Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification

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Summary

Lignocellulosic biomass is utilized as a renewable feedstock in various agro-industrial activities. Lignin is an aromatic, hydrophobic and mildly branched polymer integrally associated with polysaccharides within the biomass, which negatively affects their extraction and hydrolysis during industrial processing. Engineering the monomer composition of lignins offers an attractive option towards new lignins with reduced recalcitrance. The presented work describes a new strategy developed in Arabidopsis for the overproduction of rare lignin monomers to reduce lignin polymerization degree (DP). Biosynthesis of these ‘DP reducers’ is achieved by expressing a bacterial hydroxycinnamoyl-CoA hydratase-lyase (HCHEL) in lignifying tissues of Arabidopsis inflorescence stems. HCHL cleaves the propanoid side-chain of hydroxycinnamoyl-CoA lignin precursors to produce the corresponding hydroxybenzaldehydes so that plant stems expressing HCHL accumulate in their cell wall higher amounts of hydroxybenzaldehyde and hydroxybenzoate derivatives. Engineered plants with intermediate HCHL activity levels show no reduction in total lignin, sugar content or biomass yield compared with wild-type plants. However, cell wall characterization of extract-free stems by thioacidolysis and by 2D-NMR revealed an increased amount of unusual C6-C7 lignin monomers most likely linked with lignin as end-groups. Moreover the analysis of lignin isolated from these plants using size-exclusion chromatography revealed a reduced molecular weight. Furthermore, these engineered lines show saccharification improvement of pretreated stem cell walls. Therefore, we conclude that enhancing the biosynthesis and incorporation of C6-C7 monomers (‘DP reducers’) into lignin polymers represents a promising strategy to reduce lignin DP and to decrease cell wall recalcitrance to enzymatic hydrolysis.

Keywords: cell wall, lignin, hydroxycinnamoyl-CoA hydratase-lyase, saccharification, polymerization degree, bioenergy.

Introduction

Plant cell walls are composed of a polysaccharide-rich network of cellulose microfibrils and hemicelluloses embedded in aromatic polymers known as lignins. Lignins are mainly derived from polymerization of three hydroxyphenylpropanoid monomers (i.e. monolignols), namely p-coumaryl, coniferyl and sinapyl alcohols that give rise to the p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units (Boerjan et al., 2003). Monolignols have a C9H8O2 skeleton that consists of a phenyl ring (C6) and a propanoid (C3) side-chain. Lignin is essential for higher plants as it provides mechanical strength for upright growth, confers hydrophobicity to vessels that transport water and nutrients and acts as a physical barrier against pathogens that degrade cell walls (Boudet, 2007).

Lignocellulosic biomass is widely used as a raw material for the industrial production of pulp and paper and as a rumen livestock feed. Plant feedstocks also represent a source of fermentable sugars for the production of ethanol as well as pharmaceuticals and advanced fuels using engineered micro-organisms (Keasling, 2010). However, lignin confers recalcitrance to the processing of plant cell walls, and negative correlations exist between lignin content in plant biomass and pulp yield, forage digestibility or polysaccharide saccharifiability (Baucher et al., 2003; Chen and Dixon, 2007; Jung and Allen, 1995; Studer et al., 2011; Taboada et al., 2010). Consequently, reducing lignin content from plant feedstocks or facilitating its removal is of major interest in the field of lignocellulosic biofuels and pulping (Simmons et al., 2010).
Lignin biosynthesis has been extensively studied and was shown to be well conserved across land plants (Halpin, 2004; Umezawa, 2010; Wang and Chapple, 2010). Genetic modifications such as transcript reduction and allelic variation of specific genes involved in particular steps of this pathway have been employed to reduce lignin content in various plant species (Chen and Dixon, 2007; Leplé et al., 2007; Li et al., 2008; Xu et al., 2011). However, these approaches often result in undesired phenotypes such as dwarfism, sterility and increased susceptibility to environmental stresses owing to loss of cell wall integrity or the constitutive activation of defecse responses (Bonawitz and Chapple, 2010; Chen and Dixon, 2007; Gallego-Giraldo et al., 2011a, b; Voelker et al., 2011).

Alternatively, changing the recalcitrant structure of lignin can be achieved by modifying its monomer composition and its physico-chemical properties. For example, incorporation of coniferyl ferulate into lignin improves enzymatic degradation of cell wall polysaccharides (Grabber et al., 2008). Moreover, it has been demonstrated recently that enrichment in either S-hydroxy-G- or S-units in lignin contributes to enhanced saccharification or pulping efficiencies without significantly affecting biomass yields and lignin content (Dien et al., 2011; Fu et al., 2011; Li et al., 2010; Stewart et al., 2009).

In this study, as an alternative strategy to reduce lignin recalcitrance, we developed a dominant approach that diverts precursors from the lignin pathway and enhances production of \( \text{C}_6\text{C}_1 \) aromatics that are known as non-conventional lignin precursors from the lignin pathway and enhances production of citrance, we developed a dominant approach that diverts pre-cell wall polysaccharides (Grabber et al., 2008). Enzymatic hydrolysis or pulping efficiencies without significantly affecting biomass yields and lignin content (Dien et al., 2011; Fu et al., 2011; Li et al., 2010; Stewart et al., 2009).

In this study, as an alternative strategy to reduce lignin recalcitrance, we developed a dominant approach that diverts precursors from the lignin pathway and enhances production of \( \text{C}_6\text{C}_1 \) aromatics that are known as non-conventional lignin precursors after export to the apoplast. Compared with regular \( \text{C}_6\text{C}_1 \) monolignols, these \( \text{C}_6\text{C}_1 \) monomers have reduced polymerization properties as they lack propanoid side-chain and its conjugated double bond, disabling them from undergoing condensation at their \( \beta \)-position. Such \( \text{C}_6\text{C}_1 \) aromatics are usually found in trace amounts in most lignins and frequently form lignin end-groups (Kim and Ralph, 2010; Kim et al., 2003; Moree et al., 2004; Ralph et al., 1997, 2001, 2008). This dominant approach is designed to increase the amount of \( \text{C}_6\text{C}_1 \) monomers in lignin to reduce its DP and to be easily transferable from model plant species to crops as it does not require any particular genetic background. For this purpose, we expressed a hydroxycinnamoyl-CoA hydratase-lyase (HCHL; EC 4.2.2.101/EC 4.1.2.41) from \( \text{Pseudomonas fluorescens} \) in stems of Arabidopsis. HCHL is an enzyme that catalyses the hydration of the double bond of the lignin precursor thioesters: \( \text{p-coumaryl-CoA, caffeoyl-CoA and feruloyl-CoA, followed by a retro-aldol cleavage reaction that produces the corresponding C}_6\text{C}_1 \) hydroxybenzaldehydes and acetyl-CoA (Figure S1; Mitra et al., 1999). The promoter of a secondary cell wall cellulose synthase gene (\( \text{CesA}4 \) / \( \text{IRXS} \)) was used to restrict HCHL expression to lignifying tissues of the stem and to prevent depletion of hydroxycinnamoyl-CoAs in other tissues where they serve as precursors for hydroxycinnamate derivatives involved in plant defecse and development (Buer et al., 2010; Gou et al., 2009; Naoumkina et al., 2010). Such secondary cell wall-specific promoter was preferred to those of lignin biosynthetic genes that are known to be active in non-lignified tissues for the synthesis of phenylpropanoid-derived metabolites (Vogt, 2010). Similarly, constitutively active promoters such as \( \text{pcAMV}355 \) were not selected on the basis that high HCHL expression would be detrimental to the development and growth of non-lignified tissues (Merali et al., 2012). We show that most of the plants expressing the HCHL gene under the control of the \( \text{IRXS} \) promoter display no significant changes in lignin content, plant development or biomass yield. We also demonstrate that \( \text{C}_6\text{C}_1 \) monomers accumulate as end-groups in the lignin of HCHL transgenics, resulting in reduced lignin DP and cell walls less recalcitrant to enzymatic hydrolysis.

Results
Expression of HCHL in Arabidopsis stems
The tissue-specific activity of the \( \text{IRXS} \) promoter used to express HCHL was investigated using beta-glucuronidase (GUS) as a reporter gene (Figure S2). GUS activity was essentially detected in the xylem vessels of the stem. Prolonged incubation also revealed strong GUS activity in stem interfascicular fibres, and more moderate staining was observed in the vascular system of young seedlings, siliques, rosette and cauline leaves. No activity was detected in other organs or tissues except for the style and anthers. A codon-optimized DNA sequence encoding for HCHL from \( \text{P. fluorescens} \) was cloned downstream the \( \text{IRXS} \) promoter for preferential expression in lignifying tissues of Arabidopsis stems. The presence of HCHL transcripts in the main stem of five independent transformants was verified by RT-PCR in the T1 generation (Figure 1a). T2 plants homozygous for the \( \text{IRXS}:\text{HCHL} \) construct were used to analyse HCHL protein expression and activity. The presence of HCHL protein in stem extracts of the five selected lines was confirmed by Western blot analysis (Figure 1a), and HCHL activity in these lines ranged from 0.025 to 0.16 pkat vanillin/\( \mu \)g protein using feruloyl-CoA as substrate, whereas no activity was detected in wild-type plants (Table 1). Based on these results, four transgenic lines were selected for detailed analysis: two lines exhibiting both extremes of HCHL activity and two others with intermediate activity levels.

Growth characteristics and tissue anatomy of \( \text{IRXS:}\text{HCHL} \) lines
\( \text{IRXS:}\text{HCHL} \) plants had growth and development characteristics visually similar to those of the wild type from the early rosette stage and until senescence (Figure 1b). However, senesced mature stems from lines \( \text{IRXS:}\text{HCHL}(4) \) and \( \text{IRXS:}\text{HCHL}(5) \) were slightly shorter (22% and 13% reduction) and had lower dry weight yield (30% and 16% reduction) compared with control plants, whereas those from lines \( \text{IRXS:}\text{HCHL}(1) \) and \( \text{IRXS:}\text{HCHL}(2) \) were not significantly different (Table 2). Stem tissues of \( \text{IRXS:}\text{HCHL} \) plants were inspected using light microscopy, and transverse cross-sections stained with lignin-specific reagents such as toluidine blue O, Mäule (S-units) and phloroglucinol–HCl (hydroxycinnamaldehyde units) showed similar patterns to those of wild-type plants except for the \( \text{IRXS:}\text{HCHL}(4) \) line in which occasional sections showed a few collapsed xylem structures (Figure 1c). Overall, these data could not demonstrate drastic changes in lignin content and composition in \( \text{IRXS:}\text{HCHL} \) plants, although the observation of abnormal xylem vessels for one of the transgenic lines suggested that some modifications of cell wall integrity could sometimes occur.

\( \text{IRXS:}\text{HCHL} \) lines show enrichment in cell wall-bound \( \text{C}_6\text{C}_1 \) aromatics
Analysis of methanol-soluble fractions extracted from stems of \( \text{IRXS:}\text{HCHL} \) plants showed higher amount of various \( \text{C}_6\text{C}_1 \) aromatics compared with wild type (Tables S1–S2). Therefore, presence of these aromatics was investigated in extract-free cell wall residues (CWR) obtained from stems. Mild alkaline hydrolysis
was performed on stem CWR to release loosely bound aromatics and revealed the presence of 4-hydroxybenzaldehyde (HBAld), 3,4-dihydroxybenzaldehyde (3,4-HBAld), vanillin (Van), 5-hydroxyvanillin (5OH-Van), syringaldehyde (SyrAld), 4-hydroxybenzoic acid (HBA), vanillic acid (VA) and syringic acid (SyrA). 5OH-Van was only detected in IRX5:HCHL samples, and HBAld, SyrAld, HBA, VA and SyrA were increased by approximately 2-, 6-, 68-, 2- and 5-fold in cell walls of the different IRX5:HCHL lines compared with wild type (Table 3). These results indicate that larger amounts of C6C1 aromatics are loosely bound to cell walls in IRX5:HCHL plants. Moreover, similar amounts of ferulate and p-coumarate were quantified in cell walls of transgenics and wild type (data not shown).

Thioacidolysis reveals that lignin composition and structure are modified in IRX5:HCHL plants
Line IRX5:HCHL(2) was selected for further analyses as it showed no defective xylem structures and had biomass yields similar to wild-type plants. A preliminary screen performed on these plants using Fourier transform spectroscopy suggested no
lated monomers from lignin units involved only in labile bonds, but also from cleaved ester-linked monomers. The total yield of conventional H, G and S monomers released from lignin after thioacidolysis was reduced by 25% and 16% in the two lines of IRX5:HCHL compared with wild-type plants (Table 4). In contrast, the molar frequency of H monomers was significantly different between the wild-type and transgenic samples of coniferaldehyde (Cald), Van and VA were not significant. However, the molar frequency of G monomers was significantly higher in IRX5:HCHL plants (Table 4).

In addition to the lignin-derived conventional monomers, thioacidolysis released more unconventional C₆C₁ units such as vanillin (Van) and syringaldehyde (SyrAld) recovered as their dithio derivative products released by thioacidolysis showed on average a 20.8- and 1.65-fold increase in S/G ratio ranging between 0.34 and 0.36. However, the molar frequency of H monomers was significantly higher in IRX5:HCHL plants (Table 4).

### Table 3
Quantitative analysis of cell wall-bound aromatics in stems from extract-free senesced mature wild-type (WT) and IRX5:HCHL plants. Values are means of four biological replicates (n = 4)

<table>
<thead>
<tr>
<th>Plant line</th>
<th>HBAld μmol/g KL (%)H+G+S</th>
<th>3,4-DHBAld μmol/g KL (%)H+G+S</th>
<th>Van μmol/g KL (%)H+G+S</th>
<th>SOH-Van μmol/g KL (%)H+G+S</th>
<th>SyrAld μmol/g KL (%)H+G+S</th>
<th>HBA μmol/g KL (%)H+G+S</th>
<th>VA μmol/g KL (%)H+G+S</th>
<th>SyrA μmol/g KL (%)H+G+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>14.3 ± 1 (0.31)</td>
<td>4.3 ± 1 (0.31)</td>
<td>0.9 ± 0.3 (0.06)</td>
<td>2.6 ± 0.2 (0.25)</td>
<td>6.4 ± 1.4 (0.64)</td>
<td>18.7 ± 3.5 (1.84)</td>
<td>7.9 ± 0.3 (0.77)</td>
<td>6.4 ± 0.2 (0.67)</td>
</tr>
<tr>
<td>IRX5:HCHL</td>
<td>5.0 ± 0.1 (0.49)</td>
<td>2.6 ± 0.2 (0.25)</td>
<td>6.4 ± 1.4 (0.64)</td>
<td>0.8 ± 0.3 (0.06)</td>
<td>6.9 ± 0.1 (0.55)</td>
<td>6.2 ± 0.2 (0.50)</td>
<td>1.2 ± 0.0 (0.09)</td>
<td>2.1 ± 0.0 (0.20)</td>
</tr>
</tbody>
</table>

### Table 4
Lignin content and main H, G and S lignin-derived monomers obtained by thioacidolysis of extract-free senesced mature stems from wild-type (WT) and IRX5:HCHL(2) plants. Acidic-soluble lignin (ASL) and Klason lignin (KL) values are expressed as a relative percentage of the cell wall residue (CWR). Values are means ± SE from duplicate analyses (n = 2)

<table>
<thead>
<tr>
<th>Plant line</th>
<th>ASL% of CWR</th>
<th>KL % of CWR</th>
<th>Total yield (H+G+S) μmol/g KL</th>
<th>% H</th>
<th>% G</th>
<th>% S</th>
<th>S/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>WT</td>
<td>1.44 ± 0.04</td>
<td>20.42 ± 0.14</td>
<td>1356 ± 40</td>
<td>0.98 ± 0.00</td>
<td>73.2 ± 0.3</td>
<td>25.9 ± 0.3</td>
</tr>
<tr>
<td>IRX5:HCHL</td>
<td>1.32 ± 0.05</td>
<td>20.12 ± 0.15</td>
<td>1014 ± 5</td>
<td>1.48 ± 0.04</td>
<td>73.2 ± 0.3</td>
<td>25.2 ± 0.3</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>Culture 2</td>
<td>WT</td>
<td>1.50 ± 0.15</td>
<td>20.32 ± 0.25</td>
<td>1238 ± 13</td>
<td>1.09 ± 0.00</td>
<td>73.8 ± 0.3</td>
<td>25.2 ± 0.3</td>
</tr>
<tr>
<td>IRX5:HCHL</td>
<td>1.25 ± 0.07</td>
<td>21.29 ± 0.14</td>
<td>1041 ± 7</td>
<td>1.47 ± 0.00</td>
<td>72.7 ± 0.1</td>
<td>25.9 ± 0.1</td>
<td>0.36 ± 0.00</td>
</tr>
</tbody>
</table>

### Table 5
Minor monomers obtained by thioacidolysis of extract-free senesced mature stems from WT and IRX5:HCHL(2) plants. Values are means ± SE of duplicate analyses (n = 2). Values are expressed in μmol/g KL and as a relative percentage of the total main H, G and S monomers released by thioacidolysis

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Van alc μmol/g KL (%)H+G+S</th>
<th>Syralc μmol/g KL (%)H+G+S</th>
<th>Vanalc μmol/g KL (%)H+G+S</th>
<th>Syrald μmol/g KL (%)H+G+S</th>
<th>Cald μmol/g KL (%)H+G+S</th>
<th>VA μmol/g KL (%)H+G+S</th>
<th>SyrA μmol/g KL (%)H+G+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>WT</td>
<td>4.3 ± 1 (0.31)</td>
<td>0.9 ± 0.3 (0.06)</td>
<td>2.6 ± 0.2 (0.25)</td>
<td>6.4 ± 1.4 (0.64)</td>
<td>18.7 ± 3.5 (1.84)</td>
<td>7.9 ± 0.3 (0.77)</td>
</tr>
<tr>
<td>IRX5:HCHL</td>
<td>5.0 ± 0.1 (0.49)</td>
<td>2.6 ± 0.2 (0.25)</td>
<td>6.4 ± 1.4 (0.64)</td>
<td>0.8 ± 0.3 (0.06)</td>
<td>6.9 ± 0.1 (0.55)</td>
<td>6.2 ± 0.2 (0.50)</td>
<td>1.2 ± 0.0 (0.09)</td>
</tr>
<tr>
<td>Culture 2</td>
<td>WT</td>
<td>4.6 ± 0.7 (0.37)</td>
<td>0.8 ± 0.3 (0.06)</td>
<td>2.9 ± 0.1 (0.28)</td>
<td>6.3 ± 0.7 (0.60)</td>
<td>16.7 ± 1.9 (1.60)</td>
<td>6.8 ± 0.1 (0.66)</td>
</tr>
<tr>
<td>IRX5:HCHL</td>
<td>5.3 ± 0.1 (0.50)</td>
<td>2.9 ± 0.1 (0.28)</td>
<td>6.3 ± 0.7 (0.60)</td>
<td>16.7 ± 1.9 (1.60)</td>
<td>6.8 ± 0.1 (0.66)</td>
<td>7.0 ± 0.0 (0.65)</td>
<td>2.1 ± 0.0 (0.20)</td>
</tr>
</tbody>
</table>

Vanalc, vanillyl alcohol; Syralc, syringyl alcohol; Van, vanillin; Syrald, syringaldehyde; Cald, coniferaldehyde; VA, vanillic acid; SyrA, syringic acid; nd, not detected.
The bonding mode of the Vanalc and Syralc parent structures that are cleaved during thioacidolysis is most likely as 4-O-ethers. Moreover, when expressed in the same units (e.g. µg/g CWR), the amounts of Van, VA, Syrald and SyrA products released by thioacidolysis substantially exceed those obtained by mild alkaline hydrolysis (Table 3). Overall, these thioacidolysis data suggest that the lignin from IRX5:HCHL plants compared with wild type is enriched in resistant inter-unit bonds and in C₆C₁ monomers linked with lignin only by an ether bond at their C4-OH.

**Analysis of lignin modifications by NMR**

NMR (2D ¹³C–¹H-correlated, HSQC) spectra of whole cell wall material from IRX5:HCHL(2) stems had several new correlations compared with wild type, indicating the presence of ‘new’ components in the cell wall that are even more readily seen in the difference spectrum. These included a new oxidized S-unit (SA), an oxidized H-unit (HA) and correlations resembling those of an oxidized G-unit (Figure 2). However, upon pretreatment of the CWR with crude cellulases to remove most of the polysaccharides and enrich the lignin fraction in a so-called cellulolytic enzyme lignin (CEL), the guaiacyl-type correlations disappeared from the spectra (Figure 2d), and therefore, the identification of this component has not been pursued. The chemical shifts of the SA component are consistent with either syringate esters or syringaldehyde groups. Although the SA correlations resemble those of benzylic-oxidized syringyl β-ether units that are often observed in dicot spectra (Ralph and Landucci, 2010), they cannot be assigned to such structures as the diagnostic β-C/H correlation (at ~5.10/82.8 ppm) is not present. In absolute agreement with the above-mentioned thioacidolysis observation of a 20.8-fold increase in syringaldehyde-derived units from the IRX5:HCHL plants, an enhanced aldehyde C/H correlation is also present with approximately the correct volume integral compared with that from the S2/C₆-correlations (Figure 2). Additionally, because of the overlap between syringate ester and syringaldehyde correlations, NMR analysis cannot rule out the presence of smaller fractions of syringate esters in the lignin of transgenics. The HA component’s chemical shifts suggest that they are esters or, less likely, free acids, but do not match those of typical side-chain γ-p-hydroxybenzoates found in Salix, Populus or Palmae (Ralph and Landucci, 2010). Moreover, spec-
tra after cellulase treatment revealed that the HA unit converted into another species, which possibly assigns it as a glucoside ester (Figure 2d). Acetylation of the wall produces complex shifts that are predictable for free phenolic groups because the C/H-3/5 positions move significantly. Therefore, if HA units are linked with lignin, they must be esters and not free acids, but what monoglucoses they acylate remains to be determined. Interestingly, the core lignin relative S/G levels do not change significantly—when the S2/G values calculated from NMR were 0.21 for the wild-type and 0.23 for the IRX5:HCHL plants (vs. 0.34–0.36 from thioacidolysis); such a difference is explained by the fact that NMR measures S/G on the entire lignin, whereas thioacidolysis only release a fraction of the lignin monomers.

**Lignins from IRX5:HCHL plants have a lower degree of polymerization**

Cellulolytic enzyme lignin fractions were isolated from wild-type and IRX5:HCHL(2) stems with similar yields and used to determine their polydispersity using size-exclusion chromatography (SEC). Elution profiles acquired by monitoring UV-A absorbance (SEC UV-A250) and UV-F fluorescence (SEC UV-Fou250/ou450) of the dissolved CEL revealed differences between wild-type and IRX5:HCHL plants (Figure 3a–b). First, the total area of the largest mass peak detected between 7 and 13.5 min was significantly reduced in transgenics owing to an important diminution of the largest lignin fragments that elute between 7 and 9 min. Similarly, smaller molecular mass material that elutes later in a second peak between 13.5 and 19.5 min was more abundant (increased by 27% and 16% using UV-A and UV-F detections respectively) in IRX5:HCHL samples (Figure 3a–b). Finally, the area corresponding to the smallest lignin fragments detected between 19.5 and 26.5 min using UV-F is increased by 55% in transgenics (Figure 3b). These results demonstrate that the DP of lignins purified from IRX5:HCHL plants is reduced compared with wild type.

**IRX5:HCHL lines have similar sugar content and improved saccharification efficiency**

Monosaccharide composition was determined in stems after hydrolysis of total cell wall polysaccharides. Although both genotypes released similar amounts of total monosaccharides, which is in agreement with the FT-Raman analysis (Figure S3a), transgenics showed a reduction in glucose (~12%) and increases in xylose (+22%) and arabinose (+12%) compared with wild type (Table 6). Moreover, hemicellulosic monosaccharides released from CWR using trifluoroacetic acid were 23% higher in IRX5:HCHL stems, which corresponds to higher xylose (+23%) and arabinose (+22%) contents compared with wild type (Table 6).

To examine the impact of lignin DP reduction on cell wall digestibility, we conducted saccharification assays on biomass derived from stems pretreated with hot water, dilute alkaline, and dilute acid solutions. After a 72-h incubation with cellulase and glucosidase, pretreated biomass of IRX5:HCHL plants released more sugars compared with wild type, showing improvements of saccharification efficiency ranging between 34% and 77% after hot water, 43% and 71% after dilute alkaline, and 15% and 31% after dilute acid pretreatments (Figure 4). Considering the similar total sugar contents in stems of IRX5:HCHL and wild-type plants, these data demonstrate that the cell wall of the transgenics is less recalcitrant to enzymatic hydrolysis.
et al. compared with wild type, which is in agreement with observations in tobacco plants (including photosynthetic tissues), in contrast to the secondary cell wall cellulose synthases which are conserved across vascular plants (Ruprecht et al., 2010; Tan et al., 2010; McQuailer et al., 2005; Merali et al., 2007). In the present study, HCHL expression was restricted to the lignifying tissues using a secondary cell wall-specific promoter because our goal was to reduce the lignin DP and the risk of undesirable effects and also to enhance translation successes of this approach from Arabidopsis to various crops as the general secondary cell wall regulatory network and the lignin biosynthetic pathway are well conserved across vascular plants (Ruprecht et al., 2010). Plants transformed with the IRX5:HCHL construct were neither dwarfed nor sterile and had unchanged lignin content in contrast to previous studies (Table 4; Mayer et al., 2001; Merali et al., 2007), and their young rosette leaves did not show reduced epidermal blue fluorescence under UV which is symptomatic of alteration in phenylpropanoid-derived sinapoyl-malate pools (data not shown; Bonavitz and Chapple, 2010; Vogt, 2010). The observation of unaffected lignin contents in the transgensics analysed in this work is in contrast with previous reports in tobacco that showed lignin diminution (Merali et al., 2007). This difference could be explained by the fact that the non-tissue-specific, strong and constitutive promoter (pCaMV35S) was used to drive HCHL expression in all tissues in tobacco plants (including photosynthetic tissues), in contrast to the secondary cell wall cellulose synthase IRX5 promoter which is active in tissues developing lignified secondary cell walls. IRX5:HCHL plants had similar total sugar content and a slight reduction in glucose/xylose ratio compared with wild type, which is in agreement with observations in tobacco plants expressing constitutively HCHL (Merali et al., 2007). Although two IRX5:HCHL lines showed reduced biomass, which for one of them might be caused a reduction in cell wall integrity creating occasional collapsed xylem vessels, other IRX5:HCHL lines were comparable with wild-type plants.

As expected, the transgenic lines showed increased amounts of soluble C6C1 aldehydes (HBAld, 3,4-DHBAlald and Van) that are produced upon HCHL activity after cleavage of hydroxybenzoyl-CoA, 3,4-dihydroxybenzoyl-CoA and feruloyl-CoA (Tables S1–S2). HCHL has no activity against sinapoyl-CoA (Mitra et al., 1999), suggesting that SyrAld accumulated in transgensics is a conversion product of Van after successive hydroxylation and methylation at the C3-position of the phenyl ring, which is supported by the identification of the new intermediate SOH-Van (Tables 3 and S2). Interestingly, several genes encoding monoxygenases were up-regulated in plants expressing HCHL, but none of the known or predicted O-methyltransferase showed altered expression (Table S3). Analysis of soluble aromatics in transgensics also showed that C6C1 aldehydes are oxidized to their respective C6C1 acid forms (Tables S1–S2). Notably, several genes encoding for dehydrogenases/reductases (SDR, AKR and ALDH) are up-regulated in plants expressing HCHL, with in particular, AKR4C9 (At3g37770), which is known to metabolize a range of hydroxybenzaldehydes (Table S3; Simpson et al., 2009). In addition, soluble C6C1 aromatics predominantly accumulate as conjugates in transgensics as we showed that glucose conjugates represent ~90% of the HBA soluble pool (Tables S1–S2), presumably for vascular storage as previously described for other C6C1 aromatics (Eudes et al., 2008). Such accumulation of C6C1 acid glucosides is in agreement with previous studies performed in tobacco, sugar beet, datura and sugar cane plants over-expressing HCHL (Mayer et al., 2001; McQuailer et al., 2005; Mitra et al., 2002). Interestingly, transcript profiling of HCHL plants revealed seven up-regulated genes of the UDP-glucosyltransferase (UGT) family; among them, UGT75B1 and UGT73B4 were previously shown among the UGT75B1 and UGT73B4 were previously shown to catalyse glucose conjugation of benzoates (Table S3; Eudes et al., 2008; Lim et al., 2002).

Furthermore, we showed that some C6C1 aromatics are released from extract-free cell wall fractions of senesced stems upon mild alkaline hydrolysis. Higher amounts of HBAld, SOH-Van, SyrAld, HBA, VA and SyrA were measured in the ‘loosely wall-bound’ fraction of IRX5:HCHL lines compared with wild type, which is in accordance with observations in tobacco plants expressing constitutively HCHL (Merali et al., 2007). Although the type(s) of linkages involved is unclear, loosely attached C6C1 aromatics have also been shown to be present cell walls of Arabidopsis leaves and roots (Forcat et al., 2010; Tan et al., 2004).

The analysis of lignin from plants expressing HCHL showed increased content of C6C1 monomers upon thioacidolysis, and the characterization of its monomeric composition identified two novel units (Vanalic and Syralc) in addition to higher amounts of syringaldehyde and syringate (Table S5). These data suggest that a part of the C6C1 aldehydes is converted into C6C1 alcohols, although the latest were not detected in the soluble and ‘loosely wall-bound’ fractions from IRX5:HCHL lines (data not shown). Thioacidolysis cleaves almost exclusively 4-O–ether bonds, implying that these C6C1 monomers are most likely 4-O-linked with lignins as end-groups. However, we cannot exclude that a part of the C6C1 acids is esterified to lignins because thioacidolysis also cleaves ester bonds. In agreement with this data, NMR analysis identified higher amount of etherified syringaldehyde

![Figure 4 Saccharification of biomass from mature senesced stems of IRX5:HCHL and wild-type plants after various pretreatments. Amounts of sugars released from biomass after a 72-h enzymatic digestion are shown. Values are means ± SE of four biological replicates (n = 4).](image-url)

**Discussion**

Expression of HCHL in plants has originally been considered for in planta production of valuable and easily extractable compounds such as vanillin (Van) and p-hydroxybenzoic acid (HBA). However, owing to strong ectopic HCHL expression, adverse phenotypes such as chlorotic and senescing leaves, stunting, low pollen production, male sterility, collapsed xylem vessels and reduction in biomass were observed in transgenic tobacco and sugarcane (Mayer et al., 2001; McQuailer et al., 2005; Merali et al., 2007). This difference could be explained by the fact that the non-tissue-specific, strong and constitutive promoter (pCaMV35S) was used to drive HCHL expression in all tissues in tobacco plants (including photosynthetic tissues), in contrast to the secondary cell wall cellulose synthase IRX5 promoter which is active in tissues developing lignified secondary cell walls. IRX5:HCHL plants had similar total sugar content and a slight reduction in glucose/xylose ratio compared with wild type, which is in agreement with observations in tobacco plants expressing constitutively HCHL (Merali et al., 2007). Although two IRX5:HCHL lines showed reduced
units in lignins of transgenics, and revealed the presence of new structures resembling free phenolic hydroxybenzoate esters (Figure 2).

It is unclear whether C₄-C₃ aromatics represent more nucleation sites (‘starting points’) for lignification in the walls creating more polymer chains of lower molecular weight or whether they actually incorporate growing lignin chains. In both cases, these side-chain-truncated monomers will be relegated to end-units and decrease the average lignin DP. Indeed, owing to the absence of phenylpropanoid tail, these new monomers can only initiate a new lignin chain leading to unidirectional polymerization starting from the hydroxyphenyl ring (at its 4-O- or non-methoxylated 5-positions) to which conventional monolignols couple via 5-β- or 4-O-β-coupling or terminate a growing lignin chain by 4-0-5-coupling or, in the case of the guaiacyl monomers only, 5-5- or 5-0-4-coupling.

We postulated that the lignin DP would be reduced upon enrichment in the wall of unconventional side-chain-truncated monomers that would increase the number of nucleation sites for lignification and allow only unidirectional polymerization. Analysis of the polydispersity of lignin in plants overproducing these monomers showed significant reduction in high-molecular mass oligomers and significant increase in low-molecular mass oligomers, supporting our hypothesis (Figure 3). These observations are relevant for understanding the higher susceptibility of the biomass from HCHL lines to polysaccharide enzymatic hydrolysis (Figure 4). Although saccharification efficiency of biomass is determined by several characteristics of cell walls, the improvements observed in the IRX5:HCHL lines after different pretreatments suggest that plants with less ramified or shorter lignin polymers would have their cell wall polysaccharides less cross-linked or less embedded in the lignin matrix, thus improving the accessibility of cellulose and hemicelluloses to hydrolytic enzymes.

We conclude that the overproduction of lignin ‘DP reducers’ can be used in planta to modify the lignin structure and reduce lignin DP to reduce biomass recalcitrance and enhance its conversion efficiency into fermentable sugars. As this approach is independent of any particular plant genetic background, it should be transferable to various energy crops and compatible with several traits. Restricting the biosynthesis of these lignin ‘DP reducers’ in supporting lignified tissues (i.e. schlerenchyma fibres), as well as avoiding strong production in conductive tissues (i.e. vessels), should limit the risk of adverse effects on plant development and biomass yield.

**Experimental procedures**

**Plant material and growth conditions**

*Arabidopsis thaliana* (ecotype Columbia, Col-0) seeds were germinated directly on soil. Growing conditions were 14 h of light per day at 100 μmol/m²/s, 22 °C and 55% humidity. Selection of T1 and T2 homozygote transgenic plants was made on Murashige and Skoog vitamin medium (PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with 1% sucrose, 1.5% agar and containing 50 μg/mL kanamycin.

**Generation of a pTKan-pIRX5:HCHL plasmid and plant transformation**

A Gateway cloning cassette (Life Technologies, Grand Island, NY) was inserted between XhoI and PstI restriction sites of the binary vector pTKan+ (Schaaf et al., 2006) to produce a pTKan-GW vector. The nucleotide sequence of the IRX5 promoter was amplified by PCR from Arabidopsis (ecotype Columbia, Col-0) genomic DNA using oligonucleotides 5′-CCCGCCGCGCCGATG AAAGCATCCTCTAATCGGAA-3′ and 5′-CCGGCTAGCCGCC GAGGTACACTGAGCTCTCGGAA-3′ (NotI and NheI restriction sites underlined) and inserted between the Apal and Spel restriction sites of pTKan-GW to produce a pTKan-pIRX5-GW expression vector. A HCHL codon-optimized nucleotide sequence from *P. fluorescens* AN103 (GenBank accession number CAA73502) was synthesized without stop codon (GenScript, Piscataway, NJ) and cloned into the Gateway pDONR221-f1 entry vector (Lalonde et al., 2010). A sequence-verified HCHL entry clone was LR recombined with the pTKan-pIRX5-GW vector to generate the pTKan-pIRX5:HCHL construct that was introduced into wild-type Arabidopsis plants (ecotype Col0) via Agrobacterium tumefaciens-mediated transformation (Bechtold and Pelletier, 1998).

**RNA extraction and RT-PCR**

Total RNA (1 μg) was extracted using the Plant RNAeasy extraction kit (Qiagen, Valencia, CA) and reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). The obtained cDNA preparation was quality controlled for PCR using tub8-specific oligonucleotides (5′-GGGCTAAAGGACACTACAGT-3′ and 5′-CTCTCTGGACCT CCACCTCATTCC-3′) and used for the detection of HCHL expression using oligonucleotides 5′-ATGTCTACTACAGGAGGA AGATGG-3′ and 5′-TCTCTTGTAAAGCTGGAGATC-3′.

**Western blot analysis**

Proteins were extracted using a buffer containing 100 mM Tris–HCl pH 6.5, 2% (w/v) polyvinylpyrrolidone, 2% (v/v) β-mercaptoethanol, 1% (w/v) SDS and quantified using the Bradford method (Bradford, 1976). Proteins (5 μg) were separated by SDS-PAGE, blotted and immunodetected using the universal antibody as described in Eudes et al. (2011).

**HCHL activity**

Proteins were extracted using a buffer (EB-100 mM Tris–HCl pH 8.5, 20 mM DTT, 10 mM Na2EDTA-) containing 25 mg of polyvinylpyrrolidone. Extracts were shaken at 1400 rpm for 15 min at 4 °C and centrifuged for 30 min at 20 000 g at 4 °C. Proteins were applied to PD10 columns (GE Healthcare, Piscataway, NJ), eluted with EB and quantified using the Bradford method for HCHL activity. 5 μL of protein extract was incubated for 15 min at 30 °C with 150 μM feruloyl-CoA in 100 mM Tris–HCl pH 8.5 in a total volume of 50 μL. Total amounts of protein per reaction varied from 4 to 6.5 μg. Reactions were stopped with 50 μL of cold acidified methanol (12% glacial acetic acid / 88% methanol, v/v) and stored at -70 °C until LC-MS analysis.

**Microscopy**

Basal stem transverse sections (100-μm-thick) were obtained using a vibratome (Leica Microsystems Inc., Buffalo Grove, IL). For toluidine blue O (TBO) staining, sections were incubated in a 0.05% (w/v) solution of TBO (Sigma-Aldrich, St Louis, MO) for 30 s and rinsed with water. For Wiesner lignin staining, sections were incubated for 3 min in phloroglucinol–HCl reagent (VWR International, Brisbane, CA) and rinsed with water (Nakano et al., 1992). For Mäule lignin staining, sections were incubated...
in 4% KMnO₄ for 5 min, rinsed with water, incubated in 37% HCl for 2 min and observed after addition of a drop of aqueous ammonia. All sections were analysed using bright field light microscopy.

**Cell wall-bound aromatics extraction**

Senesced stems were ball-milled using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless steel balls for 2 min at 30 s. Extract-free CWR were obtained by sequentially washing 60 mg of ball-milled stems with 1 mL of 96% ethanol at 95 °C twice for 30 min, and vortexing with 1 mL of 70% ethanol twice for 30 s. The resulting CWR were dried in vacuo overnight at 30 °C. CWR (6 mg), were mixed with 500 μL of 2 M NaOH and shaken at 1400 rpm for 24 h at 30 °C. The mixture was acidified with 100 μL of concentrated HCl and subjected to three ethyl acetate partitioning steps. Ethyl acetate fractions were pooled, dried in vacuo and suspended in 50% (v/v) methanol–water prior to LC-MS analysis.

**LC-MS**

A Poroshell-120 column (150 mm length, 3 mm internal diameter, 2.7 μm particle size) and a 1200 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA) were used. C₆C₁ aromatics were separated using a gradient elution with mobile phase composition of 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile–water (98 : 2, v/v) as solvent B. The elution gradient was 0–5 min 13% B, 5–7 min 50% B, 7–8 min 50% B and 8–11 min 13% B using a flow rate of 0.55 mL min⁻¹. The HPLC system was coupled to an Agilent 6210 time-of-flight (TOF) mass spectrometer (MS) via a 1 : 7 post-column split. Analyses were conducted using Electrospray ionization (ESI) in the positive ion mode. Detection of [M + H]⁺ ions was carried out in full scan mode at 0.85 spectra/s and a cycle time of 1.176 s spectrum using the following parameters: capillary voltage 3500 V, fragmentor 165 V, skimmer 50 V and OCT RF 170 V, drying gas flow rate 9 L min⁻¹, nebulizer pressure 15 psig and drying gas temperature 325 °C. Quantification of aromatics was made by comparison with standard curves of authentic compounds (Sigma-Aldrich; Alfa Aesar, Ward Hill, MA).

**HPAEC-PAD monosaccharide analysis**

Cell wall residues of ball-milled senesced stems were treated with sulphuric acid as previously described for total sugar hydrolysis prior to chromatography (Sluiter et al., 2008). For hemicellulose hydrolysis, CWR of ball-milled senesced stems (5 mg) were incubated in 1 mL of 2 M trifluoroacetic acid (TFA) at 800 m M NaCl (30 mL) and incubated overnight at 4 °C. After centrifugation (2800 g, 10 min), the biomass was extracted sequentially by sonication (20 min) with 80% ethanol (three times), acetone (one time), chloroform-methanol (1 : 1, v/v, one time) and acetone (one time). The obtained CWR were ball-milled for 3 h per 500 mg of sample (in 10 min on/10 min off cycles) using a PM100 ball mill (Retsch) vibrating at 600 rpm.

**Lignin analysis**

Cell wall residues of ball-milled senesced stems were prepared using a Soxhlet apparatus by sequentially extracting the ground material with toluene/ethanol (2 : 1, v/v), ethanol and water (Sluiter et al., 2008). The determination of lignin content using the standard Klason procedure (Dence et al., 1992) and the thoiacidolysis procedure (Lapierre et al., 1995, 1999) were carried out on CWR. The lignin-derived monomers were identified by GC-MS as their trimethyl-silylated derivatives. All the lignin analyses were performed in duplicate.

**NMR sample preparation**

The whole plant cell wall gel-state samples for NMR experiments were prepared as previously described by Kim et al. (2008). The dried Arabidopsis stems were preground for 1 min in a Retsch MM400 mixer mill at 30 Hz, using zirconium dioxide (ZrO₂) vessels (10 mL) containing ZrO₂ ball bearings (2 x 10 mm). The preground cell walls were extracted with distilled water (ultrasonication, 1 h, three times) and 80% ethanol (ultrasonication, 1 h, three times). Isolated cell walls were dried and ball-milled using a Fritsch Planetary Micro pulverisette 7 planetary ball mill at 800 rpm, using ZrO₂ vessels (12 mL containing ZrO₂ ball bearings (30 x 5 mm). Each sample (100 mg) was ground for 2 h (interval: 30 min: break: 5 min, 4 cycles, total run time 2.25 h). The cell walls were collected directly into the NMR tubes and gels formed using DMSO-d₆/pyridine-d₅ (4 : 1).

**2D ¹³C–¹H HSQC NMR spectroscopy**

NMR experiments for the whole plant cell wall gel-state samples were also performed as previously described (Kim and Ralph, 2010; Kim et al., 2008). NMR spectra were acquired on a Bruker Biospin (Billerica, MA) Avance 500 MHz spectrometer fitted with a cryogenically cooled 5-mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The central DMSO solvent peak was used as internal reference (δC = 39.5, δH = 2.49 ppm). The ¹³C–¹H correlation experiment was an adiabatic HSQC experiment (Bruker standard pulse sequence ‘hsqcetgpsisp.2’ phase-sensitive gradient-edited-2D HSQC using adiabatic pulses for inversion and refocusing) (Kupek and Free- man, 2007). HSQC experiments were carried out using the following parameters: acquired from 10 to 0 ppm in F2 (¹H) with 1000 data points (acquisition time 100 ms), 200 to 0 ppm in F1 (¹³C) with 400 increments (F1 acquisition time 8 ms) of 72 scans with a 500 ms interscan delay; the d₄d₄ delay was set to 0.89 ms (1/8J, J = 145 Hz). The total acquisition time was 5 h. Processing used typical matched Gaussian apodization (GB = 0.001, LB = −0.1) in F2 and squared cosine-bell and one level of linear prediction (32 coefficients) in F1. Volume integration (uncorrected) of contours in HSQC plots used Bruker’s TopSpin 3 (Mac version) software.

**Isolation of cellulolytic enzyme lignin**

One gram of ball-milled mature senesced stems was mixed with 50 mL NaCl (30 mL) and incubated overnight at 4 °C. After centrifugation (2800 g, 10 min), the biomass was extracted sequentially by sonication (20 min) with 80% ethanol (three times), acetone (one time), chloroform-methanol (1 : 1, v/v, one time) and acetone (one time). The obtained CWR were ball-milled for 3 h per 500 mg of sample (in 10 min on/10 min off cycles) using a PM100 ball mill (Retsch) vibrating at 600 rpm.
in zirconium dioxide vessels (50 mL) containing ZrO$_2$ ball bearings (10 × 10 mm). Ball-milled walls (490 mg for wild type and 480 mg for irx5:HCHL) were digested four times over 3 days at 30 °C with crude cellulosases (Cellulysin; 60 mg/g of sample; EMD Biosciences Inc, San Diego, CA) in NaOH pH 5.0 buffer (30 mL). The obtained CEL was washed three times with deionized water and lyophilized overnight. CEL recovered were 131 mg for wild type (27.3%) and 101 mg for irx5:HCHL (20.6%). For NMR, the CEL (30 mg) was swelled in DMSO-d$_6$/pyridine-d$_5$ as described earlier. For SEC analysis, 1% (w/v) CEL lignin solutions were prepared in 1-methyl-2-pyrrolidinone (NMP)-DMSO (1 : 1, v/v) after sonication for 3 h at 40 °C.

Size-exclusion chromatography

Polydispersity of dissolved CEL was determined using analytical techniques SEC UV-F and SEC UV-A as previously described (George et al., 2011). An Agilent 1200 series binary LC system (G1312B) equipped with FL (G1321A) and DA (G1315D) detectors was used. Separation was achieved with a Mixed-D column [5 mm particle size, 300 mm i.d., linear molecular weight range of 200–400 000 g/mol (Agilent Technologies Inc.)] at 80 °C using a mobile phase of NMP at a flow rate of 0.5 mL min$^{-1}$. Absorbance of material eluting from the column was detected at 300 nm (UV-A). Excitation 250 nm and emission 450 nm were used for UV-F detection. Intensities were area normalized, and molecular mass estimates were determined after calibration of the system with polystyrene standards.

Cell wall pretreatments and saccharification

Ball-milled senesced stems (10 mg) were mixed with 340 μL of water, 340 μL of H$_2$SO$_4$ (1.2%, w/v) or 340 μL of NaOH (0.25%, w/v) for hot water, dilute acid or dilute alkaline pretreatments, respectively, incubated at 30 °C for 30 min and autoclaved at 120 °C for 1 h. After cooling down at room temperature, samples pretreated with dilute acid and dilute alkaline solutions were neutralized with 5 N NaOH (25 μL) and 1.25 N HCl (25 μL), respectively. Saccharification was initiated by adding 635 μL of 100 mM sodium citrate buffer pH 6.2 containing 80 μg/mL tetracycline, 5% w/w cellulase complex NPS0013 and 0.5% w/w glucosidase NPS0010 (Novozymes, Bagsværd, Denmark). After 72 h of incubation at 50 °C with shaking (800 rpm), samples were centrifuged (20 000 g, 3 min), and 10 μL of the supernatant was collected for reducing sugar measurement using the DNS assay and glucose solutions as standards (Miller, 1959).

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Conflict of interest

J.D.K. has financial conflict of interest in Amyris, LS9 and Lygos.

References


Supporting information

Additional Supporting information may be found in the online version of this article:

**Figure S1** Synthesis of C6C1 monomers upon HCHL activity.

**Figure S2** Organ- and tissue-specific activity of the IRX5 promoter in Arabidopsis.

**Figure S3** Spectral analysis of IRX5:HCHL and wild-type plants.

**Table S1** Quantitative analysis of soluble aromatics in stems from 5-week-old wild-type (WT) and IRX5:HCHL plants.

**Table S2** Quantitative analysis of acid-hydrolysed soluble aromatics in stems from 5-week-old wild-type (WT) and IRX5:HCHL plants.

**Table S3** List of genes differentially expressed genes in the IRX5:HCHL stems.

**Data S1** Supporting experimental procedures for supplemental data.

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