Down-Regulation of PoGT47C Expression in Poplar Results in a Reduced Glucuronoxylan Content and an Increased Wood Digestibility by Cellulase

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Xylan is the second most abundant polysaccharide in dicot wood. Unraveling the biosynthetic pathway of xylan is important not only for our understanding of the process of wood formation but also for our rational engineering of wood for biofuel production. Although several glycosyltransferases are implicated in glucuronoxylan (GX) biosynthesis in Arabidopsis, whether their close orthologs in woody tree species are essential for GX biosynthesis during wood formation has not been investigated. In fact, no studies have been reported to evaluate the effects of alterations in secondary wall-associated glycosyltransferases on wood formation in tree species. In this report, we demonstrate that PoGT47C, a poplar glycosyltransferase belonging to family GT47, is essential for the normal biosynthesis of GX and the normal secondary wall thickening in the wood of the hybrid poplar Populus alba × tremula. RNA interference (RNAi) inhibition of PoGT47C resulted in a drastic reduction in the thickness of secondary walls, a deformation of vessels and a decreased amount of GX in poplar wood. Structural analysis of GX using nuclear magnetic resonance (NMR) spectroscopy demonstrated that the reducing end of GX from poplar wood contains the tetrasaccharide sequence, β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α-D-GalpA-(1→4)-D-Xylp, and that its abundance was significantly decreased in the GX from the wood of the GT47C-RNAi lines. The transgenic wood was found to yield more glucose by cellulase digestion than the wild-type wood, indicating that the GX reduction in wood reduces the recalcitrance of wood to cellulase digestion. Together, these results provide direct evidence demonstrating that the PoGT47C glycosyltransferase is essential for normal GX biosynthesis in poplar wood and that GX modification could improve the digestibility of wood cellulose by cellulase.

Keywords: Biomass recalcitrance • Glucuronoxylan biosynthesis • Glycosyltransferase • Poplar • Secondary wall biosynthesis • Wood formation.

Abbreviations: CaMV, cauliflower mosaic virus; fra8, fragile fiber8; GalA, galacturonic acid; GlcA, glucuronic acid; GT, glycosyltransferase; GUS, β-glucuronidase; GX, glucuronoxylan; irx, irregular xylem; MALDI-TOF MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; MeGlcA, 4-O-methyl-glucuronic acid; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Rha, rhamnose; RNAi, RNA interference; Xyl, xylose.

Introduction

Wood is the most abundant biomass produced by plants. It is estimated that up to 56 billion tons of CO₂ are fixed by land plants per year (Field et al. 1998), and the bulk of this fixed carbon is stored in wood. Wood is not only the most abundant reservoir of fixed carbon in plants but is also important raw material for our daily lives. Wood is widely used for construction, pulping and paper-making, furniture and many other products. Recently, wood is considered to be an important renewable source of biofuels, although lignocellulosic wood is naturally recalcitrant to enzymatic conversion of cellulose into glucose (Himmel et al. 2007). Therefore, tremendous efforts have been put into understanding the molecular mechanisms underlying wood formation in the hope of being able to improve wood quantity and quality genetically on the basis of our needs (Mellerowicz and Sundberg 2008).

Wood is mainly composed of cellulose, hemicellulose and lignin, and the elucidation of their biosynthetic pathways is the first step toward our understanding of wood formation.
The biosynthesis of cellulose and lignin in woody tree species has been intensively studied; genes encoding cellulose synthases and genes in the lignin biosynthetic pathway have been isolated and characterized (Boerjan et al. 2003, Joshi et al. 2004). In contrast, much less is known about the genes involved in hemicellulose biosynthesis in wood. Hemicelluloses from dicot wood mainly consist of glucuronoxylan (GX), with glucomannan as a minor component (Timell 1967). Glucomannan is made of linear chains of β-1,4-linked mannose residues interspersed with glucosyl residues. The genes encoding glucomannan synthases have been cloned and characterized biochemically in both Arabidopsis and poplar (Suzuki et al. 2006, Liepman et al. 2007). GX is composed of linear chains of β-1,4-linked xylosyl residues, some of which are substituted by α-1,2-linked glucuronic acid or 4-O-methylglucuronic acid residues, and acetylated at C-2 or C-3 (Timell 1967). It has been found that the reducing end of GX from birch, spruce and Populus tremula consists of a unique tetrasaccharide sequence composed of β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α-D-GalpA-(1→4)-D-Xylp (Shimizu et al. 1976, Johansson and Samuelson 1977, Anderson et al. 1983, Pena et al. 2007). At least six glycosyltransferases are required for the biosynthesis of the GX backbone, the addition of side chains and the biosynthesis of the reducing end tetrasaccharide sequence. Currently, the functional roles of glycosyltransferases potentially involved in GX biosynthesis during wood formation in tree species have not yet been investigated.

Transcriptome profiling of genes expressed in the developing secondary xylem in poplar has identified 25 glycosyltransferases that are associated with wood formation (Aspeborg et al. 2005). Among these wood-associated glycosyltransferases, six of them, namely GT47C, GT8D, GT8E/F, GT43A/B, have close orthologs in Arabidopsis that were shown to be required for GX biosynthesis. The Arabidopsis FR48/F8H, IRX8 and PARVUS, which are orthologs of poplar GT47C, GT8D and GT8E/F, respectively, have been demonstrated to be expressed specifically in fibers and vessels in which GX is abundant (Zhong et al. 2005, Lee et al. 2007b, Pena et al. 2007, Persson et al. 2007, Lee et al. 2009). Mutations of these genes result in a reduction in GX content and a near absence of the tetrasaccharide sequence at the reducing end of GX, indicating that FR48/F8H, IRX8 and PARVUS are involved in the biosynthesis of the reducing end tetrasaccharide sequence of GX (Brown et al. 2007, Lee et al. 2007b, Pena et al. 2007, Lee et al. 2009). Overexpression of the poplar GT47C in the fra8 mutant is able to rescue the GX-deficient phenotype, suggesting that the poplar GT47C is a functional homolog of FR48 (Zhou et al. 2006). Another Arabidopsis glycosyltransferase, IRX9, which is a close homolog of poplar GT43A/B, has also been shown to be specifically expressed in GX-containing fibers and vessels (Pena et al. 2007). Mutation of the IRX9 gene leads to a significant reduction in xylan xylosyltransferase activity and a concomitant decrease in xylan chain length and content (Lee et al. 2007a, Pena et al. 2007). It is suggested that IRX9 is a xylosyltransferase essential for the elongation of the GX backbone. Poplar GT43A/B are probably functional homologs of IRX9 because overexpression of the poplar GT43B in the irx9 mutant was able to complement the GX-deficient phenotype (Zhou et al. 2007).

Although functional complementation studies of several poplar wood-associated glycosyltransferases in Arabidopsis GX-deficient mutants have provided evidence suggesting that genes involved in GX biosynthesis are functionally conserved between herbaceous Arabidopsis and woody species (Zhou et al. 2005, Zhou et al. 2007), direct proof of the functional roles of these glycosyltransferases in GX biosynthesis in woody tree species is required. Genetic modification of GX content in wood may also potentially provide a means for reduction in recalcitrance of wood to enzymatic conversion of cellulose into glucose during biofuel production because hemicellulose is considered to be one of the factors contributing to the lignocellulosic recalcitrance (Himmel et al. 2007). So far, no studies on alterations of wood formation by altering secondary wall-associated glycosyltransferases have been reported in tree species, although such studies would have important implications in tree biotechnology (Mellerowicz and Sundberg 2008). In this study, we demonstrate that RNA interference (RNAi) inhibition of PoGT47C expression in the hybrid poplar Populus alba × tremula leads to a drastic reduction in the amount of GX and cellulose during wood formation. Concomitantly, the secondary wall thickness of fibers and vessels was markedly reduced, and the vessels were severely deformed. We reveal by nuclear magnetic resonance (NMR) spectroscopy that like the GX from birch, spruce and Arabidopsis, the reducing end of GX from poplar wood also contains the tetrasaccharide sequence and its abundance is significantly reduced by RNAi inhibition of PoGT47C. We further show that the transgenic wood with a reduced GX content exhibits an increased accessibility of cellulose to cellulase digestion. Our study provides the first line of evidence that the PoGT47C glycosyltransferase is essential for normal GX biosynthesis and that GX modification could reduce the recalcitrance of wood to cellulase digestion.

Results

Generation of transgenic poplar plants with a reduction in PoGT47C expression

To investigate the functional role of PoGT47C in wood formation in poplar, we employed the RNAi inhibition approach to down-regulate the expression of the PoGT47C gene. The GT47C-RNAi construct (Fig. 1A) was introduced into poplar (Populus alba × tremula) plants, and 120 independent
transgenic lines were generated and analyzed for the degree of reduction in \( \text{PoGT47C} \) expression. Using real-time quantitative PCR analysis, we found that the expression level of \( \text{PoGT47C} \) in 11 transgenic lines was reduced to <25% of that of the wild type (Fig. 1B). In particular, the expression of \( \text{PoGT47C} \) in line GT47C-RNAi-44 was reduced to about 6% of that of the wild type. These results demonstrated that the RNAi inhibition strategy effectively down-regulated the expression of \( \text{PoGT47C} \) in transgenic poplar plants.

**Down-regulation of \( \text{PoGT47C} \) expression results in a reduction in secondary wall thickening and a collapsed vessel phenotype in wood**

We next examined the effects of RNAi inhibition of \( \text{PoGT47C} \) on wood formation. Examination of wood cell morphology in the 11 transgenic lines with down-regulation of \( \text{PoGT47C} \) expression revealed a significant reduction in secondary wall thickness and an alteration in vessel morphology. Since all 11 transgenic lines exhibited similar phenotypes, the results from five representative lines, GT47C-RNAi-7, -20, -22, -44, and -52, were shown. Wild-type wood from both stems and roots contains thick-walled xylary fibers and regular-shaped vessels (Fig. 2A, D). RNAi inhibition of \( \text{PoGT47C} \) led to a prominent reduction in the wall thickness of xylary fibers in the wood of stems and roots (Fig. 2B, C, E, F). In addition, it was observed that the vessels in GT47C-RNAi-7 were slightly deformed (Fig. 2B, E) and those in GT47C-RNAi-44 were severely deformed (Fig. 2C, F). It was noted that the deformation of vessels in these lines is not as severe as that seen in the *Arabidopsis fra8* mutant, probably due to a milder defect in their secondary walls. In addition, some of the vessels in the PoGT47C-RNAi lines appeared to be larger than those in the wild type (Fig. 2D–F). This slightly enlarged vessel sizes could be caused by a prolonged cell expansion due to a reduced secondary wall deposition. Transmission electron microscopy showed that the wall thickness of fibers and vessels in the stems and roots of GT47C-RNAi-7 and -44 was clearly reduced compared with the wild type (Fig. 3; Table 1). A similar degree of reduction in the wall thickness of xylary fibers was observed in the wood of GT47C-RNAi lines. However, it was noted that the secondary xylem in roots of the PoGT47C-RNAi lines had less density of stored ergastic substances compared with that in the wild type (Fig. 2D–F), indicating that the RNAi inhibition of \( \text{PoGT47C} \) may have some effects on the storage of ergastic substances. These results indicate that the \( \text{PoGT47C} \) gene is essential for normal secondary wall biosynthesis during wood formation.

**Decrease in GX content and LM10 antibody labeling of xylan in the wood of \( \text{PoGT47C} \)-RNAi plants**

To examine the effects of down-regulation of \( \text{PoGT47C} \) expression on the biosynthesis of secondary wall components, we analyzed the composition of cell walls from the wood of the 11 transgenic GT47C-RNAi lines (Table 2). Wood cell walls were sequentially extracted with 1 N and 4 N KOH, and the alkali extracts together with the remaining wall residues were measured for the composition of neutral sugars. It was found that in the 1 N KOH extracts of GT47C-RNAi wood, the amount of xylene (Xyl) which is the main component of GX in wood, was reduced to 43–77% of that of the wild type (Table 2). A slight reduction in the amount of xylene was also seen in the remaining wall residues after alkali extractions, whereas no reduction was found in the 4 N KOH extracts (Table 2). The total amount of xylose in the cell walls of GT47C-RNAi wood was decreased to 54–79% of that of the wild type (Fig. 5A). The amount of glucose in the remaining wall residues after alkali extractions, which are primarily composed of cellulose, was lowered to 56–87% of that of the wild type (Table 2; Fig. 5B). This is consistent with previous findings showing that a reduction in GX biosynthesis leads to a defect in cellulose biosynthesis (Brown et al. 2005, Zhong et al. 2005, Lee et al. 2007). The amount of other cell wall sugars, including mannose, galactose,
arabinose and rhamnose, was not significantly altered (Table 2). Lignin composition analysis of wood from two representative RNAi lines, GT47C-RNAi-7 and -44, showed that the amount of extractable guaiacyl and syringyl lignin was slightly elevated compared with the wild type (Fig. 5C). This elevation of lignin content could be caused by a compensatory effect in response to the reduction in cell wall polysaccharides or could simply be due to the relative decrease in the amounts of GX and cellulose.

To substantiate further the finding that PoGT47C is required for GX biosynthesis, we next examined the effect of RNAi inhibition of PoGT47C on GX deposition in wood secondary walls.
by immunodetection of GX using the monoclonal antibody LM10 that binds to 4-O-methylglucuronoxylan (McCartney et al., 2005). Sections of the secondary xylem of wild-type stems and roots exhibited intensive GX fluorescent signals in the secondary walls of xylary fibers and vessels (Fig. 6A, D). (D–F) The wall thickness of xylary fibers and vessels in the secondary xylem of roots is reduced in GT47C-RNAi-7 (E) and GT47C-RNAi-44 (F) compared with the wild type (G). ve, vessel; xf, xylary fiber. Bar in (A) = 8.8 µm for (A–F).

**Table 1** Wall thickness of xylary fibers and vessels in the secondary xylem of wild-type and GT47C-RNAi poplar plants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stem Xylary fibers (µm)</th>
<th>Stem Vessels (µm)</th>
<th>Root Xylary fibers (µm)</th>
<th>Root Vessels (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.96 ± 0.21</td>
<td>0.71 ± 0.07</td>
<td>2.50 ± 0.36</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>GT47C-RNAi-7</td>
<td>1.07 ± 0.18</td>
<td>0.61 ± 0.05</td>
<td>1.25 ± 0.43</td>
<td>0.54 ± 0.11</td>
</tr>
<tr>
<td>GT47C-RNAi-44</td>
<td>0.75 ± 0.25</td>
<td>0.36 ± 0.08</td>
<td>0.93 ± 0.32</td>
<td>0.35 ± 0.10</td>
</tr>
</tbody>
</table>

The wall thickness was measured from transmission electron micrographs of fibers and vessels.
Data are means (µm) ± SE from 15 cells.

Down-regulation of PoGT47C expression causes a reduction in the abundance of the tetrasaccharide sequence located at the reducing end of GX

The results described above clearly demonstrated that down-regulation of PoGT47 expression resulted in a reduction in GX content. To determine whether RNAi inhibition of PoGT47C also affected GX structure, KOH-solubilized GX from the wood of the wild type and the transgenic lines GT47C-RNAi-7 and -44 was digested with endoxylanase and the acidic xylooligosaccharides generated by the digestion were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The acidic xylooligosaccharides from wild-type wood displayed one major ion peak [M + Na]+ at mass-to-charge ratios (m/z) of 891 (Fig. 7A), which is attributed to xylooligosaccharides with five Xyl residues substituted with one 4-O-Me-α-L-GlcA residue (Fig. 7B). This is consistent with the known

**Fig. 3** Transmission electron microscopy of the secondary walls of fibers and vessels in the wood of the wild type, GT47C-RNAi-7 and GT47C-RNAi-44. (A–C) The wall thickness of xylary fibers and vessels in the secondary xylem of stems is reduced in GT47C-RNAi-7 (B) and GT47C-RNAi-44 (C) compared with the wild type (A). (D–F) The wall thickness of xylary fibers and vessels in the secondary xylem of roots is reduced in GT47C-RNAi-7 (E) and GT47C-RNAi-44 (F) compared with the wild type (G). ve, vessel; xf, xylary fiber. Bar in (A) = 8.8 µm for (A–F).
structure of GX from poplar wood, which mainly bears 4-O-Me-α-D-GlcA side chains with a small amount of α-D-GlcA side chains (Mellerowicz et al. 2001). The MALDI-TOF spectra of the acidic xylooligosaccharides from the wood of GT47C-RNAi-7 and -44 contain two ion peaks at an m/z of 759 and 891 (Fig. 7A), which correspond to xylooligosaccharides with four and five Xyl residues, respectively, substituted with one 4-O-Me-α-D-GlcA residue (Fig. 7B). The results demonstrated that down-regulation of PoGT47C expression did not affect the addition of the 4-O-Me-α-D-GlcA side chains.
Table 2  Monosaccharide composition of 1 and 4 N KOH extracts from the wood of wild-type and GT47C-RNAi poplar plants.

<table>
<thead>
<tr>
<th>Cell wall sample</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Arabinose</th>
<th>Rhamnose</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 N KOH sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>96.0 ± 4.1</td>
<td>6.5 ± 0.5</td>
<td>3.8 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>3.9 ± 1.1</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>GT47C-RNAi-2</td>
<td>48.1 ± 3.1</td>
<td>8.4 ± 0.8</td>
<td>4.3 ± 1.2</td>
<td>4.0 ± 1.0</td>
<td>8.5 ± 1.0</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>GT47C-RNAi-7</td>
<td>47.3 ± 1.5</td>
<td>2.3 ± 0.6</td>
<td>4.3 ± 0.4</td>
<td>3.1 ± 0.0</td>
<td>2.2 ± 0.2</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>GT47C-RNAi-17</td>
<td>45.3 ± 3.4</td>
<td>9.3 ± 3.2</td>
<td>2.9 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>4.4 ± 0.9</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>GT47C-RNAi-20</td>
<td>44.5 ± 0.1</td>
<td>12.1 ± 1.0</td>
<td>3.8 ± 0.9</td>
<td>1.6 ± 0.3</td>
<td>11.0 ± 3.8</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>GT47C-RNAi-22</td>
<td>45.2 ± 3.3</td>
<td>8.8 ± 3.0</td>
<td>3.4 ± 2.4</td>
<td>6.7 ± 3.6</td>
<td>3.7 ± 0.5</td>
<td>7.9 ± 2.0</td>
</tr>
<tr>
<td>GT47C-RNAi-25</td>
<td>45.4 ± 2.0</td>
<td>5.4 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td>2.5 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>GT47C-RNAi-33</td>
<td>44.5 ± 0.3</td>
<td>5.1 ± 0.7</td>
<td>6.0 ± 3.0</td>
<td>6.7 ± 0.2</td>
<td>3.0 ± 0.0</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>GT47C-RNAi-41</td>
<td>65.8 ± 1.7</td>
<td>11.3 ± 1.9</td>
<td>3.5 ± 1.0</td>
<td>4.4 ± 1.5</td>
<td>11.3 ± 4.1</td>
<td>10.3 ± 0.5</td>
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<tr>
<td>GT47C-RNAi-44</td>
<td>42.8 ± 4.3</td>
<td>2.2 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>4.9 ± 0.9</td>
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<td>51.9 ± 1.2</td>
<td>5.7 ± 0.8</td>
<td>4.5 ± 0.9</td>
<td>4.3 ± 0.3</td>
<td>4.4 ± 0.9</td>
<td>7.7 ± 0.3</td>
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<td>5.8 ± 0.3</td>
<td>3.7 ± 0.0</td>
<td>1.8 ± 0.4</td>
<td>3.3 ± 0.1</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>4 N KOH sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>22.6 ± 1.6</td>
<td>2.6 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>7.0 ± 0.8</td>
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<tr>
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<td>20.2 ± 0.3</td>
<td>5.1 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>4.0 ± 1.3</td>
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<tr>
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<td>36.8 ± 3.0</td>
<td>1.9 ± 0.1</td>
<td>2.7 ± 0.6</td>
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<td>4.0 ± 0.4</td>
<td>2.6 ± 0.2</td>
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<td>9.2 ± 1.5</td>
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<td>3.2 ± 1.2</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>9.7 ± 1.4</td>
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<tr>
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<td>27.9 ± 0.7</td>
<td>8.1 ± 0.4</td>
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<td>2.3 ± 0.4</td>
<td>3.4 ± 0.1</td>
<td>8.6 ± 0.1</td>
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<td>2.1 ± 0.1</td>
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<td>2.0 ± 0.1</td>
<td>1.5 ± 0.0</td>
<td>8.8 ± 1.0</td>
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<td>6.0 ± 2.1</td>
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<td>5.6 ± 2.8</td>
<td>3.9 ± 0.8</td>
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<td>3.1 ± 0.2</td>
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<td>6.4 ± 3.1</td>
<td>3.5 ± 0.2</td>
<td>11.2 ± 2.7</td>
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<td>Remaining wall residues</td>
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<td>11.0 ± 1.2</td>
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<td>6.4 ± 0.2</td>
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<td>345 ± 6</td>
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<td>GT47C-RNAi-17</td>
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<td>5.6 ± 1.1</td>
<td>4.1 ± 0.1</td>
<td>4.8 ± 0.4</td>
<td>5.2 ± 1.0</td>
<td>370 ± 43</td>
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<tr>
<td>GT47C-RNAi-20</td>
<td>21.7 ± 1.8</td>
<td>8.5 ± 2.8</td>
<td>5.4 ± 0.1</td>
<td>7.3 ± 1.0</td>
<td>4.8 ± 0.5</td>
<td>326 ± 5</td>
</tr>
<tr>
<td>GT47C-RNAi-22</td>
<td>21.3 ± 2.4</td>
<td>9.3 ± 0.9</td>
<td>6.7 ± 1.7</td>
<td>9.0 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>354 ± 3</td>
</tr>
<tr>
<td>GT47C-RNAi-25</td>
<td>25.2 ± 6.6</td>
<td>10.2 ± 0.9</td>
<td>6.8 ± 0.7</td>
<td>8.6 ± 2.7</td>
<td>4.6 ± 0.6</td>
<td>356 ± 61</td>
</tr>
<tr>
<td>GT47C-RNAi-33</td>
<td>18.0 ± 1.6</td>
<td>4.5 ± 0.8</td>
<td>14.3 ± 5.0</td>
<td>6.9 ± 0.7</td>
<td>3.5 ± 0.3</td>
<td>261 ± 10</td>
</tr>
<tr>
<td>GT47C-RNAi-41</td>
<td>16.7 ± 0.6</td>
<td>10.7 ± 1.7</td>
<td>5.3 ± 0.2</td>
<td>7.0 ± 1.5</td>
<td>6.0 ± 1.7</td>
<td>337 ± 7</td>
</tr>
<tr>
<td>GT47C-RNAi-44</td>
<td>16.4 ± 2.3</td>
<td>13.2 ± 0.4</td>
<td>12.3 ± 6.4</td>
<td>6.0 ± 1.8</td>
<td>3.4 ± 0.2</td>
<td>254 ± 1</td>
</tr>
<tr>
<td>GT47C-RNAi-52</td>
<td>18.6 ± 0.5</td>
<td>7.4 ± 0.1</td>
<td>7.1 ± 3.4</td>
<td>6.2 ± 0.3</td>
<td>3.6 ± 0.1</td>
<td>257 ± 9</td>
</tr>
<tr>
<td>GT47C-RNAi-97</td>
<td>27.0 ± 0.3</td>
<td>10.3 ± 0.8</td>
<td>7.7 ± 1.5</td>
<td>9.0 ± 0.6</td>
<td>4.6 ± 0.1</td>
<td>388 ± 11</td>
</tr>
</tbody>
</table>

The cell walls were extracted sequentially with 1 and 4 N KOH. The alkali-soluble extracts and the remaining cell wall residues after alkali extractions were used for monosaccharide composition analysis. Bold numbers highlight significant changes between the wild type and the transgenic GT47C-RNAi plants. Data are means (mg g⁻¹ dry cell wall) ± SE of two independent assays.
chains in GX although the digestibility of GX by xylanase is somewhat altered.

It has been shown that the reducing end of GX from birch, spruce and Arabidopsis contains a unique tetrasaccharide sequence that differs from the GX backbone (Shimizu et al. 1976, Johansson and Samuelson 1977, Anderson et al. 1983, Pena et al. 2007). To investigate whether the reducing end of GX from poplar wood also contains the tetrasaccharide sequence and whether its biosynthesis is affected by down-regulation of PoGT47C expression, we analyzed the acidic xylooligosaccharides using NMR spectrometry. The 1H-NMR spectrum of the acidic xylooligosaccharides from the wild-type poplar wood displayed the resonances assigned to the tetrasaccharide sequence \( \beta-D-Xyl-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow2)-\alpha-D-GalA-(1\rightarrow4)-D-Xyl \) (Fig. 8). These resonances include H1 of \( \alpha-D-GalA \), H1 of \( \alpha-L-Rha \), H1 of 3-linked \( \beta-D-Xyl \), H4 of \( \alpha-D-GalA \) and H2 of \( \alpha-L-Rha \). The spectrum also contained anomeric resonances of H1 and H5 of 4-O-Me-\( \alpha-D-GlcA \) residues and H1 of branched \( \beta-D-Xyl \) residues bearing a 4-O-Me-\( \alpha-D-GlcA \) residue, and resonances of H1 of \( \alpha-Xyl \) and \( \beta-Xyl \) residues at the reducing end of the oligoxyllosaccharides and H1 of unbranched \( \beta-Xyl \) residues (Fig. 8). The observed 1H-NMR spectrum of wild-type poplar acidic xylooligosaccharides is the same as that previously reported for wild-type Arabidopsis acidic xylooligosaccharides except for the absence of the resonances of \( \alpha-D-GlcA \) residues (Fig. 7; Zhong et al. 2005, Pena et al. 2007), which is consistent with the MALDI-TOF data showing the absence of \( \alpha-D-GlcA \) side chains in poplar wood GX (Fig. 7). Quantitative comparison of the 1H-NMR spectrum of the acidic xylooligosaccharides from the wood of the wild type, and the GT47C-RNAi-7 and -44 lines revealed an apparent reduction in the intensity of the resonances of H1 of \( \alpha-D-GalA \), H1 of \( \alpha-L-Rha \), H1 of 3-linked \( \beta-D-Xyl \), H4 of \( \alpha-D-GalA \) and H2 of \( \alpha-L-Rha \), which are assigned to the reducing end tetrasaccharide sequence \( \beta-D-Xyl-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow2)-\alpha-D-GalA-(1\rightarrow4)-D-Xyl \) (Fig. 8; Table 3). These results demonstrated that RNAi inhibition of PoGT47C led to a reduction in the abundance of the reducing end tetrasaccharide sequence in GX. In addition, examination of the 1H-NMR spectra revealed that GX from the wood of both the wild type and the GT47C-RNAi lines had one 4-O-Me-\( \alpha-D-GlcA \) side chain in every five xylosyl residues (Table 3), confirming the result of the MALDI-TOF analysis showing that RNAi inhibition of PoGT47C had no effect on the addition of the 4-O-Me-\( \alpha-D-GlcA \) side chains during GX biosynthesis.

**Reduction in GX in the PoGT47C-RNAi lines leads to an increased digestibility of wood by cellulase**

GX cross-links with cellulose in the secondary walls, and this cross-link is thought to be one of the factors contributing to the biomass recalcitrance during the conversion of cellulose.
to glucose for biofuel production (Himmel et al. 2007). Our generation of transgenic poplar trees with a reduction in xylan presented an ideal tool to test \textit{in planta} the effect of reduced GX on the digestibility of wood by cellulase. Among the five PoGT47C-RNAi lines examined, the line PoGT47C-RNAi-7 exhibited a 48\% increase in the conversion of cellulose into glucose by cellulase digestion compared with the wild type (Fig. 9). Another line, PoGT47C-RNAi-20, showed a 24\% increase, and the rest had only a slight increase in the wood digestibility by cellulase (Fig. 9). The results suggest that the alteration of GX content probably renders the wood easier to be accessed by cellulase for conversion of cellulose into glucose.

**Discussion**

Although a number of glycosyltransferases potentially involved in polysaccharide biosynthesis have been identified in poplar wood (Aspeborg et al. 2005), their roles in wood formation have not yet been investigated. Two of these wood-associated glycosyltransferases, PoGT47C and PoGT43B, were previously shown to be able to complement the GX-deficient phenotypes in the \textit{Arabidopsis} fra8 and irx9 mutants, respectively (Zhou et al. 2005, Zhou et al. 2007). Our current finding that PoGT47C is essential for GX biosynthesis in poplar wood provides direct evidence for the functional role of PoGT47C in GX biosynthesis during wood formation. GX is considered to be one of the barriers blocking the access of biomass cellulose by cellulase, and it was proposed that a direct alteration of the molecular interaction between cellulose and GX in transgenic plants may increase the digestibility of biomass cellulose by cellulase enzymes (Himmel et al. 2007). Our finding that the transgenic wood with reduced GX is easier for the conversion of cellulose into glucose by cellulase enzymes provides the first line of evidence supporting this hypothesis.
Modification of GX content alters the recalcitrance of wood to cellulase digestion

Cellulosic biofuel has been considered to be one of the most promising alternative renewable energy sources because of the abundant biomass produced by plants. However, conversion of plant biomass into sugars for biofuel ethanol production needs first to overcome the recalcitrance of biomass to saccharification (Himmel et al. 2007). The most abundant plant biomass is produced in the form of secondary cell walls, in which cellulose is wrapped by hemicelluloscs and further impregnated by the lignin polymer. Therefore, the biomass cellulose is inherently recalcitrant to enzymatic digestion due to the physical barriers formed by hemicelluloses and lignin. It has been shown that chemical or enzymatic removal of xylan and lignin increases the accessibility of biomass cellulose by cellulase and thus leads to an increased cellulase digestibility of biomass (Yang and Wyman 2004, Jeoh et al. 2007). The benefit of reduced lignin in biomass on the conversion of cellulose to glucose by cellulase enzymes was further revealed in the transgenic plants with altered lignin (Chen and Dixon 2007). We demonstrated that the transgenic wood with reduced GX exhibits an increased digestibility by cellulase enzymes compared with the wild-type wood, indicating that it might be feasible to reduce the biomass recalcitrance to saccharification by using transgenic biomass with reduced xylan. It is interesting to note that the increased wood digestibility in the transgenic poplar is not directly correlated with the severity of GX reduction. The line with the highest increase in wood digestibility has a relatively modest decrease in GX content and modest secondary wall defects. This finding indicates that a controlled modification of GX is needed to improve the accessibility of biomass cellulose by cellulase. Cellulose and GX cross-link to form a network in secondary walls, and a severe reduction in GX content may lead to a drastic alteration in cellulose deposition and organization in secondary walls and consequently no increase in the accessibility of cellulose to cellulase. A modest reduction in GX content without a severe alteration in cellulose deposition and organization may increase the exposure of cellulose to cellulase and thereby the digestibility. Our finding that a modest modification of GX increases the digestibility of wood cellulose by cellulase enzymes provides an important framework for future generation of transgenic biomass with modification of xylan for biofuel production.

PoGT47C is essential for the biosynthesis of a normal amount of GX in wood

We have demonstrated that down-regulation of PoGT47C expression by RNAi inhibition results in a marked reduction in GX content in wood. Together with the previous observations showing that PoGT47C is specifically expressed in fibers and vessels in which GX is abundant and its encoded protein is targeted to the Golgi where GX is synthesized (Zhou et al. 2005), these results provide strong evidence indicating that the PoGT47C glycosyltransferase participates in GX biosynthesis during wood formation. The GX-deficient phenotype caused by RNAi inhibition of PoGT47C is similar to that seen in the fra8 mutant (Zhong et al. 2005) although the degree of GX deficiency in the fra8 mutant is much more severe than that in the PoGT47C-RNAi lines. This difference is most probably due to the fact that while the fra8 mutation causes a complete loss of function in the FRA8 protein, RNAi inhibition of PoGT47C only results in a reduction in the amount of the PoGT47C protein. Nevertheless, down-regulation of PoGT47C...
expression causes a reduction in GX biosynthesis, thus establishing a role for PoGT47C in GX biosynthesis during wood formation. Our study also suggests that the RNAi inhibition approach is an effective means to investigate the functional roles of glycosyltransferases in wood formation.

PoGT47C is probably involved in the biosynthesis of the tetrasaccharide sequence at the reducing end of GX

Although the biochemical functions of PoGT47C and its Arabidopsis homolog FRA8 are currently unknown, previous studies on the structure of GX from the fra8 mutant revealed a near loss of the tetrasaccharide sequence located at the reducing end of GX, and therefore it was proposed that FRA8 is a glycosyltransferase involved in the biosynthesis of the tetrasaccharide sequence (Pena et al. 2007). Similar to the fra8 mutant, down-regulation of PoGT47C expression in poplar also causes a reduction in the abundance of the reducing end tetrasaccharide sequence in GX, indicating that PoGT47C is a functional ortholog of FRA8 involved in...
the biosynthesis of the tetrasaccharide sequence at the reducing end of GX. At least four glycosyltransferases are involved in the biosynthesis of the tetrasaccharide sequence, \(\beta-D-Xyl-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow2)-\alpha-D-GalA-(1\rightarrow4)-D-Xyl\). Because PoGT47C and FRA8 belong to the family GT47 glycosyltransferases that are known to have inverting catalytic mechanisms and catalyze the formation of cosyltransferases that are known to have inverting catalytic mechanisms and catalyze the formation of biosynthetic enzymes using \(\alpha\)-linked nucleotide sugars or vice versa, PoGT47C and FRA8 probably catalyze the formation of the \(\beta\)-linkage of Xyl to O3 of rhamnose using UDP-\(\alpha\)-D-Xyl as a substrate or the \(\alpha\)-linkage of rhamnose to O2 of galacturonic acid (GalA) using UDP-\(\beta\)-L-rhamnose. Our finding that PoGT47C is required for the biosynthesis of the tetrasaccharide sequence at the reducing end of GX provides a foundation for further investigation of the biochemical functions of PoGT47C.

It should be noted that RNAi inhibition of PoGT47C also resulted in a reduction in the amount of GX. A reduction in GX content has previously been observed in the Arabidopsis fra8, irx8 and parvus mutants, all of which exhibit a decrease in the abundance of the reducing end tetrasaccharide sequence (Zhang et al. 2005; Brown et al., 2007; Lee et al., 2007; Pena et al. 2007; Person et al., 2007). Because mutation of FRA8, a functional ortholog of PoGT47C, did not affect xylan xylosyltransferase activity (Lee et al. 2007), it is unlikely that PoGT47C directly participates in the biosynthesis of the GX backbone. The reduction in GX content in the PoGT47C-RNAi lines is probably an indirect effect caused by the reduced abundance of the tetrasaccharide sequence. Although it is currently unknown whether the tetrasaccharide sequence functions as a primer for initiating the GX backbone biosynthesis or a terminator to terminate the GX backbone biosynthesis, it is apparent that the reducing end tetrasaccharide sequence is essential for normal GX biosynthesis in poplar wood.

**PoGT47C is required for the normal thickening and strength of secondary walls in wood**

We have found that RNAi inhibition of PoGT47C results in a drastic decrease in secondary wall thickness and a deformation of vessels, indicating the important roles of GX in the normal thickening and strength of secondary walls during wood formation. The deformation of vessels is most probably caused by the reduced wall strength that renders the vessels unable to resist the negative pressure generated by transpiration. The observed reduction in cellulose biosynthesis in the PoGT47C-RNAi lines is probably due to an indirect effect caused by the reduced GX biosynthesis. Since cellulose and GX form a network in the secondary walls, a reduction in GX biosynthesis may result in a defect in the normal assembly of the network and thereby an impediment in cellulose biosynthesis.

In summary, our study provides direct proof that PoGT47C is essential for GX biosynthesis during wood formation and that a modest reduction in GX is beneficial for the conversion of wood cellulose to glucose by cellulase enzymes. We have found that like the GX from birch, spruce and Arabidopsis, the reducing end of GX from poplar wood also contains the tetrasaccharide sequence, \(\beta-D-Xyl-(1\rightarrow3)-\alpha-L-Rha-(1\rightarrow2)-\alpha-D-GalA-(1\rightarrow4)-D-Xyl\), and its biosynthesis requires PoGT47C. Our findings further substantiate the hypothesis that the molecular mechanisms involved in GX biosynthesis are conserved between herbaceous Arabidopsis and woody species (Zhou et al. 2006). Our study also provides the first line of evidence that genetic modification of GX in wood is feasible; however, a reduction in GX content apparently has adverse effects on the normal thickening and strength of secondary walls in wood. Further biochemical characterization of PoGT47C and functional investigation of other wood-associated glycosyltransferases will be necessary for our understanding of the complex process of wood formation, which may aid in rational designing of strategies for genetic modification of wood composition and properties.

**Materials and Methods**

**Generation of transgenic poplar plants**

The PoGT47C RNAi construct was created by cloning the PoGT47C cDNA into pBI121 in opposite orientations on both sides of the β-glucuronidase (GUS) spacer, which is located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. The PoGT47C-RNAi construct was transformed into Agrobacterium tumefaciens LBA4404 by electroporation. Stem segments from poplar (Populus alba × tremula) seedlings, a gift from Dr. Steve Strauss, were transformed with A. tumefaciens containing the PoGT47C-RNAi plasmid as described by Leple et al. (1992). The transgenic poplar seedlings were selected on Murashige and Skoog medium containing 50 mg l\(^{-1}\) kanamycin and 500 mg l\(^{-1}\) carbenicillin. After rooting, transgenic seedlings were transferred to soil and grown in the greenhouse. The wild-type control plants were transgenic poplar plants transformed with an empty vector. Each selected transgenic line used for detailed molecular and chemical analyses was clonally propagated to have 3–5 plants for further analysis.

**Gene expression analysis**

For analysis of the expression level of PoGT47C in the PoGT47C-RNAi lines, total RNA was isolated from young stems using a Qiagen RNA isolation kit (Qiagen, Valencia, CA, USA). Total RNA was treated with DNase I and used for first-strand cDNA synthesis. The first-strand cDNA was then used as a template for real-time quantitative PCR analysis with the QuantiTect SYBR Green PCR Kit (Clontech, Mountain View, USA).
CA, USA). The relative expression level of PoGT47C was determined by normalizing the PCR threshold cycle number of PoGT47C with that of a poplar actin reference gene. The expression level of PoGT47C in the wild-type control was set to 100, and the data were the average of three replicates from three separate RNA extractions.

**Histology**

Stem and root segments from 9-month-old transgenic plants were fixed with 2% glutaraldehyde in phosphate buffer at 4°C overnight. After fixation, tissues were dehydrated through a gradient of ethanol, embedded in LR White resin (Electron Microscopy Sciences, Fort Washington, PA, USA) and then subjected to sectioning with a microtome (Burk et al. 2006). Sections (1 μm thick) were stained with toluidine blue for light microscopy. For transmission electron microscopy, 85 nm thick sections were stained with uranyl acetate and lead citrate, and visualized using a Zeiss EM 902A transmission electron microscope (Carl Zeiss, Jena, Germany).

**Immunolocalization of GX**

For immunodetection of GX, sections (1 μm thick) of stems and roots were first blocked in phosphate-buffered saline (PBS; 33 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, and 140 mM NaCl, pH 7.2) containing 3% non-fat milk, and then sequentially incubated with the LM10 monoclonal antibody (1:10 dilution), which recognizes xylan (Plantprobes, Leeds, UK; McCartney et al. 2005), and with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma, St Louis, MO, USA) (1:200 dilution). After extensive rinsing in PBS, the sections were observed for fluorescence signals using a Leica TCS SP2 spectral confocal microscope. Images from single optical sections were collected and processed with Adobe Photoshop. For transmission electron microscopy, ultrathin sections (85 nm) of wood were incubated with the LM10 antibody and gold (10 nm)-conjugated secondary antibodies. The immunogold-labeled xylan signals were observed with a transmission electron microscope (Zhou et al. 2006).

**Cell wall isolation and extraction**

Stems of 9-month-old poplar plants were ground into fine powder in a mill and homogenized sequentially in 70% ethanol and 100% acetone with a polytron. The resulting cell wall residues were dried in a vacuum oven at 60°C overnight. After fixation, tissues were dehydrated through a gradient of ethanol, embedded in LR White resin and then subjected to sectioning with a microtome (Burk et al. 2006). Sections (1 μm thick) were stained with toluidine blue for light microscopy. For transmission electron microscopy, 85 nm thick sections were stained with uranyl acetate and lead citrate, and visualized using a Zeiss EM 902A transmission electron microscope (Carl Zeiss, Jena, Germany).

**Cell wall sugar composition analysis**

Cell wall sugars (as alditol acetates) were determined following the procedure described by Hoebler et al. (1989). Briefly, cell walls were incubated with 70% sulfuric acid at 37°C for 60 min followed by addition of inositol as the internal standard and dilution with water to 2 N sulfuric acid. After heating for 120 min at 100°C, the solution was cooled and treated with 25% ammonium solution. After reduction with sodium borohydride in dimethylsulfoxide, the solution was heated for 90 min at 40°C, followed by sequential treatment with glacial acetic acid, acetic anhydride, 1-methylimidazole, dichloromethane and water. The organic layer containing the alditol acetates of the hydrolyzed cell wall sugars was washed three times with water, and sugars were analyzed on an Agilent 6890N gas–liquid chromatograph (Wilmington, DE, USA) equipped with a 30 m × 0.25 mm (i.d.) silica capillary column DB 225 (Alltech Associates, Deerfield, IL, USA).

**Lignin composition analysis**

Lignin composition was determined as described by Akin et al. (1993) and Zhong et al. (2000). Ethanol-extracted cell wall residues were hydrolyzed in 4 N NaOH for 2 h at 170°C. The hydrolysate was acidified with 2 N HCl to pH 2.0. The released lignin monomers were extracted into diethyl ether and vacuum dried. The residue was dissolved in pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide and analyzed for phenolics by gas–liquid chromatography. Phenolic compounds were identified by comparison of their mass spectra with those of the authentic compounds. All samples were run in duplicate.

**Generation of xylooligosaccharides with β-endoxylanase**

The 1 and 4 N KOH-solubilized wall preparations were digested with β-xylanase M6 (Megazyme, Wicklow, Ireland) for generation of xylooligosaccharides as described (Zhong et al. 2005). The released xylooligosaccharides were desalted and separated by size-exclusion chromatography on a Sephadex G-25 column (100 × 2.5 mm). Fractions containing the oligosaccharides were determined by the phenol–sulfuric assay (DuBois et al. 1956), pooled and lyophilized.

**MALDI-TOF MS**

The acidic xylooligosaccharides released from β-xylanase digestion were analyzed using a MALDI-TOF mass spectrometer operated in the positive-ion mode with an accelerating voltage of 30 kV, an extractor voltage of 9 kV and a source pressure of approximately 8 × 10⁻⁷ Torr. The aqueous sample
was mixed (1:1, v/v) with the MALDI matrix (0.2 M 2,5-dihydroxybenzoic acid and 0.06 M 1-hydroxyisoquinoline in 50% acetonitrile) and dried on the stainless steel target plate. Spectra are the average of 100 laser shots.

1H-NMR spectroscopy

NMR spectra of the acidic xylooligosaccharides from β-xylanase digestion were acquired at 20°C on a Varian Inova 600 MHz spectrometer (599.7 MHz, 1H) using a 5 mm cryogenic triple resonance probe (Varian, Palo Alto, CA, USA). All NMR samples were prepared using 100% D2O in a 3 mm standard NMR tube. 1H chemical shifts were referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt). For all experiments, 64 transients were collected using a spectral width of 6,000 Hz and an acquisition time of 5 s. After 59 s pre-delay (relaxation delay), the residual water resonance was suppressed by a 1 s pre-saturation pulse at a field strength of 40 Hz. 1D spectra were processed using MestReC (MestReC Research, Santiago de Compostela, Spain) with 0.2 Hz apodization followed by zero-filling to 128k points. The 1H NMR assignments were done by comparison with the NMR spectra data for Arabidopsis acidic xylooligosaccharides (Zhong et al. 2005, Pena et al. 2007).

Wood digestibility analysis

The digestibility of wood cellulose by cellulase enzymes was analyzed according to Chen and Dixon (2007). Briefly, the milled, ethanol-insoluble wood cell walls (equivalent to 100 mg of glucose) were incubated with cellulase (52 U; Celuclast 1.5L from Sigma, St Louis, MO, USA) and cellobiase (16 U; Sigma) in a 10 ml 0.1 M sodium acetate buffer (pH 4.8) at 37°C for 72 h. Under the same conditions, the enzyme mixture was able to digest 90% of Whatmann No. 1 filter paper cellulose into glucose. The cellobiose released by cellobiose was converted to glucose by the addition of cellobiase in the digestion solution. After centrifugation, the supernatant was used for quantitation of neutral sugars released from wood cellulose using gas chromatography as described above. The digestion of wood with cellulase and cellobiase resulted in little release of xylose. The enzyme mixture without the addition of wood cell walls was used as a blank. All samples were assayed independently twice.

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