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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

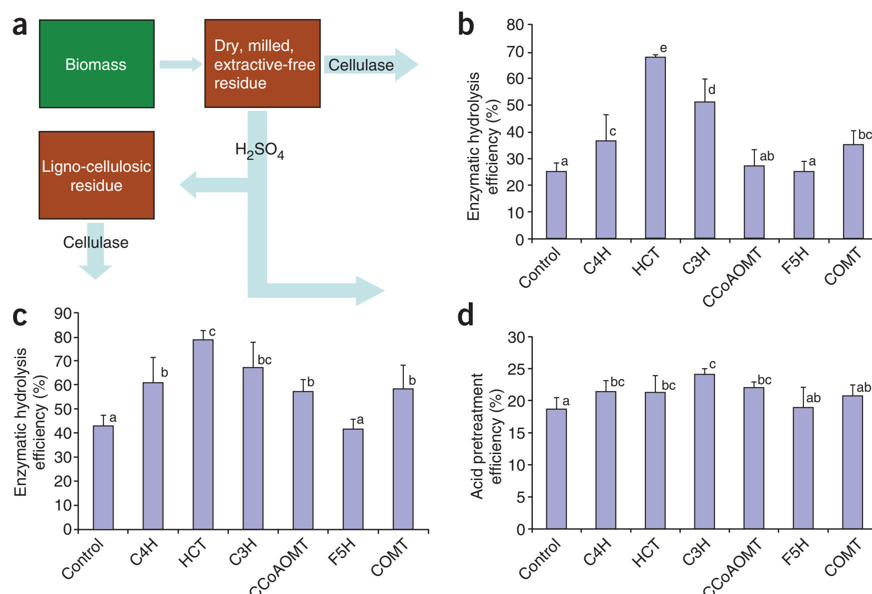

Figure 1 Sugar release from alfalfa biomass by chemical and enzymatic saccharification. Stems were from developmentally matched controls and plants with altered lignin as a result of downregulation of the genes indicated. (**a**) Scheme for treatments. (**b**) Saccharification efficiencies (total sugar 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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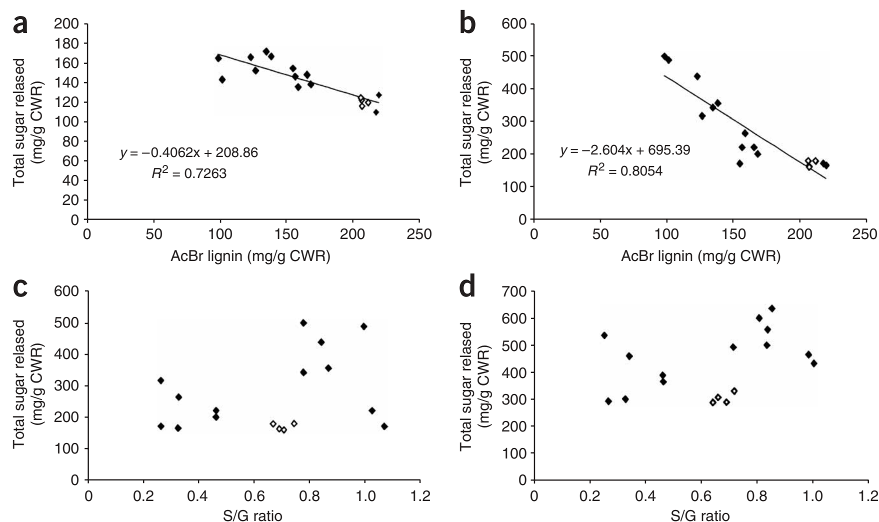

Figure 2 Relationships between lignin levels and properties and saccharification of alfalfa biomass. Each point represents an individual control (open symbol) or transgenic plant with altered expression of a monolignol pathway enzyme (closed symbol). Stem material was treated with cellulase and cellobiase 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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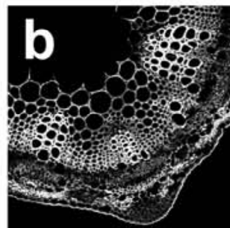

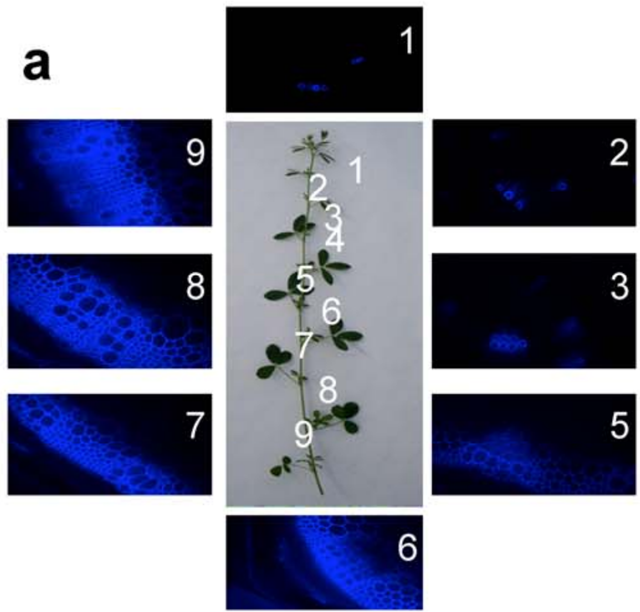

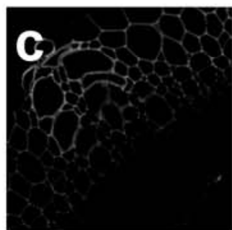

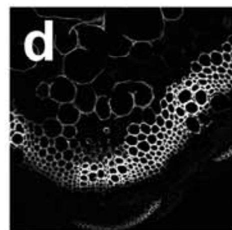

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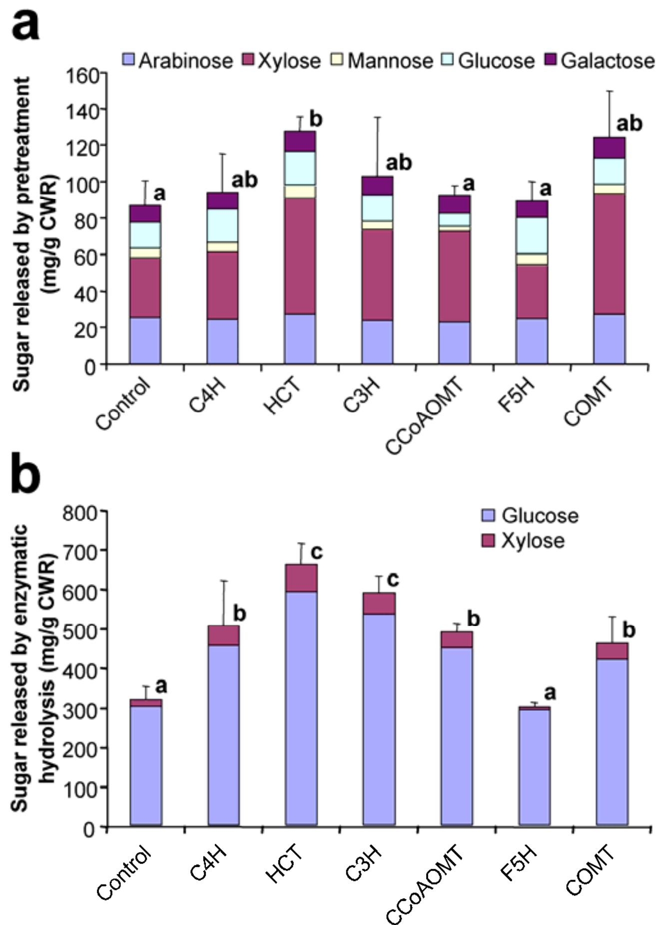
Phenylalanine
PAL
Cinnamic acids
Cinnamoyl CoAs
Cinnamyl aldehydes
Cinnamyl alcohols
cinnamic acid
C4H
p-coumaric acid
4CL
p-coumaroyl CoA
CCR
p-coumaryl aldehyde
CAD
p-coumaryl alcohol
H lignin units
HCT
shikimate
C3H
shikimate
HCT
SCoA
CCoAOMT
feruloyl CoA
CCR
coniferyl aldehyde
CAD
coniferyl alcohol
G lignin units
F5H
F5H
COMT
COMT
sinapyl aldehyde
CAD
sinapyl alcohol
S lignin units

Supplementary Figure 1. The currently accepted pathway to the H, G and S monolignol building blocks of lignin, and lignin deposition patterns in alfalfa stems. (**a**) A stem of alfalfa surrounded by cross sections of individual internodes (numbered from 1 at the top to 9 near the base) viewed under UV light. The blue fluorescence indicates the presence of lignin and wall-bound hydroxycinnamic acids. (**b-d**) UV autofluorescence showing the deposition of lignin in the stems of wild-type alfalfa (**b**) and transgenic alfalfa down-regulated in the expression of HCT (**c**) or COMT (**d**). (**e**) Pathway for monolignol biosynthesis. Enzymes are: PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl CoA: shikimate/quinate hydroxycinnamoyl transferase; C3H, coumaroyl shikimate 3-hydroxylase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; F5H, "ferulate 5-hydroxylase"; COMT, "caffeic acid 3-*O*-methyltransferase". The enzymes in red boxes were targets for antisense down-regulation.



Supplementary Figure 2. Composition of sugars released from alfalfa biomass by acid pre-treatment and enzymatic digestion. Biomass was prepared from mature lignified stems of control plants and plants with altered lignin as a result of antisense down-regulation of the enzymes indicated. (**a**) Sugar composition of hydrolysates from sulfuric acid pre-treatment. (**b**) Sugar composition of hydrolysates resulting from digestion of pre-treated biomass with a mixture of cellulase and cellobiase. Sugars were measured as their alditol acetates after derivatization and GC/MS analysis in (**a**), and by HPLC in (**b**). Different letters indicate statistically significant differences in the sums of all sugars released as determined by ANOVA.

Line	Lignin content (mg/g CWR)*				Lignin composition*									
	Untreated		Pretreated		Untreated (μmol/g CWR)					Pretreated (μmol/g CWR)				
	mean**	SD	mean	SD	H unit	G unit	S unit	Total	S/G	H unit	G unit	S unit	Total	S/G
Control	207.2 ^{a}	11.3	240.9 ^{a}	12.7	32.6	375.8	263.1	671.5^{a}	0.70 ^{a}	24.7	348.7	235.3	608.6^{a}	0.67 ^{a}
C4H	147.1^{bc}	24.7	200.2^{b}	38.3	27.6	263.4	99.8	390.7^{d}	0.36 ^{d}	19.3	252.2	95.1	366.5^{b}	0.35 ^{de}
HCT	99.8 ^{d}	2.8	126.9^{d}	4.0	93.1	16.0	14.2	123.3 ^{f}	0.88 ^{b}	104.1	16.1	13.2	133.4^{c}	0.82 ^{b}
C3H	131.7 ^{c}	8.5	171.6 ^{c}	10.6	141.2	77.4	63.7	282.3^{e}	0.82 ^{b}	95.9	55.3	44.5	195.7^{c}	0.78 ^{b}
CCoAOMT	155.3 ^{b}	4.0	193.7^{bc}	8.7	32.2	212.2	221.1	465.6^{bc}	1.04 ^{c}	25.3	196.2	194.6	416.1^{b}	0.99 ^{c}
F5H	217.9 ^{a}	6.5	250.3 ^{a}	18.2	26.2	384.2	112.6	523.0 ^{b}	0.29 ^{d}	21.8	394.7	116.4	532.8^{a}	0.29 ^{e}
COMT	161.7 ^{b}	11.9	215.1 ^{b}	9.5	28.9	285.1	113.8	427.7^{cd}	0.39 ^{d}	22.9	279.2	112.3	414.3^{b}	0.40 ^{d}

Supplementary Table 1. Lignin content and composition of alfalfa stem biomass analyzed in the present work. Plants were empty vector and non-transformed controls, or antisense down-regulated for the enzymes indicated. Dried, milled, extractive-free stems were either untreated or pre-treated with 1.3% sulfuric acid at 130 ^{o}C for 40 min. Lignin monomers (H, G, S) were determined by thioacidolysis (see Methods).

*Mean values and standard deviations (SD) are reported for duplicate analyses of n independent lines. Controls, n = 4; C3H, n = 3: C4H, HCT, CCoAOMT, F5H, COMT, n = 2.

**Means with the same letter are not significantly different at the 0.95 confidence level.

CWR, cell wall residue.

Individual lines are available from the authors as vegetatively propagated material.

Line	Pretreatment yield (% of CWR)*		Lignin released by pretreatment (% of CWR)*		Carbohydrate content (mg/g CWR)*			
					Untreated		Pretreated	
	mean**	SD	mean	SD	mean	SD	mean	SD
Control	79.2 ^{a}	2.0	0.63 ^{a}	0.01	651.5 ^{a}	37.3	698.9 ^{ab}	38.4
C4H	76.^{3bc}	3.1	0.59 ^{ac}	0.05	682.1 ^{ab}	54.7	729.7 ^{bc}	38.2
HCT	72.8 ^{d}	1.6	0.50 ^{d}	0.08	698.7 ^{b}	32.4	765.0 ^{c}	20.6
C3H	73.4 ^{d}	1.4	0.50 ^{d}	0.01	730.4 ^{b}	42.8	769.8 ^{c}	53.0
CCoAOMT	75.1 ^{cd}	2.3	0.70 ^{b}	0.04	685.3 ^{ab}	22.1	780.0 ^{c}	67.9
F5H	78.5 ^{ab}	1.6	0.53 ^{d}	0.01	634.7 ^{a}	51.0	656.5 ^{a}	27.7
COMT	76.6 ^{bc}	2.4	0.54 ^{cd}	0.03	686.5 ^{ab}	39.4	735.9 ^{bc}	58.9

Supplementary Table 2. Pretreatment parameters for control and lignin-modified alfalfa plants. Solubilized lignin was determined spectrophotometrically in the hydrolysate.

* mean values and standard deviations (SD) are reported for duplicate analysis of multiple lines (2-4 lines, see Legend to **Supplementary Table 1**).

** means with the same letter are not significantly different at 0.95 confidence level.

Sugar released	Intercept	AcBr lignin (X1)	Total monomer yield (X2)	G/T (X3)	S/T (X4)	S/G (X5)	R^2	adjusted R^2	P-value
Untreated stem									
Y	695.39	-2.60 X1					0.8053	0.7924	a
Y	523.8		-0.58 X2				0.8386	0.8279	a
Y	501.64			-454.24 X3			0.5850	0.5574	a
Y	483.31				-753.08 X4		0.5609	0.5316	
Y	174.21					142.42 X5	0.1034	0.0436	
Y	600.85			-356.65 X3	-548.63 X4		0.9228	0.9118	a
Y	682.25	-1.13 X1		-221.61 X3	-415.96 X4		0.9798	0.9752	a
Y	592.64		-0.22 X2	-260.79 X3	-335.75 X4		0.9538	0.9431	a
Pretreated stem									
Y	965.56	-2.62 X1					0.9118	0.9059	a
Y	684.92		-0.63 X2				0.8487	0.8386	a
Y	640.93			-415.49 X3			0.5060	0.4731	
Y	593.30				-568.14 X4		0.2190	0.2340	
Y	313.99					1178.2 X5	0.1402	0.0829	
Y	729.30			-359.98 X3	-400.83 X4		0.6373	0.5855	
Y	968.40	-2.38 X1		-18.57 X3	-164.75 X4		0.9310	0.9151	
Y	691.07		-0.5972 X2	-79.23 X3	71.33 X4		0.8645	0.8333	

Supplementary Table 3. Regression equations for sugar released by enzymatic hydrolysis (mg/g CWR) using lignin content (mg/g CWR), total thioacidolysis monomer yield (mmol/g CWR) and relative monomer ratios in untreated and pretreated alfalfa stems. CWR, cell wall residue.

a- the P-values for all the variables are less than 0.001.

Lignin modification overcomes recalcitrance for production of liquid biofuels

Fang Chen and Richard A. Dixon

SUPPLEMENTARY METHODS

Plant material and sample collection. Alfalfa (*Medicago sativa* cv Regen SY) plants independently harboring antisense constructs targeting each of six different enzymes in the monolignol pathway have been described previously [1]. Control plants were either transformed with an empty vector (CTR) or were non-transformed but taken through somatic embryogenesis in parallel to the transgenics (CK). Plants were vegetatively propagated in the greenhouse under standard conditions with a 16-hour day from 6:00h to 22:00h facilitated by supplementary lighting. Plant material was collected from each line when the plants had reached the late flowering stage. Mature stems were harvested (the first seven internodes from the top were discarded), dried in an oven at 55 °C, and then ground in a Wiley mill with a sieve diameter of 1.00 mm. The fines were removed by passing through a 0.05 mm diameter sieve. Soluble extractives were removed by three successive extractions with chloroform: methanol (2:1 v/v), methanol and water at room temperature.

Determination of lignin content and composition. Lignin content of stem material was determined by the acetyl bromide method using ~15 mg extractive-free material [2]. The same molar extinction coefficient of 17.2 (as determined for lignin from wild-type alfalfa) was used for samples of all the transgenic lines. Thioacidolysis methods [3, 4] were used for the determination of lignin composition. Thioacidolysis was performed using ~20 mg of extractive-free samples reacted with 3 ml of 0.2 M BF_3 etherate in an 8.75:1 dioxane/ethanethiol mixture. Lignin-derived monomers were identified by gas

chromatography mass spectrometry (GC/MS) and quantified by GC as their trimethylsilyl derivatives, as described previously [1].

The acid soluble lignin content of hydrolysates from acid-pretreatment was determined according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (LAP-019). Acid soluble lignin was measured at a wavelength of 240 nm using an absorptivity constant of 25 $L.g^{-1}.cm^{-1}$.

Chemical pretreatment. The stem material at a solid loading of 10% (w/w) was mixed with dilute sulfuric acid (final concentration 1.3% (w/w)) and pretreated in an autoclave at 130 °C for 40 min. After pretreatment, the hydrolysates were separated and collected by filtration from residual biomass and the biomass residues were washed with water.

Enzymatic hydrolysis. Celluclast 1.5 L (cellulase from *Trichoderma reesei*) and Novozyme 188 (cellobiase from *Aspergillus niger*) were purchased from Sigma Company (St Louis, MO). The enzyme cocktail was obtained by mixing equal volumes of the two enzymes. The enzymatic activity of the mixture (42.5 filter paper units (FPU) ml^{-1} for cellulase and 330 CBU ml^{-1} for cellobiase) was checked by the procedure of Ghose[5]. Enzymatic saccharification of lignocellulosic material was according to the laboratory analytical procedure of the National Renewable Energy Laboratory (LAP-009). Biomass samples equal to 0.1g equivalents of cellulose were hydrolyzed with a cellulase/cellobiase mix in a total volume of 10 ml by incubating with appropriate amounts of enzyme mixture and sodium citrate buffer (0.1 M, pH 4.8) for 72 h. The enzyme loadings were in excess (21 FPU per g cell wall residue). Enzyme blanks and Whatman #1 filter paper were digested alongside the samples. Hydrolysis of filter paper was always more than 95%.

Total sugar and sugar component analysis. The determination of total carbohydrates in untreated and pretreated biomass was according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (LAP-019). The biomass (~300mg) was first hydrolysed in 72% sulfuric acid at 30 ºC then in dilute sulfuric acid (4%) at 130 ºC for 1 h. Solubilized total sugars in extractive free cell wall residues and hydrolyzates

were estimated spectrophotometrically using the phenol-sulfuric acid assay [6]. Monomeric sugars (arabinose, galactose, glucose, xylose) in hydrolysates from acid pre-treatment were analyzed as alditol acetates by GC/MS according to TAPPI method T 249 cm-85. GC/MS was performed on a Hewlett Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60-m x 0.25-mm x 0.25-μm film thickness), and mass spectra were recorded in electron impact mode (70 eV) with 60-650 m/z scanning range. The glucose and xylose contents of enzymatic hydrolysates were determined by HPLC (Agilent 1200 Series LC System with 1200 Series Refractive Index Detector). An Aminex HPX-87P column was used at 80 °C, and the sugars were eluted with Milli-Q filtered water at a flow rate of 0.6 ml/min. Detector temperature was 50 °C. Peaks were identified and quantified by comparison to authentic standards of glucose and xylose.

Statistical analyses. Analysis of variance (ANOVA) was conducted using the GLM procedure in SAS to test the null hypothesis of no statistical differences in sugar released by acid pretreatment and enzymatic hydrolysis, or in lignin content or composition, between the individual transgenic plant lines [7]. Numbers of independent lines analyzed were 4 (controls), 2 (C4H), 2 (HCT), 3 (C3H), 2 (CCoAOMT), 2 (F5H) and 2 (COMT). For each independent line, two analytical replications were performed. The null hypothesis was rejected at the 0.05 level. Regression analysis was performed using the statistics program built into Microsoft Excel.

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