This is the published version of a paper published in Phytochemistry.

Citation for the original published paper (version of record):

Phytochemistry, 112: 210-220
http://dx.doi.org/10.1016/j.phytochem.2014.06.002

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:
http://urn.kb.se/resolve?urn=urn:nbn:se:umu:diva-103218
Expression of a fungal glucuronoyl esterase in *Populus*: Effects on wood properties and saccharification efficiency

Madhavi Latha Gandla a,1, Marta Derba-Maceluch b,1, Xiaokun Liu b, Lorenz Gerber b, Emma R. Master c, Ewa J. Mellerowicz b,c, Leif J. Jönsson a,b,*

a Department of Chemistry, Umeå University, SE-901 80 Umeå, Sweden
b Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden
c Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON, Canada

This paper forms part of a special issue of Phytochemistry dedicated to the memory and legacy of Professor (Godfrey) Paul Bolwell, MA DSc (Oxon), (1946–2012), internationally-recognised plant biochemist and Regional Editor of Phytochemistry (2004–2012). He is much missed by his friends.

**Abstract**

The secondary walls of angiosperms contain large amounts of glucuronoxylan that is thought to be covalently linked to lignin via ester bonds between 4-O-methyl-α-D-glucuronic acid (4-O-Me-GlcA) moieties in glucuronoxylan and alcohol groups in lignin. This linkage is proposed to be hydrolysed by glucuronoyl esterases (GCEs) secreted by wood-degrading fungi. We report effects of overexpression of a GCE from the white-rot basidiomycete *Phanerochaete chrysosporium*, PcGCE, in hybrid aspen (*Populus tremula L. × tremuloides Michx.*) on the wood composition and the saccharification efficiency.

The recombinant enzyme, which was targeted to the plant cell wall using the signal peptide from hybrid aspen cellulase PtcCel9B3, was constitutively expressed resulting in the appearance of GCE activity in protein extracts from developing wood.

Diffuse reflectance FT-IR spectroscopy and pyrolysis–GC/MS analyses showed significant alternation in wood chemistry of transgenic plants including an increase in lignin content and S/G ratio, and a decrease in carbohydrate content. Sequential wood extractions confirmed a massive (+43%) increase of Klason lignin, which was accompanied by a ca. 5% decrease in cellulose, and ca. 20% decrease in wood extractives. Analysis of the monosaccharide composition using methanolysis showed a reduction of 4-O-Me-GlcA content without a change in Xyl contents in transgenic lines, suggesting that the covalent links between 4-O-Me-GlcA moieties and lignin protect these moieties from degradation. Enzymatic saccharification without pretreatment resulted in significant decreases of the yields of Gal, Glc, Xyl and Man in transgenic lines, consistent with their increased recalcitrance caused by the increased lignin content. In contrast, the enzymatic saccharification after acid pretreatment resulted in Glc yields similar to wild-type despite of their lower cellulose content.

These data indicate that whereas PcGCE expression in hybrid aspen increases lignin deposition, the inhibitory effects of lignin are efficiently removed during acid pretreatment, and the extent of wood cellulose conversion during hydrolysis after acid pretreatment is improved in the transgenic lines possible due to reduced cell wall cross-links between cell wall biopolymers by PcGCE.

© 2014 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Lignocellulosic biomass is an abundant renewable resource mainly composed of carbohydrate polymers, such as cellulose and hemicelluloses (collectively represented as C₆H₁₂O₅), and lignin, a polymeric phenylpropanoid (represented as CH₁.₁₂O₀.₃₇₇) (Pauly and Keegstra, 2008; Lerouxel et al., 2006). The heterogeneity and molecular structure of the polysaccharides and the lignin make the lignocellulosic biomass recalcitrant to enzymatic hydrolysis for production of biofuels and other commodities (Vega-Sanchez and Ronald, 2010). The contribution of hemicelluloses to the recalcitrance of lignocellulose is not very well understood but reports suggest that they play an important role in this phenomenon, especially with respect to hardwood, such as wood from *Populus*...
sp. (Vega-Sanchez and Ronald, 2010), where about 20% of the cell wall is composed of hemicelluloses. The backbone of xylan, the predominant hemicellulosic carbohydrate in *Populus*, consists of Xyl units linked by β-(1→4)-glycosidic bonds. The degree of polymerization of xylan is around 120 and it is partially substituted by 4-O-methyl-α-D-glucuronic acid (4-O-Me-GlcA) residues through α-(1→2)-glycosidic linkages (Timell, 1967; Shimizu et al., 1976; Johansson and Samuelson, 1977; Andersson et al., 1983; Jacobs and Dahlman, 1981). A portion of the backbone is acetylated at either the C-2 or the C-3 position of the Xyl residues (Timell, 1967). The average molar ratio of Xyl:4-O-Me-GlcA:acetic acid in hardwood xylan is 10:1:7 (Bouweng, 1961). Studies have shown that there are potentially three types of covalent linkages between lignin and hemicelluloses in plant cell walls (Fry, 1986; Jeffries, 1990). These linkages consist of (i) p-coumaric/ferulic acid residues linked ethereally to lignin and esterically to hemicellulose sugar residues (Scalbert et al., 1985), (ii) ether bonds between hydroxyl groups of sugar residues and lignin (Watanabe et al., 1989) and (iii) ester bonds between 4-O-Me-GlcA or GlcA residues of glucuronoxylans and hydroxyl groups of lignin (Watanabe and Koshijima, 1988; Balakshin et al., 2011).

To facilitate bioconversion of lignocellulosic feedstocks to biofuels, it is relevant to understand and modify the recalcitrance of the biomass and to reduce the inhibitory effects of intermolecular linkages on cellulases and hemicellulases, including intermolecular linkages predicted to occur between lignin and polysaccharides (Vega-Sanchez and Ronald, 2010). Research is in progress to identify and modify the key enzymatic activities involved in xylan biosynthesis, especially the backbone assembly and the side-chain addition, for improving the enzymatic saccharification of lignocellulosic biomass (York and O’Neill, 2008; Wu et al., 2009; Brown et al., 2009; Lee et al., 2010; Mortimer et al., 2010; Lee et al., 2011a, b, 2012; Urbanowicz et al., 2012; Bromley et al., 2013). Interestingly, it has been found that the reduction of 4-O-Me-GlcA substitutions in glucuronoxylan increases its extractability from cell walls and its enzymatic hydrolysis to Xyl (Mortimer et al., 2010). Whole genetic modification can contribute to making plants less recalcitrant, improved methods for pretreatment and enzymatic hydrolysis of lignocellulosic biomass make bioconversion more efficient and give a better understanding of the fundamental relationship between cell-wall composition and sugar release. Pretreatment aims at reducing the complexity of the cell wall by either removing the lignin or solubilising the hemicelluloses, making the cellulose more accessible to enzymatic hydrolysis (Vilikari et al., 2007).

Glucuronoyl esterase (EC 3.1.1.-), which was originally isolated from the basidiomycete fungus *Schizopodium commune*, has been suggested to play a role in biomass degradation by hydrolysis of ester linkages between 4-O-Me-GlcA residues of glucuronoxylan and aromatic alcohol groups of lignin (Špániková and Biely, 2006). The CAZY classification has grouped glucuronoyl esterase in the Carbohydrate Esterase (CE) family 15 (Li et al., 2007). Such enzymes could potentially increase extractability of cell wall polymers and accessibility of cellulases and xylanases to their substrates since their proposed activity would remove cross-links between lignin and glucuronoxylan. The present investigation is focused on transgenic hybrid aspen (*Populus tremula* L. × *tremuloides* Michx.) expressing a glucuronoyl esterase from a basidiomycete fungus causing white rot, *Phanerochaete carnosa*, PcGCE (Tsai et al., 2012). The wood composition and the susceptibility of the lignocellulose to enzymatic hydrolysis with and without pretreatment were investigated in transgenic and wild-type hybrid aspens. Small-scale analytical saccharification studies were performed after acidic pretreatment, which is a relevant technology for pretreatment of recalcitrant forms of lignocellulose, such as wood. HPAEC (high-performance anion-exchange chromatography) was used to obtain a comprehensive and accurate view of monosaccharide formation during pretreatment and enzymatic hydrolysis. Although expression of the glucuronoyl esterase gene has been studied previously using the herb *Arabidopsis thaliana* (Tsai et al., 2012), this is the first investigation where the effects on a woody plant that is relevant for bioenergy and biorefining have been studied, revealing its specific responses to the transgene. Research in this area offers an indication of the potential benefits or disadvantages associated with specific enzyme activities in planta, for example with regard to plant growth, wood polysaccharide content, and susceptibility to pretreatment and bioconversion.

2. Results and discussion

2.1. Expression of glucuronoyl esterase in hybrid aspen

To study the importance of intermolecular linkages in plant cell walls on the development and wood properties of a hardwood species, we generated transgenic hybrid aspen overexpressing PcGCE under control of 35S CaMV promoter. This enzyme is known to be induced in *P. carnosa* when the fungus is exposed to lignified woody substrates including hardwoods and softwoods (MacDonald et al., 2011), and it is active at acidic pH (Tsai et al., 2012), typical for cell walls. To target the enzyme to cell walls, we used the signal sequence of hybrid aspen secreted cellulase, *PttCel9B3* (Rudsander et al., 2003; Takahashi et al., 2009). To confirm cell-wall targeting of the overexpressed SP<sub>35S::PcGCE</sub> recombinant protein, we prepared *Arabidopsis* transgenic plants expressing 35S::SP<sub>35S::PcGCE</sub>eGFP fusion protein, which can be visualized by confocal microscopy, and observed the localization of the eGFP signal in the root cells. After plasmolysis of cells by exposure to mannitol, the protoplasts shrunk revealing the presence of recombinant protein in cell walls (Fig. 1A). No signal was seen from control wild-type plants. This indicates the proper targeting of the SP<sub>35S::PcGCE</sub> construct for post-synthetic modification of cell walls.

35S::SP<sub>35S::PcGCE</sub> construct was transferred to hybrid aspen by agrobacterium infiltration and 24 kanamycin-resistant independent transformants were analysed by semi quantitative RT-PCR. Five most highly expressing lines were planted in a greenhouse, ten trees per line, together with wild-type trees. Trees were grown for 3 months, when they reached ca. 2 m in height. The transgenic lines were slightly but not significantly shorter than the wild-type trees, except line 22, the height of which was reduced up to 50% (Fig. 2B). All the lines had significantly reduced stem diameter and shed leaves prematurely (Fig. 2A and C). Interestingly, in *Arabidopsis* plants expressing the same enzyme, the leaf-yellowing phenotype was observed in oldest rosette leaves (Tsai et al., 2012). Semi quantitative RT-PCR (Fig. 1B) and the glucuronoyl esterase activity assays (Fig. 1C) were used to confirm transgene expression and enzyme activity in the transformants, respectively. Transcript and activity levels were lowest in line 4 and highest in line 22. No transgene expression or glucuronoyl esterase activity was observed in the wild-type plants.

Distribution of PcGCE activities in sequential protein extractions of transgenic and wild-type plants confirmed the cell wall localization of recombinant protein in aspen of transgenic plants. Most activity was recovered in the ionically-bound and apoplastic fluid fractions (Fig. 1D), indicating that the enzyme is targeted to the apoplast where it is ionically interacting with cell wall components, similar to many extracellular proteins.

2.2. Effect of PcGCE expression on wood chemistry

In order to detect modifications in wood chemistry caused by the PcGCE expression, wood of transgenic and wild-type trees was analysed by diffuse reflectance Fourier transform infrared (FT-IR) spec-
troscopy and orthogonal partial least squares discriminant analysis (OPLS-DA) (Trygg and Wold, 2002). The model showed a very clear separation of transgenic and the wild-type trees with one predictive and two orthogonal components (Fig. 3A), which was supported by the values of Q2(cum) = 0.917, R2X(cum) = 0.899 and R2Y(cum) = 0.938. FT-IR signals reflect oscillation of chemical bonds and since the same type of bonds are present in different polymers, the loading plots should be analysed for the presence of changes in several signals representing the same type of polymer (Gorzsás et al., 2011). The plots (Fig. 3B, Table S1) showed increased lignin and decreased carbohydrates content in the transgenic lines. This was indicated by reduced signals from glycosidic linkage (1150 cm\(^{-1}\)) and other linkages found in carbohydrates (likely cellulose: 900, unspecific: 1000–1100 cm\(^{-1}\) region) and higher signals assigned to bonds in lignin (1422 cm\(^{-1}\), 1462 cm\(^{-1}\), 1510 cm\(^{-1}\), 1595 cm\(^{-1}\)) (Gorzsás et al., 2011).

Pyrolysis–GC/MS (Py–GC/MS) analysis (Meier et al., 2005) was used to find differences in lignin composition and relative lignin and carbohydrate contents in transgenic lines. Although the individual lines when compared to wild-type did not always reveal significant differences (P < 5%), they showed consistent trends resulting in significant changes in transgenic plants when considered altogether (Table 1). Significant increases in syringyl (S) lignin in all transgenic lines, in guaiacyl (G) lignin in four transgenic lines, and in p-hydroxyphenyl (H) lignin in line 22 were observed (Table 1). In all transgenic lines, the S/G ratio and the total relative lignin content were increased. In contrast, the relative carbohydrates content decreased in three lines and carbohydrate to lignin content ratio decreased in all lines. Thus both Py–GC/MS and FT-IR fingerprinting methods pointed to substantial compositional change in cell walls involving a relative increase in lignin and a decrease in carbohydrates.

To quantify these changes, sequential wood extractions were performed as described by Ona et al. (1995). First, the extractives were removed by hot toluene/ethanol mixture followed by ethanol, and water. Then the hemicelluloses were extracted using KOH, and the remaining pellet was analysed for cellulose and lignin contents. The most prominent effect of PcGCE expression revealed by this analysis was a massive increase in Klasson lignin content, by 43% on the average and up to 66% in the most highly expressing line, whereas the acid soluble lignin content was not affected (Fig. 4). This was in striking contrast with the results obtained for transgenic Arabidopsis plants expressing the same enzyme, in which the acid-soluble lignin content was increased up to 30%, but the Klasson lignin content was not affected (Tsai et al., 2012). However, the in situ FTIR analysis of cell walls in interfascicular fibers of Arabidopsis plants expressing PcGCE indeed showed a higher signal at 1595 cm\(^{-1}\) indicating increased lignification in these cell types (Tsai et al., 2012). Excess lignin production has been observed as a reaction of plants to cell wall damage (Caño-Delgado et al., 2003; Denness et al., 2011). Therefore, increased lignification in PcGCE-overexpressing hybrid aspen could be a reaction to weakening of their lignified cell walls caused by the glucuronoyl esterase activity, and since lignified walls are relatively less abundant in Arabidopsis stems, this reaction to the transgene is less prominent in Arabidopsis.

Assuming that other cell wall components were not affected in the transgenic lines, the 43% increase in Klasson lignin content would lead to the decrease of remaining constituents by approximately 5% in relation to their wild-type content (Fig. 4). The cellulose content was indeed observed to decrease to this extent, whereas the contents of hemicellulose, and acid-soluble lignin were too variable to detect such a small change in their content. However, the extractives content was decreased much more than
**Fig. 2.** Morphology of transgenic hybrid aspen lines expressing 35S::SPCel9B3::PcCGE. (A) Appearance of transgenic and wild-type (WT) hybrid aspen at the age of three months. (B) Height growth of WT and transgenic hybrid aspen lines. Means ± SE, n = 8–10 biological replicates per line. (C) Stem diameter of WT and transgenic hybrid aspen measured at internode (INT) 15 and 40. Asterisks indicate P values for comparison with WT: *P < 0.05 (Student’s t-test; n = 8–10 biological replicates per line).

**Fig. 3.** FT-IR analysis of wood in transgenic hybrid aspen lines expressing 35S::SPCel9B3::PcCGE. (A) OPLS-DA models of diffuse reflectance FT-IR spectra from wood of transgenic trees (grey symbols, n = 7–10 per line, five lines) and wild-type (WT) trees (black symbols, n = 18). The model shows the separation between the transgenic and WT plants with one predictive and two orthogonal components. (B) Correlation-scaled loadings plot for Predictive Component 1, showing factors separating transgenic and WT trees. The marked positive bands are more intense in WT and related to cellulose and other carbohydrates. The marked negative bands are more intense in transgenic plants and are mostly related to lignin. The major separating factors that are marked with arrows in the Loadings plots are summarized in Supplementary Table S1.

**Table 1**
Pyrolysis–GC/MS analysis of wood from wild-type (WT) and PcGCE-overexpressing plants.

<table>
<thead>
<tr>
<th>Line</th>
<th>S (%)</th>
<th>G (%)</th>
<th>H (%)</th>
<th>S/G ratio</th>
<th>L (%)</th>
<th>C (%)</th>
<th>C/L ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 4</td>
<td>18.62 ± 0.49**</td>
<td>9.99 ± 0.14*</td>
<td>0.12 ± 0.02</td>
<td>1.86 ± 0.03*</td>
<td>28.72 ± 0.62**</td>
<td>60.84 ± 0.89</td>
<td>2.13 ± 0.08**</td>
</tr>
<tr>
<td>Line 10</td>
<td>20.09 ± 0.38***</td>
<td>10.17 ± 0.16**</td>
<td>0.13 ± 0.02</td>
<td>1.98 ± 0.05***</td>
<td>30.39 ± 0.43***</td>
<td>58.97 ± 0.65**</td>
<td>1.95 ± 0.05***</td>
</tr>
<tr>
<td>Line 21</td>
<td>18.30 ± 0.72**</td>
<td>9.78 ± 0.18</td>
<td>0.14 ± 0.02</td>
<td>1.87 ± 0.07*</td>
<td>28.22 ± 0.83*</td>
<td>60.89 ± 1.01</td>
<td>2.18 ± 0.11*</td>
</tr>
<tr>
<td>Line 22</td>
<td>20.05 ± 0.74***</td>
<td>10.47 ± 0.44***</td>
<td>0.10 ± 0.01*</td>
<td>1.92 ± 0.04**</td>
<td>30.61 ± 1.14***</td>
<td>58.58 ± 1.33**</td>
<td>1.94 ± 0.13***</td>
</tr>
<tr>
<td>Line 23</td>
<td>19.33 ± 0.42***</td>
<td>10.17 ± 0.21**</td>
<td>0.12 ± 0.01</td>
<td>1.90 ± 0.04**</td>
<td>29.62 ± 0.57***</td>
<td>59.66 ± 0.65*</td>
<td>2.02 ± 0.06**</td>
</tr>
<tr>
<td>WT</td>
<td>16.15 ± 0.59</td>
<td>9.29 ± 0.22</td>
<td>0.16 ± 0.02</td>
<td>1.73 ± 0.04</td>
<td>25.99 ± 0.79</td>
<td>62.80 ± 0.87</td>
<td>2.52 ± 0.12</td>
</tr>
</tbody>
</table>

| WT vs PcGCE plantsb | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 | P < 0.0001 |

a Comparison of identified peaks areas, values are percentages of the total peak area. S, peaks assigned to syringyl lignin; G, peaks assigned to guaiacyl lignin; H, peaks assigned to p-hydroxyphenyl lignin; C, peaks assigned to carbohydrates; L, combined peaks assigned to lignin. Wild-type (n = 20 biological replicates), line 4, 10 and 21 (n = 10 biological replicates), line 23 (n = 9 biological replicates), line 22 (n = 7 biological replicates). Asterisks indicate significant differences from the wild type at P < 5% (*), 1% (**), and 0.1% (***) according to Student’s t-test.

b P-values obtained by comparison wild-type plants versus all transgenic plants with Student’s t-test. Means ± SE.
expected, exceeding a 20% reduction in most affected lines (Fig. 4). The extractives of hardwoods comprise a variety of compounds of different nature, such as terpenes, fats, flavonoids and phenolic compounds. They also include water-soluble compounds, including water-soluble polysaccharides, such as pectin, arabinoxylan, and mannan. Therefore, to further investigate the compositional changes in matrix polysaccharides, we carried out cell-wall monosaccharide compositional analysis by TMS (trimethylsilyl) derivatization (Table 2). This analysis showed few distinct effects of PcGCE expression, including a decrease in Ara and Rha, and 4-O-Me-GlcA. A decrease in Rha and Ara without any change in GalA suggests a compositional change in pectin contents in the transgenic lines, which could have contributed to the strong decrease in extractives (Fig. 4). Such change is difficult to interpret in terms of characterized PcGCE activity using model substrates, and probably represents an indirect effect of the transgene expression. A clear decrease in the content of 4-O-Me-GlcA was observed that correlated with enzyme activity in the different lines. The lower content of 4-O-Me-GlcA residues and lignin, exposes these residues to hydrolytic activities residing in cell walls.

Thus in sum, the wood chemical analyses revealed profound changes in cell wall composition in PcGCE-expressing plants, including a severe increase in lignin content and a significant decrease in extractives including water-soluble compounds, such as pectins. The analysis also indicated a lower extent of glucuronoxylan branching consistent with the PcGCE enzyme acting on suggested lignin-4-O-Me-GlcA ester linkages.

2.3. Effects of PcGCE expression on saccharification

To investigate the effect of PcGCE expression on the susceptibility to enzymatic saccharification, small-scale analytical experiments were performed both with and without acid pretreatment. While saccharification without pretreatment generated an enzymatic hydrolysate, saccharification with pretreatment generated a pretreatment liquid and an enzymatic hydrolysate that were analysed separately (Table 3). The pretreatment liquid, which also can be referred to as a hemicellulose hydrolysate, is the result of a thermochemical hydrolysis process catalysed by sulphuric acid, while the other two hydrolysates are the result of the actions of carbohydrate-degrading enzymes.

Table 2

Monosaccharide composition of wood from wild-type (WT) and PcGCE-overexpressing lines.a

<table>
<thead>
<tr>
<th>Line</th>
<th>Ara</th>
<th>Rha</th>
<th>Fuc</th>
<th>Xyl</th>
<th>4-O-Me-GlcA</th>
<th>Man</th>
<th>Gal</th>
<th>GalA</th>
<th>Glc</th>
<th>GlcA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 4</td>
<td>0.51 ± 0.03***</td>
<td>0.87 ± 0.02</td>
<td>0.06 ± 0.06</td>
<td>67.69 ± 1.01</td>
<td>4.83 ± 0.08</td>
<td>0.59 ± 0.13</td>
<td>1.02 ± 0.11</td>
<td>4.68 ± 0.17</td>
<td>19.77 ± 0.81</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Line 10</td>
<td>0.81 ± 0.09***</td>
<td>0.82 ± 0.07</td>
<td>0.06 ± 0.06</td>
<td>66.39 ± 1.18</td>
<td>4.94 ± 0.15</td>
<td>0.71 ± 0.22</td>
<td>0.95 ± 0.09</td>
<td>4.57 ± 0.37</td>
<td>21.00 ± 1.50</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>Line 21</td>
<td>0.84 ± 0.09**</td>
<td>0.83 ± 0.03</td>
<td>0.06 ± 0.04</td>
<td>66.39 ± 1.18</td>
<td>4.94 ± 0.15**</td>
<td>0.89 ± 0.24</td>
<td>1.35 ± 0.15</td>
<td>4.32 ± 0.18</td>
<td>21.03 ± 1.70</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Line 22</td>
<td>0.70 ± 0.03</td>
<td>0.84 ± 0.05</td>
<td>BDL</td>
<td>68.95 ± 1.22</td>
<td>4.53 ± 0.24</td>
<td>0.90 ± 0.24</td>
<td>1.21 ± 0.21</td>
<td>4.35 ± 0.10</td>
<td>18.46 ± 1.05</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Line 23</td>
<td>0.62 ± 0.05*</td>
<td>0.83 ± 0.04</td>
<td>0.02 ± 0.02</td>
<td>64.49 ± 1.19</td>
<td>4.51 ± 0.11**</td>
<td>0.18 ± 0.1</td>
<td>1.08 ± 0.05</td>
<td>4.89 ± 0.29</td>
<td>23.39 ± 1.40</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>WT</td>
<td>0.73 ± 0.04</td>
<td>0.93 ± 0.04</td>
<td>BDL</td>
<td>67.19 ± 2.91</td>
<td>5.07 ± 0.23</td>
<td>0.63 ± 0.23</td>
<td>1.15 ± 0.14</td>
<td>4.66 ± 0.22</td>
<td>19.63 ± 2.67</td>
<td>0.02 ± 0.02</td>
</tr>
</tbody>
</table>

a Mol % of monosaccharide composition of AIR (alcohol insoluble residue) treated with α-amylase and hydrolyzed in 2 M HCl/MeOH. Ara, arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; 4-O-Me-GlcA, 4-O-methylated glucuronic acid; Man, mannose; Gal, galactose; GaLA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid. Asterisks indicate significant differences from the wild-type at P ≤ 0.05 (**), 0.01 (***), and 0.001 (****) according to Student’s t-test. Wild type (n = 4 biological replicates), line 4, 10, 21 and 23 (n = 4 biological replicates), line 22 (n = 2 biological replicates).

b P-values obtained by comparison wide-type samples versus all transgenic samples using Student’s t-test. Means ± SE. BDL: below detection limit.
Table 3

<table>
<thead>
<tr>
<th>Line</th>
<th>Sugar yield (g g\textsuperscript{-1})</th>
<th>(V_{\text{GCE}})</th>
<th>(V_{\text{wt}})</th>
<th>(V_{\text{wt}}/V_{\text{GCE}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Line 4</td>
<td>0.030 ± 0.002 (230%)</td>
<td>0.020 (100%)</td>
<td>0.022 (100%)</td>
<td></td>
</tr>
<tr>
<td>Line 10</td>
<td>0.022 (100%)</td>
<td>0.012 ± 0.002 (60%)</td>
<td>0.014 (70%)</td>
<td></td>
</tr>
<tr>
<td>Line 23</td>
<td>0.019 ± 0.007 (35%)</td>
<td>0.009 ± 0.004 (50%)</td>
<td>0.011 (60%)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.003 (100%)</td>
<td>0.002 ± 0.001 (60%)</td>
<td>0.003 (70%)</td>
<td></td>
</tr>
</tbody>
</table>

In hydrolysates without pretreatment

| Line 4 | 0.036 ± 0.002 (260%)                   | 0.021 (100%)      | 0.023 (100%)      |                               |
| Line 10| 0.022 (100%)                          | 0.012 ± 0.002 (60%) | 0.014 (70%)      |                               |
| Line 23| 0.007 ± 0.007 (75%)                   | 0.004 ± 0.004 (50%) | 0.005 (60%)      |                               |
| WT     | 0.002 (100%)                          | 0.002 ± 0.001 (60%) | 0.003 (70%)      |                               |

In the pretreatment liquid after acidic pretreatment

| Line 4 | 0.037 ± 0.002 (270%)                   | 0.021 (100%)      | 0.023 (100%)      |                               |
| Line 10| 0.022 (100%)                          | 0.012 ± 0.002 (60%) | 0.014 (70%)      |                               |
| Line 23| 0.007 ± 0.007 (75%)                   | 0.004 ± 0.004 (50%) | 0.005 (60%)      |                               |
| WT     | 0.002 (100%)                          | 0.002 ± 0.001 (60%) | 0.003 (70%)      |                               |

Enzymatic hydrolysis of \(PcGCE\)-expressing transgenic hybrid aspen without pretreatment resulted in a statistically significant decrease in the yield of Glc for three lines and in the yield of Xyl for four lines (Table 3). The yields of Ara, Gal and Man were low and for the individual lines they did not change significantly compared to those of the wild-type (Table 3). The average yields of Ara, Gal, Glc, Xyl and Man in the enzymatic hydrolysates of the transgenic lines were 0.003, 0.012, 0.143, 0.019 and 0.012 g g\textsuperscript{-1}, respectively. When the average yields of monosaccharides from the transgenic lines were compared with those of the wild-type, there were significant differences for Gal (41% lower), Glc (44% lower), Xyl (27% lower), and Man (8% lower).

The monosaccharide contents of the pretreatment liquids (Table 3) show that Xyl was the predominant sugar as expected for hemicellulose hydrolysates of hybrid aspen. The Xyl yields of the transgenic lines, however, were not significantly different from that of the wild-type. Small amounts of Gal, Glc, Man and Ara were also released by the pretreatment. For all five transgenic lines, the Glc yield in the pretreatment liquid was significantly lower than that of the wild-type. The average Glc yield of the transformants was 0.016 g g\textsuperscript{-1}, which was only 23% of the Glc yield of the wild-type.

As expected, Glc was the predominant sugar detected after enzymatic hydrolysis of pretreated hybrid aspen (Table 3). For the wild-type, the Glc yield in the pretreatment liquid amounted to 16% of the Glc yield of the enzymatic hydrolysis. The Glc yields of the transgenic lines were slightly higher than those of the wild-type, but the difference was not statistically significant. The yields of the other monosaccharides were low, which is expected as the pretreatment targets the hemicelluloses. The total sugar yields, i.e. the combined yields of the pretreatment liquids and the enzymatic hydrolysates, are shown in Table 4. Table 4 also shows the yields of hexose (Gal, Glc and Man) and pentose (Ara and Xyl) sugars, and the total monosaccharide yield (all five monosaccharides determined). The transgenic hybrid aspens exhibited lower yields of Gal, Glc and Man than the wild-type, and also slightly lower yields of hexoses and total monosaccharides (Table 4). However, these differences were not statistically significant (\(P \leq 0.05\)). The cellulose content of the wild-type, 46.5% (Fig. 4), would result in a theoretical maximum Glc yield from cellulose of 0.52 g Glc per g wood. In addition to that Glc could be obtained by hydrolysis of hemicellulose glucans. Table 4 suggests that the total sugar yields were nearly quantitative.

Lower sugar yields from the transgenic lines compared to the wild-type, as observed for Glc and Xyl without pretreatment (Table 3), might be due to decreased susceptibility to enzymatic hydrolysis and/or to lower carbohydrate content. Different analytical techniques including FT-IR (Fig. 2), Py–GC/MS (Table 1) and sequential extraction (Fig. 4) consistently point towards lower cellulose and carbohydrate content and higher lignin content for the transgenic plants. However, neither TMS analysis (Table 2) or sequential extraction (Fig. 4) support a decrease of the main hemicellulose xylan. Therefore, the decreased Xyl yield obtained for transgenic hybrid aspen without pretreatment can be attributed to decreased susceptibility to xylanases in the enzyme mixture. This can be explained by the higher lignin content, which increases the recalcitrance of lignocellulosic biomass by preventing hydrolytic enzymes from efficiently reaching the carbohydrates and also by causing catalytically unproductive binding of carbohydrate-degrading enzymes including xylanases (Pareek et al., 2013.). The lower Glc yields obtained from transgenic plants after enzymatic hydrolysis of wood that was not pretreated (Table 3) are probably also correlated with decreased susceptibility due to higher lignin content, since only a minor part of the cellulose was converted. Considering the Glc yields expressed per gram of available cellulose, 0.34 g of Glc was obtained from the lignocellulose of
wild-type trees without pretreatment. The transgenic lines showed a 30% reduction to approximately 0.26 g (Fig. 5). As the pretreatment resulted in about five times higher and almost quantitative sugar yields in all genotypes (Table 3), the difference between the transgenic lines and the wild-type would be more difficult to discern, but the results obtained with pretreated hybrid aspen nevertheless show that after pretreatment the yields per gram of cellulose are increased by about 12% by the PcGCE-expressing lines. The yield of glucose in g per g of cellulose in hydrolysates (Table 4) are not indicated.

### Other factors
Other factors, such as low carbohydrate-to-lignin ratio, were obviously more important. However, the higher S/G ratio could possibly be another factor, beside reduced intermolecular cross links, that increased cellulose conversion after pretreatment.

### Pretreatment and enzymatic hydrolysis results
Pretreatment and enzymatic hydrolysis results in formation of acetate through hydrolysis of acetyl groups in xylan. The yield of acetic acid may change as a consequence of modifications of the content or the composition of xylan. Other aliphatic acids, such as formic acid and levulinic acid, are formed as thermal degradation products during acidic pretreatment (Jonsson et al., 2013). Acetic acid was the quantitatively dominant acid in the pretreatment liquid (Fig. S1B). The yield of acetic acid in the pretreatment liquid is shown in Fig. S1A. The yield of acetic acid in the enzymatic hydrolysate (Fig. S1B) was about one order of magnitude lower than the yield in the pretreatment liquid (Fig. S1A), and there was no significant difference between the transgenic lines and the wild-type. This is consistent with the TMS analysis and the sequential extraction, which indicated no decrease in xylan or total hemicellulose content for the transgenic lines.
3. Conclusions

PcGCE expression in a woody species provided new insights on the in planta activity of the enzyme, on plant reaction to such expression, and on effects of its action on biomass saccharification. The decreased ratio of 4-O-Me-GlcA to Xyl in transgenic plants supports the notion of the enzyme acting on ester linkage involving 4-O-Me-GlcA side chain of glucuronoxylan. Secondary effects of PcGCE expression were observed in hybrid aspen and involved a massive increase in Klassen lignin, a decrease in extractives, most likely including pectic water-soluble substances, and a decrease in cellulose. This suggests that PcGCE expression induces stress responses in hybrid aspen as also observed in Arabidopsis. Considering the massive increase in lignin, the saccharification resulted in surprisingly good yields, not different from the wild-type after acid pretreatment, while the yields without pretreatment were expectedly decreased. Considering yields of Glc per cellulose, the transgenic plants showed lower yields without pretreatment but higher yields after acid pretreatment compared to wild-type. This indicates that the contribution to recalcitrance of the increased lignin content of the transgenic lines expressing PcGCE was removed by the acid pretreatment and is consistent with better extractability in transgenic lines due to reduced cross-linking. Although expression of PcGCE provided interesting effects on saccharification, it was also obvious that there was a negative impact of expressing this gene on plant growth. The reasons for such effects are presently unclear, and they would need to be clarified before such enzymes could be used in biotechnology programs to reduce biofuels.

4. Experimental

4.1. Vector construction, hybrid aspen transformation, gene expression and enzyme localization and activity analysis

4.1.1. Vector construction

The Phanerochaete cariosa glucuronoyl esterase cDNA clone (PcGCE, NCBI accession: JQ972915; Tsai et al., 2012) was used to create the expression vector. Its native signal peptide sequence was exchanged to the corresponding sequence from PttCel9B3 gene (alias PttCel9B) form Populus tremula x Populus tremuloids (GenBank accession AY660968.1; Rudsander et al., 2003) using PCR. Briefly, PttCel9B3 signal peptide sequence with adaptor sequence (underlined) was amplified using the primers OC9Bf1 (5’ caccATGAGAAGGGGACCTTCTTGGCCTTG 3’) and OC9Br6 (5’ CGACTGGGCTTTTGTTggttaattgggtttGGCTTGGACAAAACC 3’), and PcGCE cDNA without signal peptide sequence but including the same adaptor sequence was amplified using primers FC6f1 (5’ aaacccaatcaacAAGAACCGCCACTGTGTTGGCTGCCTCCACG 3’) and FC6r1 (5’ ATGAGAAACGTGGGACCTTGGTGTCAGTTGATC 3’). The purified products were used as templates for the final amplification, with OC9Br6 and FC6r1s (5’ ttaATGAGAAACCTGGG GTGTCAGTTGATC 3’) primers. The product (SP_PcGCE) was cloned into pENTR/D-TOPO vector (pENTR™ Directional TOPO® Cloning Kits, Invitrogen), sequenced and subsequently subcloned into binary vector pK2WG7.0 (Karimi et al., 2002) using Gateway® System (Invitrogen). The vector containing SP_PcGCE was transformed into competent Agrobacterium tumefaciens strain GV3101 using electroporation.

4.1.2. Hybrid aspen transformation

Hybrid aspen, Populus tremula L. x tremuloides Michx., trees (clone T89) were transformed by Agrobacterium tumefaciens as described in Gray-Mitsumune et al., 2008. Kanamycin resistant plants were regenerated, clonally propagated in vitro and planted in the greenhouse in a commercial soil/sand/fertilizer mixture (Yrkes Plantjord; Weibulls Horto, http://www.weibullshorto.se) at 22 °C (15 °C (light/dark) with a 18-h photoperiod and a relative humidity of at least 70%. Trees were watered daily and fertilized once per week with an approximately 150 ml 1% Rika-S (N/P/K 7:1:5; Weibulls Horto). The transgenic trees were grown together with the wild-type (WT) trees for 3 months with a weekly rotation of the site in the greenhouse to minimize positional effects. At harvest, two types of samples were collected:

1. for gene expression and enzyme activity analysis, the internodes 20–39 (counted from the top) were collected, debarked, and exposed developing wood was scraped with a scalpel directly into liquid nitrogen, ground to a fine powder in a mortar, and stored at −80 °C (Gray-Mitsumune et al., 2004).
2. for wet chemistry, Py–GC/MS, Fourier transform infrared spectroscopy (FT-IR), saccharification and total carbohydrate analysis, the internodes 44–60 were collected, debarked, and the wood without pith was freeze-dried for 36 h.

4.1.2.1. Gene expression and enzyme activity analysis

Total RNA was extracted using an Aurum RNA extraction kit (Bio-Rad, http://www.bio-rad.com). DNA was removed by DNA-free kit (Ambion, USA). 1 µg of total RNA was used for reverse transcription using the iScript cDNA biosynthesis kit (Bio-Rad). Purity of DNA was confirmed by DNA-template PCR reaction with reference gene primers. Fc6seq1 (5’ CTGCTAAACACAAACAGCTT 3’) and Fc6r1s (5’ ttaATGAGAAACCTGGG GTGTCAGTTGATC 3’) primers were used to amplify 899 bp fragment of PcGCE and gUBQL for (5’ tagatcaagacagctgtggtt 3’) and gUBQL_rev (5’ cctcctacgaagcaacagcaacagcaacagcaacagggaagctttcctgg 3’) primers were used to amplify 152 bp fragment of polyubiquitin gene (NCBI AF244045.1), used as a reference gene.

Glucuronol esterase activity was measured as previously described by Španíková and Biely (2006). Briefly, total proteins of developing wood were extracted in 50 mM sodium phosphate buffer, pH 6.0, containing 2 mM EDTA, 4% PVP mw 360,000, 1 M NaCl, and protease inhibitor cocktail (complete, Roche) for 1 h at 4 °C with stirring. After centrifugation (20,000g, 10 min), the protein contents were determined in supernatants (Bradford, Bio-Rad), and aliquots containing 5 µg of protein were used for the glucuronol esterase activity assays. Reactions were prepared in ELISA-plates in a volume of 40 µl in 50 mM sodium phosphate buffer pH 6.0, containing 5 mM methyl 4-O-methyl-α-D-glucopyranuronate, as substrate, received from Prof. Peter Biely (Institute of Chemistry, Slovak Academy of Sciences), incubated for 1 h at 30 °C, and ester bonds remaining in the reaction were quantified according to Hestrin (1949).

Apoplastic fluids were isolated from all aerial parts of 6-week-old plants grown in vitro as described in Pogorelko et al. (2011) with modifications. Namely, 50 mM Na-phosphate buffer with 50 mM EDTA pH 6.0 was used for vacuum infiltration. Remaining material was frozen and ground in the same buffer for isolation soluble proteins. After centrifugation 20 000g for 10 min, the supernatant containing soluble proteins was collected, and the pellet was resuspended in Na-phosphate buffer containing 1 M NaCl to release ionically-bound proteins. Glucuronol esterase activity was measured as described above and expressed per 1 g of dry material used for sequential extraction.

4.1.3. Intracellular localization of PcGCE recombinant protein

SP_PcGCE was subcloned from entry clone (pENTR/D-TOPO vector) into binary vector pK7FWG2.0 (Karimi et al., 2002) using Gateway® System (Life Technologies™). For expression of SP_PcGCE, PcGCE:GFP. Arabidopsis thaliana plants were transformed...
using the floral dip method (Clough and Bent, 1998). Seeds collected from the transformed plants were germinated on ½ MS plates with kanamycin (50 µg ml⁻¹). T2 Seeds were germinated on ½ MS plates, and 7-day-old seedlings were mounted on glass slides, plasmolyzed in 20% manniitol and immediately analysed using a Leica TCS SP2 confocal microscope employing an argon laser for excitation at 488 nm and detecting emission between 498 and 530 nm by sequential line scanning. To verify that the signal emitted by the cells had the spectrum of GFP, the lambda scan was performed every 10 nm between 480 and 630 nm and compared to that of GFP.

4.2. Wood chemistry analysis

Freeze-dried wood was milled using an A11 Basic Analytical Mill (IKA, Staufen, Germany) followed by grinding in Ultra Centrifugal mill ZM 200 (Retsch, Haan, Germany) equipped with a 0.5 mm ring sieve to obtain the rough wood powder. The rough powder was subsequently ground to a fine powder in 10 ml stainless steel jars with one 12 mm grinding ball at 30 Hz for 2 min, using an MM400 bead mill (Retsch).

4.2.1. FT-IR spectroscopy

Samples of seven to ten trees of selected lines and 18 WT trees were individually examined. Ten mg of fine wood powder were mixed with 390 mg potassium bromide (KBr, infrared spectroscopy quality; Merck, Darmstadt, Germany) and ground using an amethyst mortar and pestle before measurements. A diffuse reflectance 16-sample holder carousel accessory was used (Harrick Scientific Products, Pleasantville, NY, USA) to run 15 samples, and pure KBr for background correction. FT-IR spectra were recorded under vacuum (4 mbar), using a Bruker IFS 66/S spectrometer (Bruker Optik GmbH, Ettlingen, Germany). Spectra were collected with 128 scans at a resolution of 4 cm⁻¹ between 400 and 5200 cm⁻¹ and converted to data point tables using OPUS (V7.0.122; Bruker Optik). Data for the region 800–2000 cm⁻¹ were used in the subsequent multivariate analysis. Spectra were baseline corrected (2-point linear fit at 792 and 1865 cm⁻¹), and normalized over the same spectral range using custom-build software in Matlab (V 8.2. The MathWorks, Inc.) and then exported to the SIMCA-P software (version 11.0.0.0, Umetrics AB, Sweden, with built-in options) for multivariate analysis. The initial PCA analysis was carried out with 118 observations, using custom-build software in Matlab (V 8.2; The MathWorks, Inc.) and then exported to the SIMCA-P software (version 11.0.0.0, Umetrics AB, Sweden). The pyrolysate was separated and analysed by HPLC (HPAEC). The pyrolysate was separated and analysed according to Gerber et al. (2012).

4.2.2. Pyrolysis coupled to gas chromatography/mass spectrometry (Py-GC/MS)

Fine wood powder, 50 µg (±10 µg) was applied to a pyrolyser equipped with an auto sampler (PY-2020ID and AS-1020E, Frontier Lab, Japan) connected to a GC/MS (7890A/5975C; Agilent Technologies AB, Sweden). The pyrolysate was separated and analysed according to Sweeley et al. (1966). Silylated monosaccharides were separated on a J&W DB-5MS column (30 m length, 0.25 mm diameter, 0.25 µm film thickness) (Agilent Technologies) with the oven program: 80 °C followed by a temperature increase of 20 °C/min to 140 °C for 2 min, then 2 °C/min to 200 °C for 5 min, then 30 °C/min to 250 °C for 5 min. The total run time was 47 min.

4.4.2.4. Wet chemical analysis

Extractives-free wood was prepared as described by Ona et al. (1995) starting with 100 mg of fine wood powder pooled for all 10 trees per line. Duplicate technical replicates per line were prepared. Extractives were removed by sequential extractions with ethanol:toluene (1:2; v:v) for 6 h, then 95% ethanol for 4 h, and finally with water for 2 h. All extractions were carried in a Soxhlet apparatus. The pellet was dried and hemicelulloses were removed from extractives-free wood samples by sequential extraction at 4 °C for 1 h each in 0.1 M KOH, 1 M KOH and 6 M KOH followed by washing with water. The remaining pellets were used to determine the crystalline cellulose content according to Updegraff (1969) where the Glc content was determined with the anthrone method (Scott and Melvin, 1953), and the acid-insoluble lignin (Klason lignin) content according to Theander and Westerlund (1986).

4.3. Pretreatment and enzymatic hydrolysis

The rough wood powder was prepared as for wood chemical analyses and was then sieved using an analytical sieve shaker AS 200 (Retsch). The fraction with a particle size between 0.1 and 0.5 mm was collected. This fraction from two different plants of each line was pooled as one biological sample. For each line, 4–5 biological replicates were analysed, with the exception of Line 22, where 2 technical replicates from a single sample consisting of a pool of 10 plants were analysed. Fifty mg of wood sample in a reaction mixture with a total weight of 1000 mg was pretreated using a single-mode microwave system (Initiator Exp, Biotage, Uppsala, Sweden) using an acid catalyst [1% (w/w) sulphuric acid]. The pretreatment was performed for 10 min at 165 °C. The combined severity (Chum et al., 1990) of the pretreatment was 2.2. The solid and liquid fractions were separated by centrifugation for 15 min at 14,100g in preweighed tubes. The liquid fraction, referred to as the pretreatment liquid, was collected for analysis, while the solid fraction was washed twice with one ml of deionized water and once with one ml of sodium citrate buffer (50 mM, pH 5.2) prior to enzymatic hydrolysis. The weight of the residual washed solids from the pretreatment was determined. Then, sodium citrate buffer (50 mM, pH 5.2) and 50 mg of an enzyme cocktail consisting of equal proportions of Celluclast 1.5 L and Novozyme 188 (obtained from Sigma–Aldrich (St. Louis, MO, USA)) were added so that the total weight of the reaction mixture was 1000 mg. Reaction mixtures with wood that had not been pretreated consisted of 50 mg of milled wood, 900 mg of the sodium citrate buffer, and 50 mg of the enzyme cocktail. The reaction mixtures were incubated for 72 h at 45 °C in an orbital shaker (Ecotron incubator shaker, Infors, Bottmingen, Switzerland) set at 170 rpm. The liquid remaining after 72 h was analysed using high-performance anion-exchange chromatography (HPAEC).

by 2 M HCl/MeOH at 85 °C for 24 h in 6 ml glass tubes. The tubes were cooled down and the solvent was evaporated at 40 °C under the stream of nitrogen. After 3 rounds of washing with methanol and evaporation in the stream of nitrogen, silylation was carried out using Tri-sil reagent (3-3039, SUPELCO) at 80 °C for 20 min. Solvent was evaporated under a stream of nitrogen and pellet was dissolved in 1 ml hexane and filtered through glass wool. This filtrate was evaporated to the final volume of 200 µl of which 0.5 µl was analysed by GC/MS (7890A/5975C; Agilent Technologies) according to Sweeley et al. (1966). Silylated monosaccharides were separated on a J&W DB-5MS column (30 m length, 0.25 µm diameter, 0.25 µm film thickness) (Agilent Technologies) with the oven program: 80 °C followed by a temperature increase of 20 °C/min to 140 °C for 2 min, then 2 °C/min to 200 °C for 5 min, then 30 °C/min to 250 °C for 5 min. The total run time was 47 min.
4.4. Analysis of hydrolysates using HPAEC

The concentrations of sugars (Ara, Gal, Glc, Xyl and Man) in the pretreatment liquid and released after 72 h of enzymatic hydrolysis were determined by using an HPAEC system with pulsed amperometric detection (Ion Chromatography System ICS-3000, Dionex, Sunnyvale, CA, USA). The separation was performed using a CarboPac PA20 column (3 x 150 mm) (Dionex) equipped with a CarboPac PA20 guard column (3 x 30 mm) (Dionex). Prior to injection the samples were filtered through 0.2 µm nylon filters (Millipore). A volume of 10 µl was loaded. Elution of sugars was performed with a 2 mM solution of sodium hydroxide during 27 min, followed by regeneration with 100 mM sodium hydroxide for 5 min, and equilibration with 2 mM sodium hydroxide for 15 min. The flow rate was 0.4 ml min⁻¹. Pulsed amperometric detection (PAD) of monosaccharides was performed with the detector set on Gold Standard PAD waveform and with Ag/AgCl as reference electrode. Peaks were identified and quantified by comparison of standards containing Ara, Gal, Glc, Xyl, and Man (monosaccharides were purchased from Sigma–Aldrich). The sugar yields in the pretreatment liquid and in the enzymatic hydrolysate are reported as g of sugar per g (dry weight) of wood after pretreatment and after 72 h of enzymatic hydrolysis, respectively.

The concentrations of aliphatic acids (acetic acid, formic acid and levulinic acid) in the pretreatment liquid, and acetic acid in enzymatic hydrolysate were determined by using the ICS-3000 system and the conductivity detector (Dionex). Separation was performed with an AS15 (4 x 250 mm) separation column equipped with an AG15 (4 x 50 mm) guard column (Dionex). The mobile phase consisted of a 35 mM solution of sodium hydroxide (Sodium Hydroxide Solution for IC, Sigma–Aldrich), and the flow rate was 1.2 ml min⁻¹.

4.5. Statistical analyses

To analyse the difference between individual lines and the wild-type, the significance was tested at P < 0.05 using two-tailed, heteroscedastic (unequal variance) Student's t-test in Excel, or using modelling by Standard Least Squares, followed by the t-test in JMP® 9.0.2 (SAS Institute).

Acknowledgements

We are grateful to Prof. Peter Biely (Institute of Chemistry, Slovak Academy of Sciences) for the gift of substrates, the Plant Cell Wall and Carbohydrate Analytical Facility at UPSC/SLU, supported by Bio4Energy and TC4F project for help in cell wall analyses and Dr. András Gorzsás and the Vibrational Spectroscopy Platform at the Chemical-Biological Centre (KBC) for assistance with the FTIR spectroscopic analysis. The funding from Formas (including HemiPop), the Swedish Research Council (VR) (621-2011-4388), Bio4Energy (www.bio4energy.se), the Swedish Energy Agency (35367-1), and the Swedish Governmental Agency for Innovation Systems (VINNOVA) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.06.002.

References


