floral dip method (Clough & Bent, 1998). Detection of recombinant protein was carried out as previously described (Latha Gandla et al., 2014; Pawar PAM et al., in revision).

**Detection of endoxylanase and xylan endotransglycosylase activities in plants**

**Protein extraction.** Secondary-walled developing xylem from transgenic and WT (T89) hybrid aspen was scraped and ground in liquid nitrogen. Soluble and wall-bound proteins were isolated as previously described (Biswal et al., 2014). All buffers contained complete protease inhibitor cocktail (Roche). Transfected Arabidopsis protoplasts were harvested by centrifugation and proteins were extracted using buffer comprising 0.1 M Na succinate (pH=4.7) and 10 mM CaCl₂.

**Thin-layer chromatography for detecting products of xylanase and xylan endotransglycosylase activities.** Protein extracts containing either 1.25 mM or 6.25 mM
Xylo₆ (Megazyme, Ireland) were incubated at 40°C for 48 h. Volumes corresponding to the same amounts of substrates were analysed on TLC Silica Gel 60 Glass plates as described by Franková & Fry (2011).

**Quantitative analysis of xylan endotransglycosylase using a fluorogenic substrate.** To prepare the fluorogenic substrate, 20 µg of Xylo₆ (Megazyme, Ireland) was labelled with 8-aminonaphthalene-1, 3, 6-trisulfonic acid (ANTS, Invitrogen) according to (Kosik et al. 2012). The NaCNBH₃ was quenched with a two-fold molar excess of hydrochloric acid, re-neutralised with NaOH to pH 7, dried in vacuum and re-dissolved in a small amount of MilliQ water. The solution containing Xylo₆-ANTS was then spotted onto a dry column packed with Silicagel 60 (Merck, Germany). The excess ANTS was washed out with iso-propanol:NH₄OH:water (5:1:1, v/v/v) and 0.5 mL flow-through fractions were collected and dried down. Xylo₆-ANTS was eluted off the column with 40% ethanol (v/v), dried down in vacuum, reconstituted in a small amount of 3 M urea and electrophoresed on a PACE gel together with the flow through fractions to ensure the purity of the product. The concentration of Xylo₆-ANTS was estimated from absorbance measured at 365 nm and calculated using a calibration curve.

An endotransglycosylation assay was performed according to Kosik et al. (2011) with the following modifications: extracted proteins were incubated in 0.15% birch xylan (Sigma) and 165 µM Xylo₆-ANTS at RT for 1 h. Reactions were loaded onto circles of Whatman 3MM chromatographic paper in ELISA UV plates, then washed and the bound fluorescence measured using a Spectra Max Gemini (Molecular Devices) micro-plate reader at 355 nm excitation and 538 nm emission. For the blank samples, reactions without Xylo₆-ANTS were used. Product bound to 3MM paper was treated with 1 U of either endo-1,4-beta-xylanase M1 from *T. viride* (Megazyme) or pectate lyase from *C. japonicus* (Megazyme) used as an example of xylan-inert enzyme in extraction buffer or in the extraction buffer only, for 1 h at 40°C. The reaction was stopped by washing three times with 66% ethanol. Fluorescence of the remaining product was measured as above.

**Xylanase activity.** Azo-Xylan (from birchwood, Megazyme) was used for the measurement of endoxylanase activity. The reaction was performed according to the manufacturer’s instructions for 20 h at 40°C in 0.1 M succinate buffer, pH 5.5. β-
xylanase M1 from *Trichoderma viride* (Megazyme) was used to construct the standard curve.

To quantify hydrolytic activity by reducing ends, 0.25% (w/v) birchwood xylan (Sigma) was incubated with extracted proteins in 0.1 M Na-succinate buffer, pH 5.5 at 40°C for 20 h. The reaction was stopped by boiling for 5 min in PAHBAH reagent (1.5% *p*-hydroxybenzoic acid hydrazide in 0.5 M NaOH) and absorbance was measured at 410 nm after cooling (Lever, 1972). Xylose (2 mM to 0.0078 mM) was used to construct the standard curve and the data were calibrated to units of β-xylanase M1 from *Trichoderma viride* (Megazyme).

**Expression of PtxtXyn10A in Arabidopsis cells**

Full length *PtxtXyn10A* cDNA was amplified (primers listed in Table S6). The products were cloned into the pENTR/D-TOPO vector (pENTR™ Directional TOPO® Cloning Kits, Invitrogen), sequenced and subsequently subcloned into the binary vector pK2WG7.0 (Karimi *et al.*, 2002) using the Gateway® system (Invitrogen).

An *Arabidopsis* cell suspension derived from roots was used for protoplast isolation and transient protoplast transformations according to Dóczi *et al.* (2011) with slight modifications: 5x10⁵ protoplasts were used for each transformation with 5 µg of plasmid DNA without any carrier. After transformation, protoplasts were incubated in the dark for 24 h and harvested by centrifugation at 300 g for 8 minutes.

**Generation of transgenic antisense aspen**

For the antisense construct, cDNAs of C-terminal fragments covering the whole catalytic module of *PtxtXyn10A* and 28 bp of the 3’ end of CBM22_3*PtxtXyn10A* were amplified, cloned into the binary vector pPCV702.kana and transferred to hybrid aspen as described previously (Gray-Mitsumune *et al.*, 2008) using *Agrobacterium tumefaciens*.

**FT-IR spectroscopic analysis**

Wood at internode 44 from five to seven trees of selected lines and the WT were individually examined as previously described (Latha Gandla *et al.*, 2014). The initial PCA analysis was carried out with 28 observations and 624 variables on UV scaled pre-
treated spectra. After excluding outliers, OPLS-DA analyses (Trygg & Wold, 2002) were performed to identify wavenumbers that distinguished different classes based on cell wall composition. The OPLS-DA model was based on 21 observations and approximately 624 variables from Pareto-scaled pre-treated spectra using two classes (WT and transgenic).

**Physicochemical wood analyses**
Transgenic antisense lines carrying the antisense *PtxtXyn10A* construct (lines 2, 3 and 32) and a WT line (clone T89) were each represented by a minimum of 5 trees. Basal internodes from trees approximately 2 m tall, taken below internode 46, were frozen in liquid N and stored at -80°C. The samples were thawed, debarked, hand chipped and dried before analysis.

**Wet chemistry of wood.** Bulk wood samples were analysed for lignin content and carbohydrate composition as described in method AH 23-18 (Theander & Westerlund, 1986). The method involves full hydrolysis of samples, followed by derivatisation of liberated monomers and gas chromatography. Lignin content (Klason and acid soluble) was determined according to SCAN-C.1. The hemicellulose molecular weight was determined using size-exclusion chromatography (SEC) according to Jacobs & Dahlman (2001). Hemicellulose was isolated by extraction using 24% w/v KOH after delignification of samples using chlorite and fractionated using hydrogel columns (120, 250, 500). An alkaline solution containing 0.2 M NaOH and 0.1 M acetate was used as eluent. The molecular weight was calculated based on standard curves obtained from fractionation and MALDI-TOF measurements (Jacobs & Dahlman, 2001).

The glucuronoxylan branching pattern was determined by PACE using glucuronoxylanase GH30 as described in Bromley *et al.* (2013). Briefly, wood powder was treated with 4 M NaOH, then neutralized with HCl, buffered with 0.1 M ammonium acetate pH 6.0 and digested with glucuronoxylanase (*Bo*GH30; Bacova_03432, Rogowski *et al.*, 2014) for 2 h at room temperature. Digestion products, along with no enzyme and enzyme only controls, were labelled with ANTS, separated by polyacrylamide gel electrophoresis as described in Goubet *et al.* (2002) and visualised with a G-box UV gel documentation system (Syngene).
X-ray diffraction. Aspen stem segments without bark and pith were divided into three classes according to xylem thickness. Four replicates from the WT and three replicates from each of the transgenic antisense lines were measured in each size class after drying at 300 K. The X-ray diffraction experiments and data analysis for the determination of cellulose crystallite size, microfibril angle distribution and crystallinity index were conducted using CuKα₁ radiation (1.54 Å) as explained in Svedström et al. (2012).

Slit pit angle
Wood from internodes 42-43 was macerated as previously described (Gray-Mitsumune et al., 2008). Cells were examined under an Axioplan 2 microscope (Zeiss). To measure the cellulose microfibril angle, the angle of slit pits was measured in three to five pits for each fibre, and at least 50 fibres were measured for each of three randomly selected trees per line.

Field-emission scanning electron microscopy
FAA-fixed stem segments from internode 30 were washed with water and sectioned to small cubes with a cryomicrotome (Microm HM 505E), treated with 0.1% (v/v) sodium hypochlorite for three min and dehydrated in an ethanol series prior to critical point drying. The specimens were mounted on an aluminium stub and coated with 5 nm iridium. For imaging, a Zeiss Merlin field emission SEM was used with 4 kV accelerating voltage.

Microarray analysis
RNA and array preparation was carried out according to UPSC-BASE standardized procedures (Sjödin et al., 2006). The different scan levels for each slide were merged using restricted linear scaling (RLS) followed by step-wise normalization before further analysis. B-statistics were calculated against line 2 and 32 and the two lists of array elements were compared. Genes in antisense lines were considered differentially regulated if $B \geq 0$ and $P \leq 0.05$ compared to a reference WT. Genes selected from the ranking list of B-statistics were annotated against the Populus genome (Phytozome 9.0).
Statistical analysis

Univariate data were subjected to analysis of variance followed by post-hoc tests as indicated using the JMP 7 program (SAS Inc., USA). Multivariate data analysis was performed using SIMCA-P software (version 11.0.0.0, Umetrics AB, Sweden).

Results

Molecular cloning and bioinformatic analysis of PttxXyn10A

The full-length PttxXyn10A cDNA sequence was cloned from a cDNA library of developing xylem in hybrid aspen (GenBank accession number AY935501). The predicted PttxXyn10A peptide lacks a signal sequence and contains three carbohydrate-binding modules family 22 (CBM22) followed by a Xyn10 catalytic domain (Fig. 1a). Five N-glycosylation sites and one processing site after R-329, releasing the mature peptide with molecular weight 65.3 kDa and pI = 5.77, were predicted by the sequence analysis.

Populus GH10 gene family

The Populus trichocarpa genome contains eight GH10 genes, i.e. PtXyn10A - PtXyn10H, of which seven have been previously identified (Geisler-Lee et al., 2006) and one, PtXyn10H, was found in the region directly upstream of PtXyn10A on chromosome 2 (Fig. S1; Table S1). PtXyn10A and PtXyn10H have been merged into one model in the current version of Phytozome (9.1), but our rapid amplification of cDNA ends (RACE) and polymerase chain reaction (PCR) experiments confirmed the existence of two loci (Fig. S1; Table S1).

PtXyn10B lacks part of the catalytic domain and a corresponding truncated gene is also found in Arabidopsis thaliana. Phylogenetic analysis of the remaining genes of P. trichocarpa and A. thaliana (Henrissat et al., 2001) has revealed four well-defined clades with members in both species (Fig. S2). The clade to which PtXyn10A belongs, together with its closest paralog PtXyn10H and four Arabidopsis genes, including AtXyn1, is characterised by the presence of 2-4 CBMs. Other clades include genes with one CBM or none.
Xyn10A is the main GH10 transcript in secondary-walled developing xylem

Reverse transcription-PCR (RT-PCR) revealed a high abundance of \textit{PttxXyn10A} transcripts in stems and roots with secondary growth and low abundance in the apical bud and mature leaves (Fig. S3a), consistent with its localisation to developing secondary-walled xylem seen in microarray studies (Hertzberg \textit{et al.}, 2001; Aspeborg \textit{et al.}, 2005).

To determine in which xylem cell types \textit{PttxXyn10A} is expressed, we performed \textit{in situ} RT-PCR in stem sections using gene-specific nested primers (Gray-Mitsumune \textit{et al.}, 2004). \textit{Xyn10A}-specific signals were detected in all cell types in developing wood, fibres, vessel elements and ray cells, with the highest expression observed during the early stages of secondary wall deposition (Fig. S3b).

To compare the expression pattern of \textit{Xyn10A} in wood-forming tissues to that of other \textit{Populus} GH10 family members, we examined the relative transcript abundance of GH10 genes in cambium/phloem versus secondary wall developing xylem by RT-qPCR. Transcripts of \textit{Xyn10A}, \textit{Xyn10D}, \textit{Xyn10E} and \textit{Xyn10G} were detected in developing xylem, but only \textit{Xyn10A} was highly upregulated in the secondary wall forming xylem (Fig. S4a). Moreover, based on \textit{Populus} microarray data (http://bar.utoronto.ca/; Wilkins \textit{et al.}, 2009), the expression levels of other GH10 genes were found to be several orders of magnitude lower than those of \textit{Xyn10A} in developing wood (Fig. S4b).

\textbf{Xyn10A protein is present in xylem cell walls as a 68 kDa peptide}

To detect the Xyn10A protein in wood-forming tissues, the polyclonal antibody named “Kamisa” was raised against the C-terminal part of the \textit{PttxXyn10A} peptide. Soluble proteins were removed from crude plant extracts using a low ionic strength buffer, and proteins bound to the remaining pellet, including cell wall bound proteins, were extracted with a sodium dodecyl sulphate (SDS) containing buffer. A clear band at approximately 68 kDa was detected in the cell wall fraction from developing xylem consistent with the expected processed 65 kDa protein together with predicted glycosylations (Fig. 1b). No signal was detected in the soluble fraction from this tissue or in any fraction from the apical bud tissues in which the gene was lowly expressed (Fig. S3a).
Although PxtXyn10A lacks a predicted signal peptide (Table S1), the SecretomeP 2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/) predicted its target to be the apoplasm. To verify the cellular localization experimentally, a 35S::PxtXyn10A:eGFP construct was expressed in Arabidopsis. The GFP signal was detected in cell walls, but it was weak and labile, probably due to the acidic pH of cell walls. However, the fusion protein was clearly immunolocalised in cell walls after protoplast plasmolysis (Fig. 1c).

_Aspen xylem wall-bound proteins exhibit xylan endotransglycosylase and xylanase activities in a substrate concentration-dependent manner_

Both xylanase and xylan endotransglycosylase activities have been detected in plant tissues, latter activity requiring higher substrate concentration (Franková & Fry, 2011; Johnston et al., 2013). A similar dependence of activity on substrate concentration is also known for microbial GH10 enzymes (Charnock et al., 1997). Therefore, we investigated if such activities could be detected in proteins extracted with high ionic strength buffer from developing xylem (where PxtXyn10A is expressed as the main GH10 enzyme) when using xylohexaose as substrate. Under these conditions, it was expected that hydrolytic activity would yield products with DP 1-5, whereas endotransglycosylase activity would give a mixture of products with larger and lower DP than 6. When 1.85 mM xylohexaose was incubated with aspen protein extracts, products with DP 1 to DP 5 accumulated in a time-dependent manner, indicative of hydrolysis (Fig. 2a). However, when the concentration of xylohexaose was increased to 4 mM (data not shown) or 6.125 mM, products with DP values ranging from 8 to 10 were additionally detected, indicative of endotransglycosylation (Fig. 2a). The main products had DP 9 and DP 3, corresponding to cleavage in the middle of xylohexaose followed by transglycosylation. This shows that developing xylem cells exhibit xylan hydrolase and xylan endotransglycosylase activities and that the endotransglycosylase activity requires a higher substrate concentration than hydrolase (> 1.85 mM xylohexaose in the present experimental setup).

To establish a quantitative assay for xylan endotransglycosylation in high ionic strength protein extracts of developing wood, the extracts were incubated with birchwood xylan
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(391) and 8-aminonaphthalene-1,3,6-trisulfonic acid-labelled Xylo6 (Xylo6-ANTS; the acceptor). Xylan endotransglycosylation resulted in a high molecular weight ANTS labelled product, which was bound to cellulose and detected by fluorimetry (Fig. 2b). This product was significantly more susceptible to xylanase M1 than to pectate lyase or reaction buffer, confirming that it was xylan-labelled with ANTS (Fig. 2c). Decreased fluorescence observed after incubation in the buffer at 40°C was attributed to the instability of ANTS.

**PttxXyn10A expression in Arabidopsis cells strongly increases xylan endotransglycosylation but not xylan hydrolysis**

To investigate which activity (xylan hydrolase or xylan endotransglycosylase) was encoded by PttxXyn10A, Arabidopsis protoplasts were transfected by either PttxXyn10A or an empty vector and proteins were extracted from the samples expressing the transgenes, as verified by RT-qPCR. Xylan endotransglycosylation detected by ANTS assay was significantly increased in cells expressing PttxXyn10A compared to the empty vector control (Fig. 3a).

The same samples were tested for xylanase activity using either the endo-1,4-β-xylanase assay measuring solubilisation of AZO-xylan or the reducing end assay. Weak xylanase activity (four orders of magnitude lower than that of M1 xylanase from Trichoderma viride used for normalisation) was detected by these two assays in extracts for the empty vector and PttxXyn10A expressing cells, and no significant differences between these extracts were detected (Fig. 3b and c). These results indicate that PttxXyn10A-encoded enzyme exhibits xylan endotransglycosylase rather than xylanase activity in vitro.

**Suppression of PttxXyn10A expression in transgenic aspen**

To study the physiological role of PttxXyn10A, ten transgenic antisense lines of hybrid aspen were generated and two or three most highly affected antisense lines were selected for subsequent analyses. PttxXyn10A transcript levels were decreased to approx. 50% of the WT level in the selected lines (Fig. 4a) and western blotting using Kamisa antibody showed a corresponding reduction of PttxXyn10A protein in extracts obtained from xylem with high ionic strength buffer (Fig. 4b). The xylan endotransglycosylation by
these extracts was significantly lower in transgenic antisense lines compared to WT (Fig. 4c), but the xylanase activity was not affected (Fig. 4d). These data indicate that the suppression of PttxYn10A affects xylan endotransglycosylase but not xylanase activity in developing wood.

The transgenic antisense lines exhibited increased growth in height, internode number and leaf size (Fig. 5). Stem diameters and petiole lengths were also recorded, but no clear changes were detected for these variables. To investigate if the increased growth was caused by a change in the primary cell wall plasticity, thereby affecting cell size, casts were prepared from the adaxial leaf epidermis and the cell surface area was determined by microscopy. No significant difference in cell surface area between the transgenic antisense lines and WT was found (Fig. 5). Thus, PttxYn10A suppression does not increase leaf growth by increasing primary wall plasticity but by stimulating cell division.

**Effects of PttxYn10A on xylan structure**

The two putative activities of PttxYn10A were expected to differentially affect xylan chain length: xylanase would decrease it, whereas xylan endotransglycosylase may increase, decrease or not affect it depending on the length of input acceptor; without any input, the average length of xylan should not be altered by endotransglycosylation. Size exclusion chromatography of a 24% (w/v) KOH-extracted hemicellulose fraction containing mainly glucuronoxylan (Jacobs & Dahlman, 2001) gave similar weight-averaged molecular weights ($M_w$) for WT (15 600) and transgenic antisense lines (15 100 to 15 500); the polydispersivity index ($M_w/M_n$) ranged between 1.13 and 1.14 for all the lines (Table S2). Thus, no major change in glucuronoxylan molecular weight was detected, consistent with PttxYn10A acting as an endotransglycosylase *in vivo*.

To determine if the (Me)GlcA branching pattern of glucuronoxylan was affected by the endotransglycosylase activity, we used glucuronoxylanase BoGH30, which acts specifically on the xylan backbone and cleaves only when it is substituted with (Me)GlcA at the –2 position. The lengths of the digestion products thus corresponded to intervals of (Me)GlcA substitutions on the xylan backbone. PACE analysis of the labelled digestion products gave similar profiles for the transgenic antisense lines and WT aspen (Fig. 6).
The detected bands agreed with those previously described in *Arabidopsis xylan* (Bromley *et al.*, 2013) and corresponded to oligosaccharides arising from two xylan domains: evenly spaced longer (DP 6 to DP over 20) oligosaccharides of the major domain and shorter (DP 5 to 7), odd or even length fragments derived from the minor domain. The presence of both types of products in all examined lines indicates that both xylan domains are present in aspen wood and are not affected by the xylan endotransglycosylase activity.

**Effects of PtxtXyn10A on wood cell wall composition**

To determine whether reduced xylan endotransglycosylase activity affects other polymers in cell wall, diffuse reflectance Fourier-transformed infrared (FT-IR) spectra of milled wood of transgenic and WT plants were analysed. Orthogonal projections to latent structure discriminant analysis (OPLS-DA) showed separation of the transgenic and WT spectra, indicating alterations in cell wall composition (Fig. 7 a). Among the bands contributing to the separation, there were several vibrations corresponding to different forms of lignin. The 1460 cm\(^{-1}\) band (C-H deformation/bending in aromatics; Dokken *et al.*, 2005) and 1506/1595 band ratio, which reflects the condensed and cross-linked lignin structure (Akin *et al.*, 1993; Stewart *et al.*, 1997), were increased in the transgenic lines (Fig. 7 b). The ratio of absorbance at 1506 cm\(^{-1}\) to that at 900 cm\(^{-1}\) (representing the anomeric C-O stretch in cellulose (Zhong *et al.*, 2000)) was significantly higher in the transgenic lines, suggesting that they may have more lignin relative to cellulose. The observation that the entire spectral region between 1000–1100 cm\(^{-1}\) (sugar-ring vibrations) was more intense in the WT also indicates a higher lignin to cellulose ratio in the transgenic lines as the corresponding carbohydrates were less likely to be hemicelluloses or pectins because their intensity negatively correlated with that of the 1730 cm\(^{-1}\) –C=O vibration (Fig. 7 b).

To support these conclusions, we performed wet chemical analyses on wood material from the transgenic lines and WT. These analyses showed that the content of galactose and glucose was decreased, whereas the content of xylose and lignin was increased in the transgenic lines compared to WT (Table 1). Thus, the analysis confirmed the increased
lignin to cellulose ratio in the transgenic lines seen by FT-IR and additionally revealed increased xylan and reduced galactan content. Low xylan and high galactan contents are diagnostic for gelatinous fibres found in tension wood in aspen (Mellerowicz & Gorshkova, 2012), and therefore the observed changes in cell wall monosaccharide composition suggest a decreased tension wood content in the stems of transgenic lines as compared to WT.

**Effects of altered PttxTyn10A expression on cell wall architecture**

Based on the results for PttxTyn10A expression (Figs S4; Aspeborg et al., 2005; Andersson-Gunnerås et al., 2006; Winzell et al., 2010), we anticipated its involvement during secondary cell wall formation. Therefore, wood cross sections were analysed in transgenic and WT plants by light and transmission electron microscopy (Fig. S5). However, no major changes were detected. Light microscopy of isolated wood cells revealed a small reduction in fibre diameter and length and a corresponding small increase in wood density (Fig. S6). We also noted a significant change in the slit pit angles in the fibres (Fig. 8a). Since the slit pit orientation corresponds to the cellulose microfibril orientation in the S2 layer (Donaldson, 2008), we tested whether the reduced endotransglycosylase activity affected the cellulose microfibril angle (MFA) in the transgenic lines by performing X-ray diffraction analysis of the transgenic and WT wood samples. To remove the potential effects of variable tension wood content in these samples, the cellulose crystallite width was used to identify samples containing tension wood. Whereas the majority of WT and transgenic antisense samples had an average crystallite width between 29 and 31 Å, within the range typically reported for *Populus* wood (Yamamoto et al., 2010; Leppänen et al., 2011), approximately one third of the samples exhibited crystallite widths greater than 31 Å, typical for tension wood (reviewed by Mellerowicz & Gorshkova, 2012). These samples were set aside as tension wood enriched samples. Cellulose MFA in the remaining normal wood samples was clearly reduced in the transgenic antisense lines (Fig. 8b), indicating that Xyn10A activity is needed to orient cellulose microfibrils at large angles to the fibre axis in secondary walls.

To investigate if the morphology of cellulose microfibrils was also affected, the topology
of cell walls was visualized by field emission scanning electron microscopy (FE-SEM). The images revealed an ordered, strictly parallel, dense array of microfibrils in the S2 layer in all genotypes (Fig. 8c). The microfibrils had a “Z” orientation, similar to the pattern previously reported for conifers (Abe et al., 1992). No major change in microfibril topology was observed in the transgenic antisense line, except for a more axial microfibril orientation as compared to WT.

**Analysis of global gene expression pattern in transgenic antisense plants**

To investigate the effects of reduced *PtxtXyn10A* expression on the transcriptome, the global transcript profiles of developing xylem stem tissues of lines 2, 32 and WT were analysed using a 25K *Populus* (POP2) microarray (Sterky et al., 2004). 123 genes were affected (*P* ≤ 0.05 and *B* ≥ 0) in both antisense lines, of which 76 were downregulated (Tables S3 and S4). More genes were significantly affected in line 2 than 32, in agreement with the stronger *PtxtXyn10A* suppression in this line. 61 of the affected genes could be assigned functional categories, which included signal transduction, carbohydrate metabolism and cell wall, transcription and translation, energy, cell division and cellular transport. Among the signal transduction related transcripts, several stress perception and ethylene-signalling transcripts were affected, including ones possibly involved in mechano-perception (leucine-rich receptor-like kinases, Ca-signalling related proteins, microtubule-associated proteins), which were upregulated (Table 2). Within the cell wall related category, the most striking change was the downregulation of cellulose biosynthesis related genes, including *COBRA-LIKE 4*, *FRUCTOKINASE* and *CEL9A1* (*Populus* ortholog of *KORRIGAN1*), and fasciclin-like arabinogalactan proteins, which are the markers of tension wood formation (Lafarguette et al., 2004; Andersson-Gunnerås et al., 2006). In contrast, the expression of *PHENYLALANINE AMMONIA-LYASE* (*PAL1*) responsible for the first step of the lignin biosynthetic pathway was increased (Table 2). Over 60% of the genes downregulated in transgenic antisense lines (Table S2) were upregulated during the tension wood response (Andersson-Gunnerås et al., 2006), strongly indicating that downregulation of *PtxtXyn10A* affects the tension wood and stress responses and that tension wood formation was inhibited in the transgenic antisense lines.
Discussion

**PtxtXyn10A encodes a xylan endotransglycosylase**

GH10 xylanases follow a retaining catalytic mechanism similar to that of xyloglucan endotransglucosylases (Henrissat *et al.*, 2001), which allows both xylanase and xylan endotransglycosylase activities. In plants, xylanase activity of GH10 enzymes was demonstrated in cereals during caryopsis germination and pollen development (Bih *et al.*, 1999; Caspers *et al.*, 2001; Wu *et al.*, 2002). Recently, several vascular plants were shown to have extractable xylanase and xylan endotransglycosylase activities, the latter activity increasing at high 1,4-β-xylo-oligosaccharide concentration (Franková & Fry, 2011; Johnston *et al.*, 2013). Here, we have shown that these activities are also present in developing wood of aspen (Fig. 2). Four lines of evidence indicate that PtxtXyn10A is responsible for the observed xylan endotransglycosylase activity in developing wood. First, PtxtXyn10A is the main GH10 enzyme expressed in this tissue (Fig. S 4). Second, the heterologous expression of PtxtXyn10A in *Arabidopsis* protoplasts increased xylan endotransglycosylase, whereas xylanase activity was not affected (Fig. 3). Third, the downregulation of PtxtXyn10A in hybrid aspen suppressed xylan endotransglycosylase activity without affecting xylanase activity (Fig. 4). Fourth, the suppression of PtxtXyn10A activity in hybrid aspen did not result in an increased glucuronoxylan molecular weight as would be expected if it were an endoxylanase (Table S2).

**PtxtXyn10A undergoes proteolytic processing and is transported to the apoplast via a non-classical pathway**

The size of the PtxtXyn10A protein detected in the cell wall bound protein fraction provides clues to its processing, which according to the predicted cleavage site would leave CBM22_3 and the catalytic domain in the mature protein (Fig. 1). The role of the processing is presently unclear, but it does not seem to be a limiting step in the protein biosynthesis as we never observed the full-length peptide even after overexpressing the full length cDNA (data not shown), indicating that the processing step must be very rapid. Several plant xylanases have been reported to undergo proteolytic processing, and in those cases, a single CBM22 and sometimes an additional short peptide at the C-
terminal end are removed (Caspers et al., 2001; Wu et al., 2002; Chen & Paull, 2003; Van Campenhout et al., 2007; De Backer et al., 2010). Processing has been suggested to increase xylanase activity and facilitate secretion to the apoplasm (Caspers et al., 2001; De Backer et al., 2010), but the responsible protease and mechanism of transport facilitation are still unknown.

All known plant GH10 enzymes are active in the cell wall and mechanisms of their transport to this compartment vary. Aleurone layer and tapetum xylanases accumulate in cytoplasm and are released to endosperm or developing pollen grains, respectively, following programmed cell death and disintegration of their source cells (Bih et al., 1999; Caspers et al., 2001). In contrast, papaya CpaEXY1 is secreted via the classical ER-Golgi route with participation of the signal peptide (Chen & Paull, 2003). Although both PtxtXyn10A and its orthologue AtXyn1 lack the predicted signal peptide, they accumulate in the cell wall (Fig. 1; Suzuki et al., 2002) after being transported via a non-classical pathway (Agrawal et al., 2010).

**Suppression of PtxtXyn10 activity stimulates primary growth**

The stimulatory effects of PtxtXyn10A suppression on stem elongation, leaf expansion and the number of internodes observed in this study are intriguing (Fig. 5). The lack of accompanying cell size increase as found for leaf epidermis and xylem cells strongly indicates that these effects are not mediated by primary wall plasticity but rather related to metabolism of xylan in secondary walls. The observed growth stimulation could be mediated by mechanical or oligosaccharide signalling. It is also possible that the suppression of xylan endotransglycosylase activity in spirally thickened protoxylem elements changes their mechanical properties, such that they are more easily stretched during organ growth, which in turn most likely leads to increased primary organ expansion (Paolillo & Rubin, 1991).

**PtxtXyn10 activity regulates MFA in secondary walls, probably by affecting mechano-perception**

PtxtXyn10A is co-regulated with xylan biosynthesis genes during secondary wall formation (Mellerowicz & Sundberg, 2008) and induced by PtMYB021, the transcription
factor responsible for secondary wall initiation (Winzell et al., 2010). It is also strongly
downregulated during tension wood formation (Andersson-Gunnerås et al., 2006). These
expression patterns indicate that the encoded xylan endotransglycosylase plays a specific
role during secondary wall biosynthesis.

Our results show that although PtxtXyn10A activity neither affects xylan chain length
(Table S2) nor its branching pattern (Fig. 6), its suppression affects many aspects of plant
development, i.e. cellulose orientation in secondary walls (Fig. 8), tension wood
formation (Tables 1 & 2) and plant primary growth (Fig. 5). We propose that analogous
to xyloglucan endotransglucosylase activity, which releases tension in primary cell wall
during growth, xylan endotransglycosylase activity may release tension in secondary
walls. Such tension is envisaged to arise during self-assembly of the cellulose-xylan
network, during which cellulose microfibrils are deposited along rigid cortical
microtubules that orient the cellulose network (Baskin, 2001; Gardiner et al., 2003;
Funada, 2008; Li et al., 2012) and are immediately coated by negatively charged
glucuronoxylan (Stevanic & Salmén, 2009). It has been suggested that the
glucuronoxylan coat on the surface of cellulose microfibrils creates repulsive electrostatic
forces (Reis & Vian, 2004). The fact that such repulsive forces operate in cell wall can be
deduced from the behaviour of negatively charged cellulose crystals in solution; at high
concentrations, the cellulose fibrils arrange spontaneously in regular patterns forming
liquid crystals (Reis et al., 1991; Lagerwall et al., 2014). Another source of tension
stresses is the cross linking of microfibrils by xylan.

If the role of PtxtXyn10A is to relieve such growth stresses, its suppression would result
in excessive build-up of stresses that would trigger mechano-perception reactions.
Although very little is known about mechano-perception in plants, the emerging picture
points towards cortical microtubules as effectors of tensional stress signals (Jacques et
al., 2013; Landrein & Hamant, 2013). Cortical microtubules assume either a random or
parallel orientation under control of Katanin and SPIRAL2 (Wightman et al., 2013), and
can reorient within hours following different stimuli (Lindeboom et al., 2013), resulting
in a change in MFA. Thus, we suggest that PtxtXyn10A suppression may trigger
mechano-perception, which in turn re-orients cortical microtubules, resulting in the
reduction of MFA (Fig. 9).
Since tension wood induction is thought to involve mechano-perception, another prediction that follows from our hypothesis is that the suppression of xylan endotransglycosylase activity may interfere with the tension wood response. The observed effects in aspen (Tables 1 & 2) are compatible with this hypothesis. Other factors affecting cell wall self-assembly, for example pectin metabolism (Yoneda et al., 2010) or fasciclin-domain arabinogalactan proteins FLA11 and FLA12 (MacMillan et al., 2010), are believed to influence the orientation of cellulose microfibrils. Several authors have suggested that glucuronoxylan plays a role in determining MFA (Reis & Vian, 2004; Ruel et al., 2006). Its abundance and MFA in different secondary wall layers were shown to be correlated in radiata pine (Donaldson & Knox, 2012). Moreover, in white spruce, the $Xyn10$ locus was found to be associated with MFA (Beaulieu et al., 2011). Here, we have provided empirical evidence that $PxtXyn10A$ affects MFA in wood fibres in hybrid aspen and proposed a mechanism for such regulation.

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Supporting Information:

Fig. S1. Clarification of Phytozome gene models for PtXyn10A and PtXyn10H.

Fig. S2. Phylogenetic analysis of the GH10 family in Populus trichocarpa and Arabidopsis thaliana.

Fig. S3. PtxtXyn10A expression analyses.

Fig. S4. Populus GH10 family gene expression analysis.

Fig. S5. Effects of PtxtXyn10A expression on wood anatomy and ultrastructure.

Fig. S6. Relationship between wood cell dimensions, volumetric mass and expression level of PtxtXyn10A.

Supplemental Tables:

Table S1. GH10 gene models of P. trichocarpa, v 7.0 (http://www.phytozome.net/).

Table S2. Size-exclusion chromatography parameters of hemicellulose distributions in the transgenic antisense lines and WT.

Table S3. List of genes significantly ($P \leq 0.05$ and $B \geq 0$) downregulated in both transgenic antisense lines 2 and 32.

Table S4. List of genes significantly ($P \leq 0.05$ and $B \geq 0$) upregulated in both transgenic antisense lines 2 and 32.

Table S5. List of primers used for RT-qPCR analysis.

Table S6. List of primers used for cloning.
**Fig. captions**

**Fig. 1.** *PttXyn10A* protein accumulates in cell walls.

(a) Modular structure of *PttGT10A* showing three carbohydrate-binding modules (CBM22) and a catalytic domain (Xyn10). Predicted N-glycosylation sites are marked, along with the predicted processing site shown by an arrow.

(b) Western blotting of soluble and cell wall bound protein fractions extracted from developing xylem and apical bud tissues and probed with the antibody Kamisa raised against the C-terminal fragment of *PttXyn10A*. A 68 kDa peptide (arrow) was detected in the cell wall bound protein fraction from developing xylem.

(c) Immunolocalisation of *PttXyn10A* protein fused with eGFP and stably expressed in *Arabidopsis*. The root cells were plasmolyzed with 20% v/v mannitol, fixed, immunolabelled against GFP protein and observed by confocal microscopy. The arrowhead shows the signal from cell wall and the arrow shows the plasmolysed protoplast. The negative control was assay without primary antibodies. Bar = 20 µm.

**Fig. 2.** Xylanase and xylan endotransglycosylase activities are present in cell wall bound protein fractions extracted from developing xylem.

(a) Xylanase and xylan endotransglycosylase activities detected by thin-layer chromatography of products after incubation of extracted proteins with xylohexaose (Xylo$_6$) at either 1.25 mM or 6.25 mM concentrations, as indicated in the figure, for 1-42 h at 40°C. Extracts boiled for 10 min were used as negative controls. At low substrate concentration, the main products had DP 1-5, indicating hydrolysis, whereas at high substrate concentration, products corresponding to DP 8-10 were additionally detected, indicating xylan endotransglycosylase activity. The DP of xylooligosaccharides was determined from standards containing Xylo$_1$ to Xylo$_6$ either directly (black triangles) or by interpolation (white triangles). Volumes corresponding to the same amounts of substrates were loaded in each lane.

(b) Xylan endotransglycosylation activity detected by ANTS assay as described in the Material and Methods. The SE bars show variability among technical replicates. The control reaction lacked protein extracts.
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(c) Susceptibility of fluorescent product detected by ANTS assay for 1 h incubations with pectate lyase (PL), xylanase M1 (XylM1) or buffer (Buf; no enzymatic treatment) at 40°C.

Fig. 3. Expression of *Ptxt*Xyn10A in *Arabidopsis* protoplasts results in strong upregulation of xylan endotransglycosylase but not xylanase.

Protoplasts were transfected with either Xyn10A or an empty vector, and transgene expression was verified by RT-qPCR. Mean enzymatic activity ± SE, *n*=4 biological replicates, *P* values correspond to the probability of the null hypothesis in the Fisher test.

(a) Xylan endotransglycosylase specific activities determined by ANTS assay as described in the Material and Methods.

(b) Xylanase specific activities determined by Azo-Xylan solubilisation.

(c) Xylanase specific activity determined by the formation of reducing ends.

Fig. 4. Downregulation of *Ptxt*Xyn10A reduces xylan endotransglycosylase activity in developing wood. Three independent transgenic antisense lines (2, 3 and 32) are compared to WT.

(a) *Ptxt*Xyn10A transcript levels in transgenic antisense lines and WT determined by RT-qPCR. Data were calibrated to CYP and normalized to the WT level. Means of 3-9 biological replicates ± SE.

(b) *Ptxt*Xyn10A protein levels detected by the antibody Kamisa in the cell wall bound protein extracts. The signal from an unrelated cell wall localised protein (*Ptxt*Cel9B3) was used as a loading control for the transgenic antisense lines.

(c) Xylan endotransglycosylase activity in cell wall bound protein extracts from the transgenic antisense lines and WT. Means of 3 biological replicates ± SE.

(d) Xylanase activity in transgenic antisense lines determined by the formation of reducing ends following incubation of the cell wall bound protein extracts with xylan. Means of three biological replicates ± SE. The effect of genotype was not significant (ANOVA, *P* ≤10%).

* indicates values significantly different from WT (*t*-test, 5%)
Fig. 5. Downregulation of \textit{PtxtXyn10A} increases plant primary growth.

Stem height, diameter, number of internodes, internode length, leaf length and width were measured in trees after three months of growth in the greenhouse. \textbf{Average cell surface area was determined for the leaf adaxial epidermis from nail polish casts.} Means of ten biological replicates ± SE. * indicates values significantly different from WT (\textit{t-} test, 5%).

Fig. 6. Glucuronoxylan [Me]GlcA substitution pattern in transgenic antisense lines with reduced xylan endotransglycosylase activity (2 and 3) and in wild-type aspen (WT).

Milled wood was hydrolysed with \textit{Bo}GH30 to completion and the resulting digestion products were analysed using polysaccharide analysis by carbohydrate gel electrophoresis (PACE). Standards X1 to X6 (S), enzyme (E only) and wood material only (no E) are shown. * indicates non-specific labelling product.

Fig. 7. OPLS-DA models of diffuse reflectance Fourier-transform infrared spectra.

(a) Scores plot showing the separation of transgenic antisense plants (empty symbols; squares: line 2, triangles: line 3, diamonds: line 32) from wild type (filled dots), using five to six individual plants per line.

(b) Corresponding loadings plot showing factors responsible for the separation. Bands that are referred to in the text are labelled. Bands that are positive (\textit{i.e.} more intense in the transgenic antisense lines) are labelled with black regular fonts, bands that are negative (\textit{i.e.} more intense in the wild type) are labelled with black italic fonts. The 1595 cm$^{-1}$ band was unchanged and is labelled with grey regular font. The model has the following details: 1 + 1 components (predictive + orthogonal), R2X(cum) = 0.642, R2Y(cum) = 0.598; Q2(cum) = 0.254.

Fig. 8. Suppression of \textit{PtxtXyn10A} affects orientation of cellulose microfibrils in secondary walls of wood fibres.

(a) Orientation of slit-pits relative to fibre axis in isolated wood fibres from transgenic antisense lines and WT visualized using Nomarski optics. Example of slit pits and their angle is shown beside the graph.
(b) Cellulose microfibril angle (MFA) measured by X-ray diffraction in samples from transgenic antisense lines and WT representing primarily normal wood after exclusion of tension wood samples based on crystallite size.

For (a) and (b) Means of 3 biological replicates ± SE. The $P$ value for the difference between both transgenic antisense lines and the WT is indicated above the line.

(c) Appearance of cellulose microfibrils in the transgenic antisense line 2, 3 and WT visualised by SEM. The fibres were oriented vertically and observed from the inside. The cell wall appearance was similar in the transgenic antisense lines and WT, but the microfibril angle (traced with white lines) was larger in the WT. Scale bar = 200 nm.

Fig. 9. Proposed hypothesis of $PtxtXyn10A$ action on cellulose microfibril angle.

The main function of $PtxtXyn10A$ is proposed to be the elimination of tension in cell wall that arises during cell wall assembly. The suppression of $PtxtXyn10A$ causes the build-up of tension in the cell wall, which is sensed by mechanical sensors (tension gated $\text{Ca}^{++}$ channels, and/or receptor leucine-rich kinases) that activate the mechanosensing signal transduction pathway, causing re-organization of cortical microtubules and subsequent re-orientation of cellulose microfibrils.
Tables:

Table 1. Sugar composition and lignin content in the transgenic antisense lines and WT.

Five trees were pooled per line, two technical replicates. Neutral sugar and lignin contents are expressed as % of the total measured yield. The yield was over 865 mg/g.

<table>
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<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>Man</th>
<th>Klasson lignin</th>
<th>Acid soluble lignin</th>
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<tr>
<td></td>
<td>%</td>
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<td>19.9*</td>
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<td>4.09</td>
<td>23.0</td>
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</table>

$P_{WT \text{ vs. } Xyn10A \text{ AS}}$ $^1$: 0.620 0.004 0.001 0.001 0.049 0.001 0.001 0.001

* $P \leq 5\%$ (t test);

$^1P_{WT \text{ vs. } Xyn10A \text{ AS}}$ values correspond to the difference between all transgenic lines and the WT.
Table 2. Cell wall and stress signalling related transcripts significantly affected in both transgenic antisense lines, 2 and 32, as compared to the WT.

<table>
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<tr>
<th>Class</th>
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<td>Potri.003G156600</td>
<td>similar to GALACTAN SYNTHASE 2</td>
<td>-2.472, -0.805</td>
<td>AT5G44670</td>
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<td>Potri.001G052300</td>
<td>pectate lyase PpPL_26</td>
<td>0.604, 1.072</td>
<td>AT4G13710</td>
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<td>Potri.015G087800</td>
<td>similar to probable pectate lyase 22</td>
<td>0.491, 1.077</td>
<td>AT5G63180</td>
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<td>xylan</td>
<td>Potri.008G108100</td>
<td>similar to beta-D-xilosidase</td>
<td>1.049, 0.841</td>
<td>AT5G49360</td>
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<td>xyloglucan</td>
<td>Potri.018G095100</td>
<td>PnXTH16_17</td>
<td>-0.835, -0.884</td>
<td>AT5G25810</td>
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<td>Potri.007G101300</td>
<td>O-fucosyltransferase family protein</td>
<td>-1.839, -0.637</td>
<td>AT5G15740</td>
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<td>mannan</td>
<td>Potri.013G304000</td>
<td>similar to ENDO-BETA-MANNASE 2</td>
<td>1.363, 1.037</td>
<td>AT2G20680</td>
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<td>AGP</td>
<td>Potri.013G153000</td>
<td>fasciclin-like AGP PfLFA12K</td>
<td>-2.692, -1.097</td>
<td>AT5G60490</td>
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<td>Potri.012G015000</td>
<td>similar to fasciclin-like FLA 11</td>
<td>-1.942, -1.005</td>
<td>AT5G30170</td>
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<td></td>
<td>Potri.009G012200</td>
<td>fasciclin-like AGP PfLFA12V</td>
<td>-1.786, -0.878</td>
<td>AT5G60490</td>
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<td>Potri.013G151500</td>
<td>fasciclin-like AGP PfLFA12J</td>
<td>-2.138, -1.179</td>
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<td>Potri.019G123200</td>
<td>similar to fasciclin-like FLA 11</td>
<td>-2.187, -1.157</td>
<td>AT5G30170</td>
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<td>Potri.015G013300</td>
<td>fasciclin-like AGP PfLFA12E or F</td>
<td>-2.090, -1.236</td>
<td>AT5G30170</td>
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<td>Potri.009G121100</td>
<td>fasciclin-like AGP PfLFA12G, P or Q</td>
<td>-2.242, -1.313</td>
<td>AT5G30170</td>
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<td>Potri.001G450200</td>
<td>beta-galactosyltransferase PnGT31_32</td>
<td>-1.286, -0.617</td>
<td>AT1G32930</td>
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<td>Potri.013G151400</td>
<td>similar to fasciclin-like FLA 11</td>
<td>-2.293, -1.174</td>
<td>AT5G30170</td>
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<td>Potri.006G144500</td>
<td>Similar to glycosyl hydrolase family 35</td>
<td>0.588, 0.557</td>
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<td>Potri.005G161100</td>
<td>similar to AGP5</td>
<td>-1.109, -0.705</td>
<td>AT1G35230</td>
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<td>cellulose</td>
<td>Potri.002G340000</td>
<td>Fruktokinase</td>
<td>-2.399, -0.729</td>
<td>AT1G19600</td>
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<td>Potri.004G112700</td>
<td>similar to COBRA-like COB14</td>
<td>-2.845, -0.971</td>
<td>AT5G15630</td>
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<td>Potri.003G151700</td>
<td>similar to KORRIGAN1 PfCel9A1</td>
<td>-1.436, -0.727</td>
<td>AT5G49720</td>
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<td>lignin</td>
<td>Potri.008G038200</td>
<td>phenylalanine ammonia-lyase1 (PAL1)</td>
<td>1.333, 0.752</td>
<td>AT2G37040</td>
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<td>Potri.018G106500</td>
<td>cinnamoyl-CoA reductase related</td>
<td>1.147, 0.598</td>
<td>AT2G23910</td>
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<td>microtubule</td>
<td>Potri.008G137000</td>
<td>microtubule associated MAP65-like</td>
<td>1.795, 1.214</td>
<td>AT2G01910</td>
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<td>Potri.014G088500</td>
<td>similar to AtMPK4</td>
<td>0.745, 0.783</td>
<td>AT4G01370</td>
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<td>Potri.006G180000</td>
<td>similar to AtMAP70-5</td>
<td>1.443, 1.383</td>
<td>AT4G17220</td>
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<td>LRK</td>
<td>Potri.008G140500</td>
<td>Similar to BRL2</td>
<td>0.816, 0.550</td>
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<td>Potri.012G287000</td>
<td>Leucine-rich repeat protein kinase family</td>
<td>0.739, 0.992</td>
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<td>Potri.019G122700</td>
<td>Leucine-rich repeat receptor-like protein</td>
<td>-1.469, -0.556</td>
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<td>calcium</td>
<td>Potri.001G055000</td>
<td>Calcium binding protein involved in cryptochrome and phytochrome coaction</td>
<td>1.624, 1.495</td>
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<td>Potri.016G049100</td>
<td>CalB domain, plant phosphoribosyltransferase family protein</td>
<td>0.861, 0.858</td>
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<td>Potri.009G052200</td>
<td>similar to calcium-dependent protein kinase 1; MSCK1</td>
<td>0.899, 0.651</td>
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<td>vesicle</td>
<td>Potri.001G278800</td>
<td>clathrin heavy chain</td>
<td>2.885, 2.942</td>
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<td>ethylene, stress</td>
<td>Potri.017G010800</td>
<td>similar to S-adenosylmethionine decarboxylase.</td>
<td>-2.343, -1.119</td>
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<td>ethylene receptor EIN4-like</td>
<td>1.184, 1.134</td>
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<td>similar to ACC oxidase</td>
<td>-1.038, -1.225</td>
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<td>-1.193, -0.706</td>
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</table>
integration of mechanical signals from cell wall:
- transduction of the signals (Ca\(^{2+}\) and / or phosphorylation pathway?)
- activation of mechanosensing effectors (KATANIN, SP2, MAP65, Rbohs and others)