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# Lignin modification improves fermentable sugar yields for biofuel production

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**Recalcitrance to saccharification is a major limitation for conversion of lignocellulosic biomass to ethanol. In stems of transgenic alfalfa lines independently downregulated in each of six lignin biosynthetic enzymes, recalcitrance to both acid pretreatment and enzymatic digestion is directly proportional to lignin content. Some transgenics yield nearly twice as much sugar from cell walls as wild-type plants. Lignin modification could bypass the need for acid pretreatment and thereby facilitate bioprocess consolidation.**

Accessibility of plant cell wall polysaccharides to chemical, enzymatic and microbial digestion is limited by many factors, including the presence of the phenylpropanoid polymer lignin in vascular tissues and fibers[1–3] (**Supplementary Fig. 1a,b** online). Genes encoding the enzymes leading to the hydroxyphenyl (H), guaiacyl (G) and syringyl (S) building blocks of lignin (**Supplementary Fig. 1**) have been identified[4]. Relationships between lignin content or composition and efficiency of lignocellulose use for pulping of trees[5] and digestibility of forages[6] have been revealed by downregulating some of these genes in transgenic plants. However, the relationships between lignin and saccharification of plant biomass for bioethanol production are less well understood, with studies relying on comparisons of different species or different developmental stages[7,8].

To determine relationships between lignin content/composition and chemical/enzymatic saccharification, we analyzed previously generated[9] alfalfa lines expressing antisense constructs for downregulating lignin biosynthesis independently at six different steps: cinnamate 4-hydroxylase (C4H); hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT); coumaroyl shikimate 3-hydroxylase (C3H); caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H); or caffeic acid 3-*O*-methyltransferase (COMT) (**Supplementary Fig. 1** and **Supplementary Table 1** online). Mature stems were harvested at late-flowering stage. Lignin content of untreated stems decreased in the order: F5H and control (most lignin) > COMT and CCoAOMT > C4H, C3H and HCT (lowest lignin level at <50% of the wild-type value) (**Supplementary Table 1**). Lignin S/G ratios varied from about 0.3 to 1.0 depending on the targeted gene, with a high proportion of H units in HCT and C3H transgenics (**Supplementary Table 1**).

Dried, milled, extractive-free stems were pretreated with 1.3% $H_2SO_4$ at 130 °C for 30 min (**Supplementary Methods** online), and the residue and hydrolysate separated (**Fig. 1**). Levels of acetyl bromide lignin from acid-pretreated material increased slightly on a

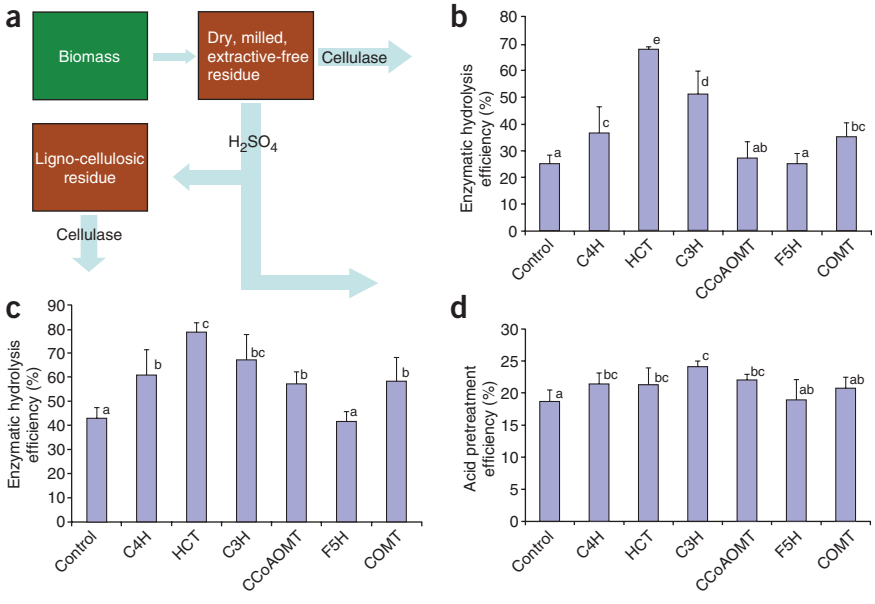


**Figure 1** Sugar release from alfalfa biomass by chemical and enzymatic saccharification. Stems were from developmentally matched controls and plants with altered lignin as a result of downregulation of the genes indicated. (**a**) Scheme for treatments. (**b**) Saccharification efficiencies (total sugar released as a percentage of total sugar in the cell wall residue) for biomass subjected to enzymatic hydrolysis with cellulase and cellobiase without acid pretreatment. (**c**) Enzymatic saccharification efficiencies for material that had been first subjected to acid pretreatment. (**d**) Saccharification efficiencies for acid pretreatment. Different letters indicate significant differences in saccharification efficiency by ANOVA. C4H, cinnamate 4-hydroxylase; HCT, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase; C3H, coumaroyl shikimate 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid 3-*O*-methyltransferase.

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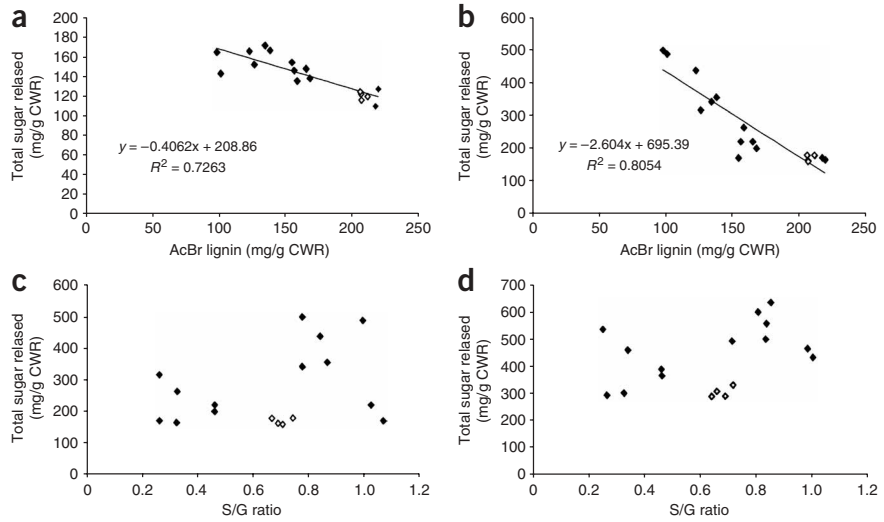


**Figure 2** Relationships between lignin levels and properties and saccharification of alfalfa biomass. Each point represents an individual control (open symbol) or transgenic plant with altered expression of a monolignol pathway enzyme (closed symbol). Stem material was treated with cellulase and cellobiase for 72 h. (**a**–**d**) Total sugar released is shown as a function of lignin content of untreated stems (**a**), lignin content of pretreated stems (**b**), lignin S/G ratio of untreated stems (**c**) and lignin S/G ratio of pretreated stems (**d**). Statistical analysis is shown in **Supplementary Table 3**. CWR, cell wall residue. AcBr, acetyl bromide.

gram cell wall residue basis. Very little soluble lignin was detected in the hydrolysates, and the S/G ratio was essentially unaffected by acid pretreatment (**Supplementary Table 2** online).

Plants with the least lignin had the highest total carbohydrate levels in untreated biomass (**Supplementary Table 2**), reflecting compensation for the reduction in lignin level on a mass balance basis. The amount of carbohydrate released by acid pretreatment increased in proportion to the reduction in lignin levels (**Fig. 2a**). Acid pretreatment efficiency (percentage of total cell wall sugar) increased slightly from an average of 18.7% in control lines to an average of 24.1% in the C3H lines (**Fig. 1d**). Sugars present in the acid hydrolysates comprised, in order of abundance, xylose, arabinose, glucose and galactose (**Supplementary Fig. 2a** online), mainly representative of hemicellulosic and pectic cell wall polymers. Improved release of hemicellulosic sugars as a function of reduced lignin amount was not observed in a study comparing alfalfa stems, reed canarygrass and switchgrass at different developmental stages[8].

Large differences were observed in the enzymatic saccharification efficiencies of acid-pretreated cell walls of the various lines (**Fig. 1c**). After 72 h incubation, saccharification efficiency was 67–79% in C3H and HCT lines, compared to 43% in controls. More than 90% of the released sugar from most lines was glucose (**Supplementary Fig. 2b**), indicating enzymatic hydrolysis of cellulose. Enzymatic hydrolysis released more xylose from transgenic lines than from control lines (**Supplementary Fig. 2b**), suggesting that lignin modification increases the accessibility of residual hemicellulose to degradative enzymes.

There was a strong negative correlation between lignin content and sugar released by enzymatic hydrolysis (**Fig. 2b** and **Supplementary Table 3** online). Because of the high proportion of H lignin in the HCT and C3H lines, S/G ratio alone did not correlate with the amount of sugar released (**Fig. 2 c,d**). Including lignin composition parameters in the multiple regression model improved the prediction (adjusted $R^2 = 0.98$) (**Supplementary Table 3**). However, after pretreatment, enzymatic sugar release correlated only with lignin content. Thus, for untreated stem material, both lignin content and composition might affect cellulose substrate-enzyme interactions during enzymatic hydrolysis, whereas, for pretreated stems, lignin composition (over the ranges measured) may not be a major factor affecting hydrolysis. Generation of additional plant lines with altered lignin contents but identical lignin compositions, and vice versa, would help to better evaluate this hypothesis.

Our results identify lignin as probably the major factor in recalcitrance of cell walls to saccharification, particularly during enzymatic hydrolysis. Moreover, they demonstrate that genetic reduction of lignin content effectively overcame cell wall recalcitrance to bioconversion. This could obviate the need for acid pretreatment; saccharification efficiency of untreated biomass of the HCT and C3H transgenics (**Fig. 1b**) is greater than that of pretreated biomass of control plants (**Fig. 1c**). Harsher chemical pretreatment might make the positive effects of lignin modification on pretreatment efficiency less obvious. However, products from the acid hydrolysis of hemicellulose inhibit the later fermentation step[10,11], and harsh chemical pretreatment makes it impossible to take advantage of *in planta* expression of enzymes to increase enzymatic processing efficiency[12]. Genetically reducing lignin can therefore facilitate bioprocess consolidation. The economic benefits of this may balance or outweigh the loss of the sugar specifically released by acid pretreatment, particularly if the hemicellulosic fraction from the transgenic lines now becomes accessible to enzymatic release.

Downregulating COMT or CCoAOMT does not affect plant yield[13], whereas strongly downregulating C3H or HCT reduces biomass (by a maximum of 40%) accompanied, in HCT transgenics, by increased branching[6,14]. A 166% increase in sugar production would offset a 40% reduction in overall biomass yield. The increased enzymatic hydrolysis of the HCT lines therefore reflects a significant theoretical improvement in fermentable glucose production on a per plant basis in spite of the yield reduction.

The biosynthetic pathways to lignin monomers are conserved across the plant kingdom[1]. The genes targeted in the present work are therefore candidates for improving saccharification potential in bioenergy crops such as poplar, switchgrass and *Miscanthus* hybrids.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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**AUTHOR CONTRIBUTIONS**

F.C. designed and conducted experiments and assisted with writing of the manuscript; R.A.D. designed experiments, analyzed results and wrote the manuscript.

**COMPETING INTERESTS STATEMENT**

The authors declare no competing financial interests.

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