Supporting Information

Van Acker et al. 10.1073/pnas.1321673111

SI Materials and Methods

Materials. Poplars (*Populus tremula* × *Populus alba* cv. "717–1B4") transformed with the vectors pFS-*CCR* and pFAS-*CCR* have been described in Leplé et al. (1). Transgenic lines FS3, FS40, and FAS13 were used in this study.

Belgian Field Trial. The transgenic lines FS3 and FS40, and WT poplar (all in *P. tremula* × *P. alba* cv. "717–1B4") were simultaneously micropropagated in vitro, and 120 ramets of each line were grown in the greenhouse. After 9 mo of growth, the stems were cut 10 cm above soil level. After 10 more days in the greenhouse, the coppiced trees were transferred to the field in May 2009, under a genetically modified field trial authorization (B/BE/07/V2) provided by the Belgian competent authorities after a positive ruling from the Belgian Biosafety Advisory Council. FS3 and FS40 correspond to the names WT52-3 and WT52-40 in the B/BE/07/2 document. The field consisted of six randomized blocks for each line, and each block consisting of 20 clonally propagated trees (Fig. S2*A*). Trees were planted in rows with alternating distance, 0.75 m and 1.5 m. In a single row, plants were planted 0.75 m from each other. A border of WT trees surrounded the field, but these plants were not included in the experimental analyses. The border trees were at least 2.5 m from the fence protecting the field. The height of the main stem and the diameter, just above the position of coppicing before planting, of every tree were measured in January 2010. After 10 mo of growth in the field, beginning of March 2010, the trees were coppiced. The bottom ∼20 cm of the stems was harvested, debarked, and immediately photographed. Both stems and bark were stored at room temperature until used for further analyses. Pictures from the debarked, bottom ∼20 cm of the stems were imported into Image J. Red-colored spots on the stem xylem were manually selected, and the surface was measured in squared pixels, as well as the total surface of the stem. Based on the percentage of surface that had a red coloration, individual stems were categorized into six different redness classes (class 0, fully white; class 1, 0–25% red; class 2, 25–50% red; class 3, 50–75% red; class 4, 75–100% red; and class 5, fully red).

French Field Trial. The transgenic lines FS3 and FAS13 as well as WT were simultaneously micropropagated in vitro, and 120 ramets of each line were grown in the greenhouse. They were planted in the field in July 2008, after obtaining suitable authorization (Application B/FR/07/06/01, Authorization 07/015 from the "Direction Générale de l'Alimentation" from the French "Ministère de l'Agriculture et de la Pêche" (on September 21, 2007 for a 5-y period) after a positive ruling from the French "Commission du Génie Biomoléculaire." FS3 and FAS13 correspond to the names WT52-3 and WT62-13 in the B/FR/07/06/01 document. The 120 plants of each line were dispatched in five different randomized blocks (with 24 trees per block planted in two double rows) (Fig. S2*B*). The plant density was chosen according to short-rotation coppice practice: the space between trees of one double row was 0.55 m whereas the interspace between the two double rows was 1.5 m, and the planting distance within a row was 1 m. To prevent edge effects, the experimental plantation was bordered with one row of WT trees. During the growing season, the poplars were drip irrigated. In March 2010, the stems were coppiced. An ∼20-cm segment at the base of each stem was harvested, debarked, and photographed.

Saccharification of Greenhouse-Grown Samples. FS3, FS40, and FAS13 transgenic trees were micropropagated in vitro and, along with WT, transferred to the greenhouse. After 6 mo of growth, the stems of five ramets of FS3, FS40, FAS13, and WT were cut 10 cm above soil level, debarked, left to air-dry, and ground to powder in liquid nitrogen. Another seven ramets of FAS13 and two WT were harvested and debarked. For the latter trees, red and white xylem of FAS13, located next to each other, as well as WT xylem, were scraped along the debarked stem with a scalpel and immediately frozen in liquid nitrogen. After grinding in liquid nitrogen, the scraped xylem of red and white zones and WT was dried under vacuum.

Biomass (10 mg) was saccharified without pretreatment and with acid pretreatment as described (2).

Saccharification of Field-Grown Samples. For the Belgian field trial, every transgenic tree belonging to redness class 3, 4, and 5 was saccharified, along with 18 WT (three randomly chosen trees from each of the six blocks). For the French field trial, the five most red trees for line FS3 and FAS13 were selected from each of the five blocks, together with five randomly chosen WT. The poplar samples, taken from the ∼20-cm basal part of the harvested stems, were air dried and extensively ground and weighed with the iWALL custom-designed robot (Labman Automation Ltd.). The implementation of the iWALL system has been described in detail (3). The dilute base pretreatment solutions consisted of 6.25 mM NaOH and 62.5 mM NaOH. The dilute acid pretreatment solution was 0.4 M H_2SO_4, as described in detail (3).

For saccharifications including bark, wood and bark were ground separately and recombined based on the relative weight proportion of wood and bark in a stem. These saccharifications were performed as described (2).

Simultaneous Saccharification and Fermentation. Before pretreatment of biomass samples and the subsequent simultaneous saccharification and fermentation (SSF), total solids were determined with an automatic infrared moisture analyzer (Precisa XM60) according to the National Renewable Energy Laboratory procedure (4). Approximately 10 g of poplar biomass was pretreated with lime in the presence of water with a modified procedure from Chang et al. (5, 6); i.e., 10% (wt/vol) biomass was mixed with 1% (wt/vol) $Ca(OH)_2$ and incubated at 121 °C for 6 h. After pretreatment, samples were cooled to room temperature and collected by centrifugation at 89.44 × *g* for 3 min and washed three times with 2 mL of distilled water per gram dry biomass. The pretreated slurry was saccharified, fermented simultaneously at a substrate concentration of 8% (wt/wt), and mixed with 1% (wt/wt) yeast extract (DSM Food Specialties), 2% (wt/wt) bactopeptone in 50 mM (pH 4.8) citrate buffer. The reaction mixture contained 0.3 g/g biomass Accellerase 1500 (Genencor International) and 0.1% (wt/wt) Ethanol Red dry alcohol yeast (Fermentis). The endoglucanase and β-glucosidase activity of Accellerase 1500 were between 2,200 and 2,800 carboxymethyl cellulose U/g and 450 and 775 *para*-nitrophenylglucose U/g, respectively. Before the addition of the yeast and the enzyme complex, the fermentation broth was autoclaved. Fermentations were run in capped bottles at 37 °C and shaken at 2.01 × *g* to prevent sedimentation of the substrate and the yeast.

The concentrations of glucose, cellobiose, and ethanol were determined on a Prostar HPLC system (Varian) with an Aminex HPX-87H column (Bio-Rad) at 65 °C, equipped with a 1-cm reversed-phase precolumn and with 5 mM H_2SO_4 (0.6 mL/min) as mobile phase. Detection was done on a differential refractive index detector (LaChrom L-7490; Merck).

Wood Compositional Analysis. Lignin content was determined using the acetyl bromide (7) and Klason (8) method, lignin composition by thioacidolysis (9, 10), crystalline cellulose content by the Updegraff method (11), and compositional analyses of the matrix polysaccharides by the alditol-acetate assay (11).

Metabolomics. The outer xylem of debarked stems was scraped with a scalpel, ground in liquid nitrogen, and extracted with 1 mL of methanol. To correct for the amount of plant material extracted, the pellet was dried, and the recalculated amount of MeOH, according to the dry weight measured, was subjected to solid-phase extraction. The eluate was lyophilized and dissolved in 50 µl of water. An 8-µl aliquot was subjected to liquid chromatography (LC)-mass spectrometry (MS) and LC-tandem MS with the Acquity ultraperformance LC system (Waters) connected to a Synapt High Definition Mass Spectrometry Q-TOF mass spectrometer (Waters). Gradient elution and MS analysis were performed as described (12). The data were recorded using Masslynx 4.1, and the data were statistically analyzed using ANOVA in TransOmics (Waters).

Statistical Analyses. For the Belgian field trial, first analyses of saccharification yield, diameter, and height were performed on WT trees and on transgenic trees belonging to redness class 3, 4, and 5. Because these redness classes are not present in WT, a new categorical variable was created encompassing the line and the redness class. Differences between the means of the transgenic lines of redness class 5 and the WT were estimated. Associated *P* values were adjusted with the Dunnett test (two-sided). Block and the interaction term block*line were included as random factors. All other analyses were done on the best saccharifying trees. These analyses did not require the block*line interaction term. Nested designs were used for those analyses where several measurements were done on the same plant. The nested term was added as random term in the model. The significances of a random term were assessed with a likelihood ratio test.

The saccharification on the French field trial was performed at one time point on all individual trees. A mixed model was fitted with line, pretreatment condition, and line*pretreatment as fixed effects. The random terms block, block*line, and the nested term were not significant at the 0.05 significance level. Differences were estimated within each line between each pretreatment condition and the control treatment (= none), and within each pretreatment condition between the transgenic lines and WT. *P* values associated with these estimates were adjusted by using the linear step-up method of Benjamini and Hochberg (13). Other analyses were done on pools of five trees, two pools per block. These pools were considered as a random factor nested within line and block. All random effects were assumed to be normally distributed. For these analyses, there was only one fixed term: line. Differences between the means of the transgenic lines and the WT line were estimated. Associated *P* values were adjusted with the Dunnett test (two-sided).

In analyses, such as some saccharification analyses and all fermentation analyses, multiple observations over time were taken. These data were analyzed as repeated-measurements data. For all models, five covariance structures were fitted to the data: unstructured, autoregressive, heterogeneous autoregressive, compound symmetry, and heterogenous Toeplitz. Time was considered as a classification variable, unless line plots gave indications for a linear spline model. Fixed effects were line, time, and time*line. All random effects were assumed to be normally distributed. Observations on different plants were assumed to be independent. Variances and covariances of measures on a single plant were assumed to be the same within each line. Differences between the means of the transgenic lines and WT were estimated at particular times. Associated *P* values were adjusted by using the linear step-up method (13). Residual analysis was carefully examined.

The output of all analyses was generated using SAS/STAT software, Version 9.3 of the SAS System for windows (2011, SAS Institute).

1. Leplé J-C, et al. (2007) Downregulation of cinnamoyl-coenzyme A reductase in poplar: Multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell* 19(11):3669–3691.
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4. Sluiter A, et al. (2008) *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples: Laboratory Analytical Procedure (LAP)* (National Renewable Energy Laboratory, Golden CO), Technical Report NREL/TP-510-42621.
5. Chang VS, Kaar WE, Burr B, Holtzapple MT (2001) Simultaneous saccharification and fermentation of lime-treated biomass. *Biotechnol Lett* 23(16):1327–1333.
6. Chang VS, Nagwani M, Kim C-H, Holtzapple MT (2001) Oxidative lime pretreatment of high-lignin biomass. *Appl Biochem Biotechnol* 94(1):1–28.
7. Foster CE, Martin TM, Pauly M (2010) Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass). Part I: Lignin. *J Vis Exp* 37:e1745.
8. Dence CW (1992) *Methods in Lignin Chemistry*, eds Lin SY, Dence CW (Springer, Berlin), pp 33–61.
9. Ralph J, et al. (2008) Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency). *Plant J* 53(2):368–379.
10. Yue F, Lu F, Sun R-C, Ralph J (2012) Syntheses of lignin-derived thioacidolysis monomers and their uses as quantitation standards. *J Agric Food Chem* 60(4): 922–928.
11. Foster CE, Martin TM, Pauly M (2010) Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass). Part II: Carbohydrates. *J Vis Exp* 37:e1837.
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13. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 57(1): 289–300.

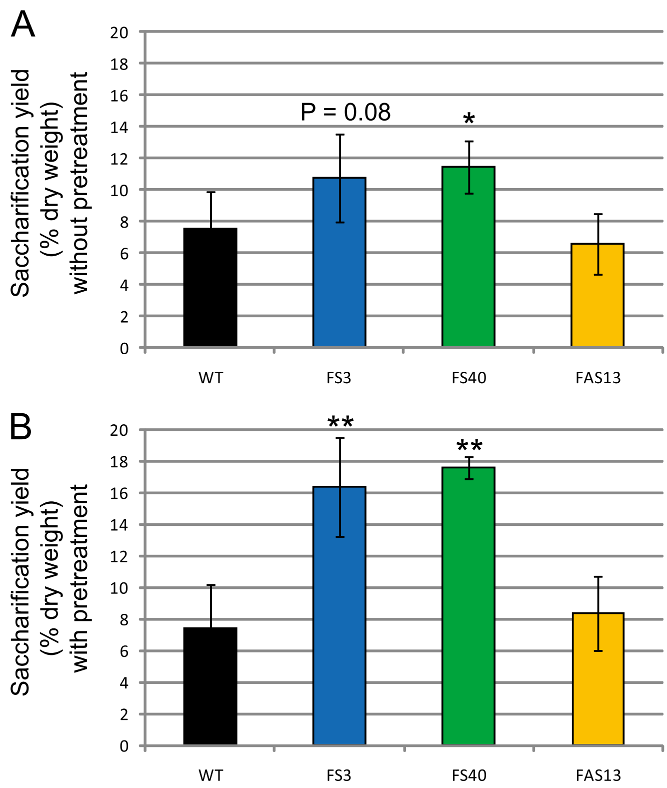


Fig. S1. Saccharification yields of greenhouse-grown transgenic trees. Saccharification yield (expressed as % dry weight) of greenhouse-grown debarked wood from FAS13, FS3, FS40, and WT ($n = 5$) after 48 h of saccharification (*A*) without pretreatment and (*B*) with acid pretreatment. Error bars represent SDs. *$0.01 < P < 0.05$; **$P < 0.01$.

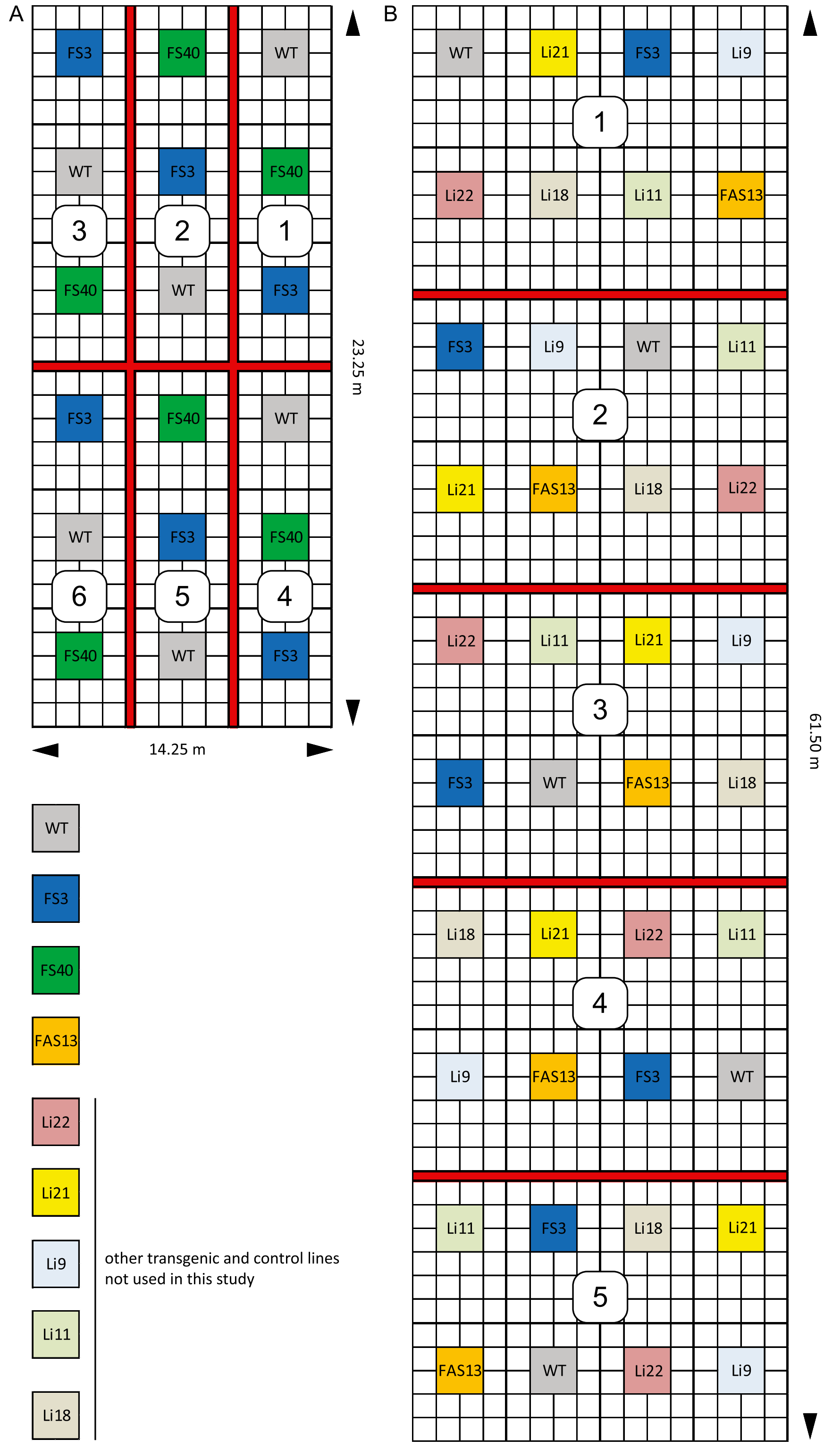


Fig. S2. Plan of the field trials. (*A*) Plan of the Belgian field trial. The field is divided into six blocks, each block containing 20 clonal replicates per line. (*B*) Plan of the French field trial. The field is divided into five blocks, each block containing 24 clonal replicates per line. Each block is referred to by a number in the rounded rectangles.

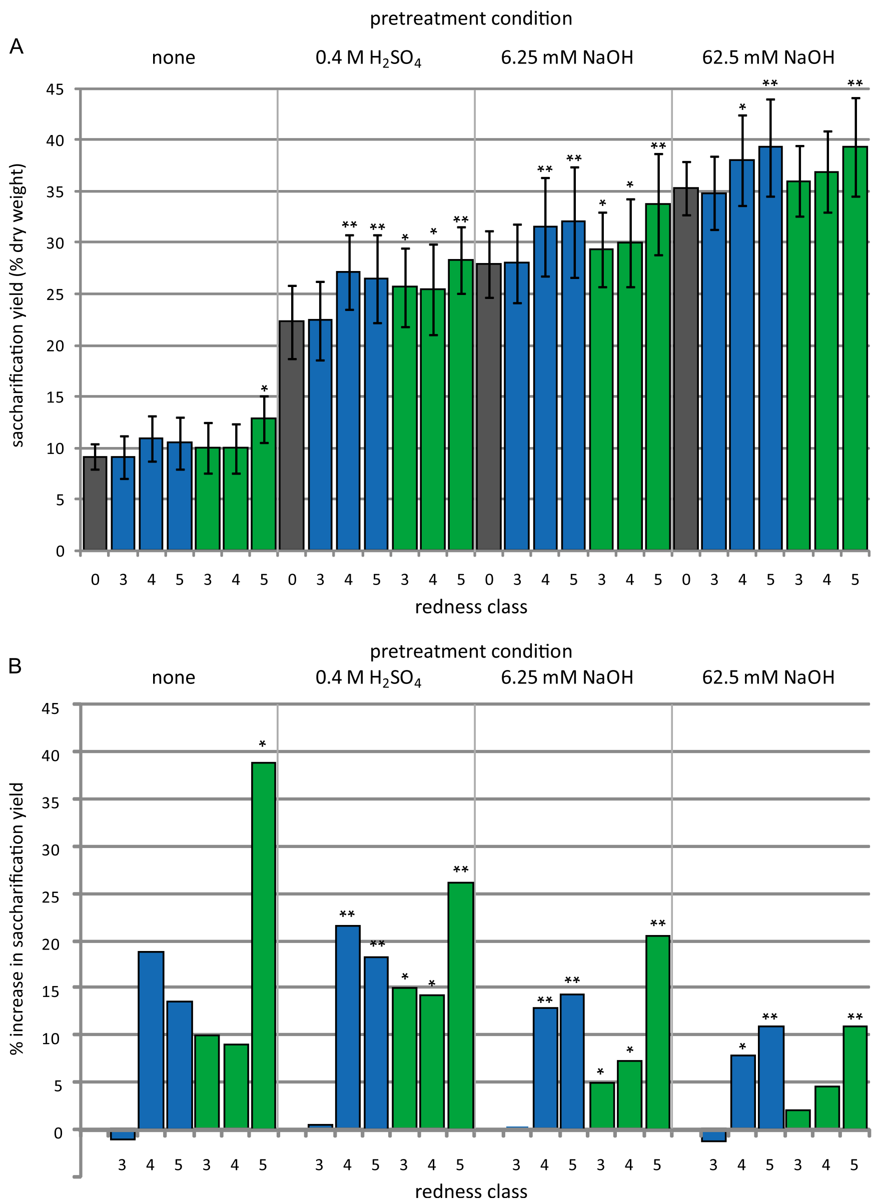
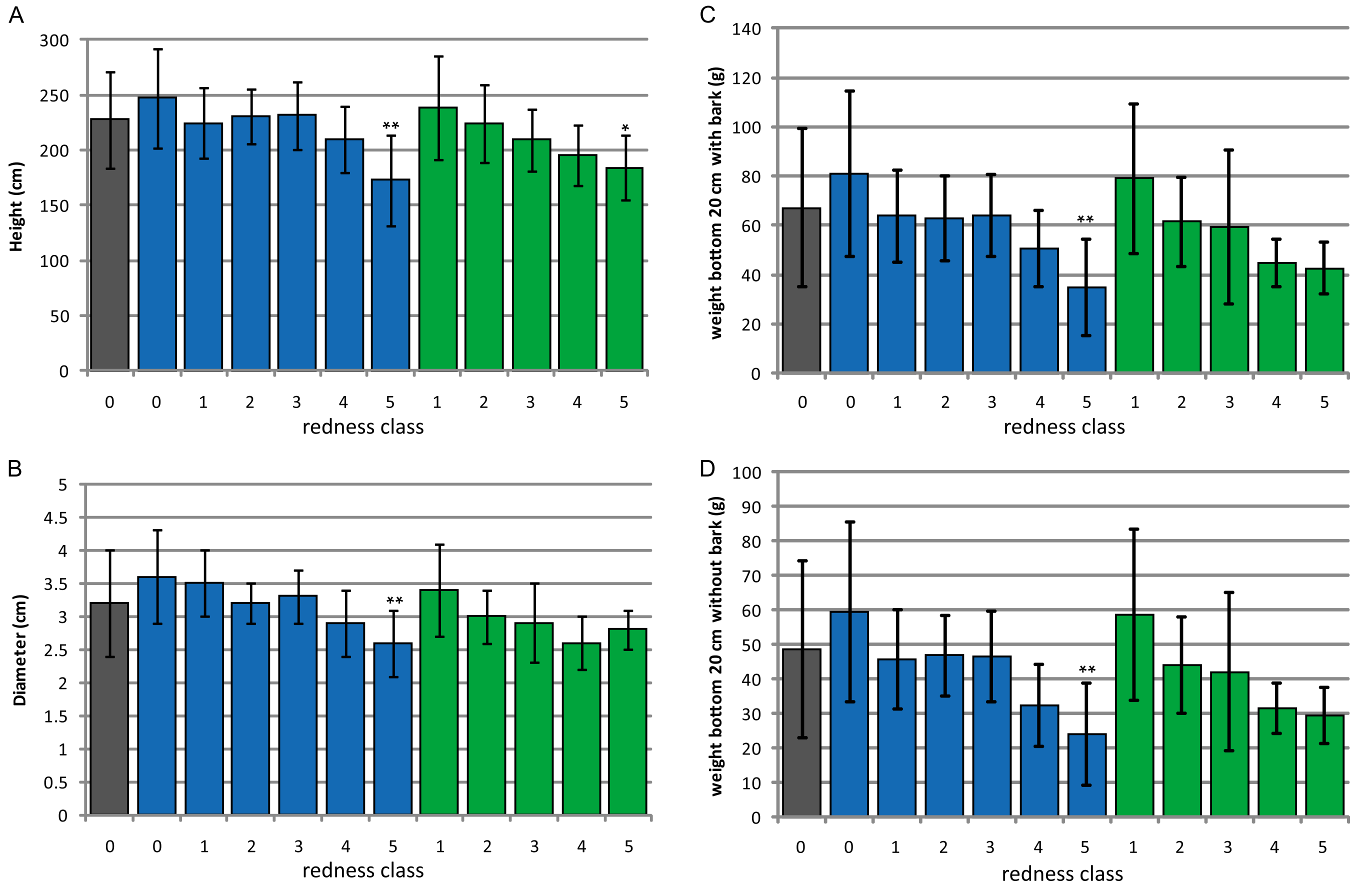


Fig. S3. Saccharification yields for different redness classes under different pretreatment conditions. (*A*) Saccharification yield (expressed as % dry weight) for different saccharification pretreatment conditions for WT (dark gray bars) and redness classes 3, 4, and 5 for both transgenic lines FS3 (blue) and FS40 (green). (WT, $n = 18$; FS3, redness class 3, $n = 12$; FS3, redness class 4, $n = 15$; FS3, redness class 5, $n = 15$; FS40, redness class 3, $n = 18$; FS40, redness class 4, $n = 13$; FS40, redness class 5, $n = 13$). Error bars represent SDs. (*B*) Average increase in saccharification yield for the different redness classes for both FS3 and FS40 compared with WT within the same pretreatment. *$0.001 < P < 0.01$; **$P < 0.001$ (significantly different from WT within the same pretreatment condition).



E

line	block	N	average biomass per tree (g) ± SD	increase/decrease compared to WT (%) per block	average biomass per tree (g) ± SD	increase/decrease compared to WT (%) whole field
FS3	1	22	259 ± 128	- 10	224 ± 107	**- 16**
	2	24	199 ± 95	+ 30		
	3	24	204 ± 117	**- 38**		
	4	24	255 ± 114	- 16		
	5	24	209 ± 65	- 19		
FAS13	1	23	212 ± 159	- 26	201 ± 122	**- 24**
	2	21	141 ± 118	- 8		
	3	24	238 ± 116	**- 27**		
	4	24	213 ± 84	**- 30**		
	5	23	192 ± 112	**- 26**		
WT	1	24	287 ± 226		266 ± 155	
	2	24	153 ± 127			
	3	24	327 ± 128			
	4	24	303 ± 110			
	5	24	258 ± 92			

Fig. S4. Growth phenotypes of trees grown in the Belgian and French field trials. (*A*) Height and (*B*) diameter, expressed in cm, and biomass for the bottom 20-cm stem segments (*C*) with bark and (*D*) without bark for trees belonging to different redness classes, for both transgenic trees and WT of the Belgian field trial. Height and diameter of 7-mo-old plants were measured in January 2010. Biomass was determined when harvested in March 2010. From left to right, WT, redness class 0–5 for FS3, redness class 1–5 for FS40. Error bars represent SDs. Dark gray, WT; blue, FS3; green, FS40. *$0.01 < P < 0.05$; **$P < 0.01$. (*E*) Average biomass (± SD) per transgenic line and WT per block and for the whole field of the French field trial. For some blocks, the number of measured trees was less than the originally planted 24 trees because some trees had died. Bold, significantly decreased compared with WT.

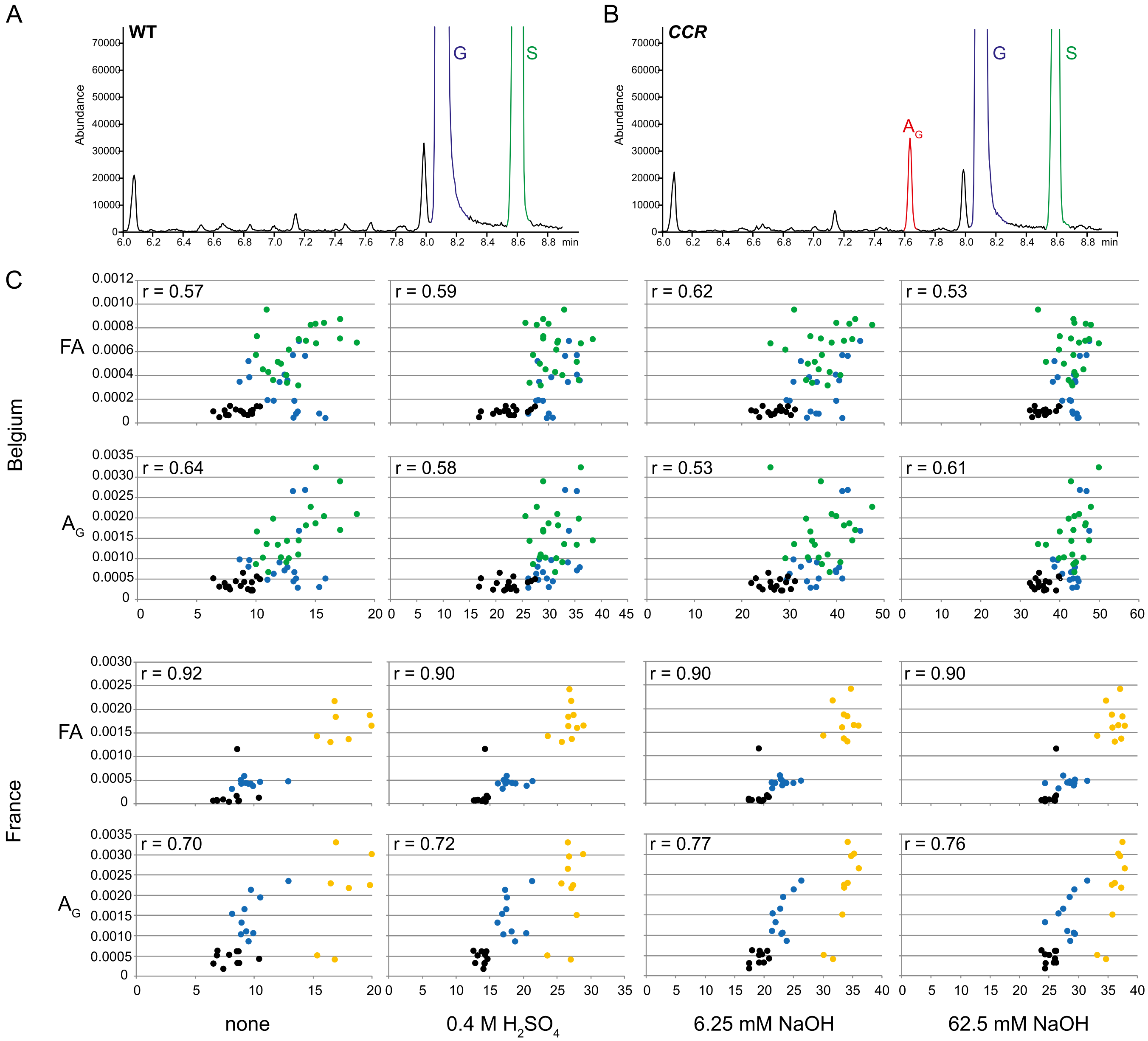


Fig. S5. Detection and incorporation of ferulic acid (FA) and the cinnamoyl-CoA reductase (CCR) marker. (*A*) The CCR marker (A_G) was detectable in trace amounts in WT poplar, but (*B*) was elevated in *CCR*–down-regulated poplars. (*C*) The incorporation of FA and the CCR marker into the lignin polymer correlated with the saccharification yield, independently of the applied pretreatment condition. On the *y* axis of the scatterplots, the peak area, normalized for the internal standard and amount of the cell-wall residue (mg), of FA or A_G is shown; on the *x* axis is the saccharification yield, in % dry weight, for the different pretreatment conditions. The Pearson correlation coefficient (r) is shown in the left upper corner of each scatterplot. The upper two rows of *C* correspond to the Belgian field trial, the lower two rows to the French field trial. G, guaiacyl unit; S, syringyl unit; black, WT; blue, FS3; green, FS40; orange, FAS13.

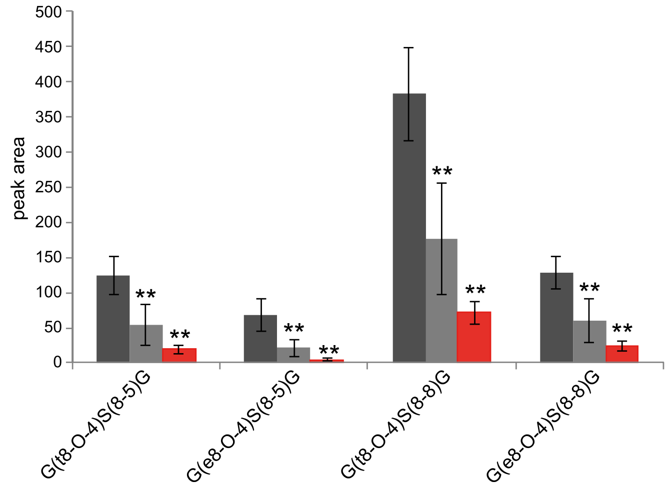


Fig. S6. Metabolomics on xylem of field-grown transgenic trees. Metabolite profiling data from red and white scraped xylem of FS3, FS40, and WT from the Belgian field trial. Averaged peak areas for G(8-O-4)S(8-5)G and G(8-O-4)S(8-8)G oligolignols, both *erythro*- and *threo*-isomers. These oligolignols are a few of the most abundant oligolignols present in poplar wood. Red, red xylem from both FS3 and FS40 (n = 11); gray, white xylem from both FS3 and FS40 (n = 11); black, xylem of WT (n = 5). $**P < 0.01$.

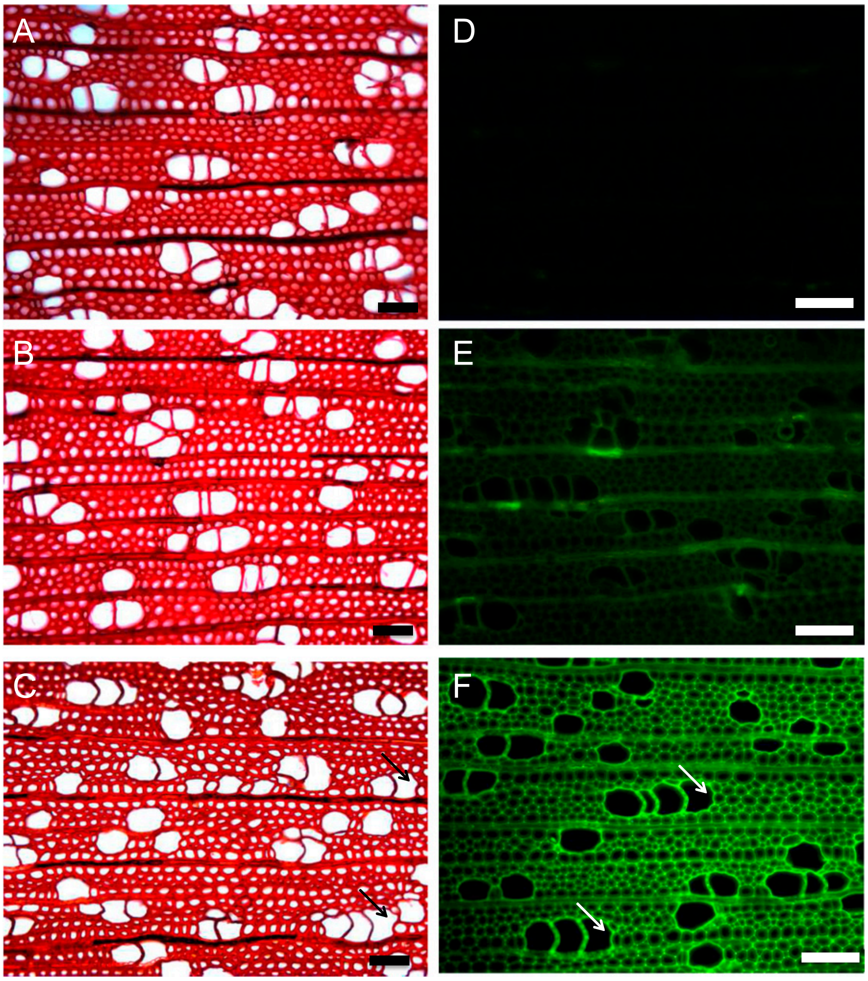


Fig. S7. Histochemical phenotyping of FAS13 *CCR*–down-regulated poplar. Safranin staining of a stem section of field-grown (*A*) WT and *CCR*–down-regulated poplar (FAS13) with (*B*) white-colored xylem and (*C*) red-colored xylem. Collapsed vessels are mainly present in the red-colored sample (representative examples are indicated with arrows). Blue-excited autofluorescence (450–490 nm) of (*D*) WT, (*E*) white-colored xylem of FAS13, and (*F*) red-colored xylem of FAS13. As already observed in Leplé et al. (1), the autofluorescence was greatly increased in the red-colored xylem and mainly in the cell wall of the vessels and in the middle lamella and/or S1 layers of fibers. White-colored xylem shows a weak autofluorescence in the vessels and fibers but also within ray cells. The collapsed vessels are also visible by blue-excited autofluorescence of the red-colored xylem (*F*). Exposure time was 675 ms. (Scale bars: 100 μm.)

1. Leplé J-C, et al. (2007) Downregulation of cinnamoyl-coenzyme A reductase in poplar: Multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell* 19(11):3669–3691.

Table S1. Selection of trees for SSF

Belgian field trial				French field trial				
Line	ID_tree	Pooled	Block	Line	ID1	ID2	ID3	Block
FS3	K12R11	a	1	FS3	L7C61	L8C59		1
FS3	K10R12	a	1	FS3	L14C44	L14C45		2
FS3	K13R8		2	FS3	L14C31	L17C30	L16C31	3
FS3	K14R26		3	FS3	L8C18	L9C17		4
FS3	K15R24		5	FS3	L10C10	L12C13		5
FS3	K11R18		5	FS3	L8C57	L8C58		1
FS3	K10R21		6	FS3	L14C46	L16C46		2
FS40	K8R19		4	FS3	L15C30	L14C26	L16C31	3
FS40	K4R17		4	FS3	L7C16	L7C19		4
FS40	K9R15		5	FS3	L10C8	L10C11		5
FS40	K7R19		5	FAS13	L2C55	L5C55	L4C53	1
FS40	K5R23	b	6	FAS13	L10C43	L11C42		2
FS40	K5R25	b	6	FAS13	L8C28	L9C30		3
FS40	K5R26		6	FAS13	L12C17	L10C17		4
WT	K10R36		1	FAS13	L14C3	L15C6		5
WT	K11R29		2	FAS13	L4C54	L4C52	L4C53	1
WT	K10R40		3	FAS13	L11C39	L11C40		2
WT	K13R34		4	FAS13	L6C26	L8C31		3
WT	K13R37		5	FAS13	L13C18	L10C19		4
WT	K15R37		6	FAS13	L14C5	L16C6		5
				WT	L15C57	L15C61		1
				WT	L7C46	L9C46		2
				WT	L13C28	L13C31		3
				WT	L2C18	L3C18		4
				WT	L10C4	L12C3		5
				WT	L15C60	L16C58		1
				WT	L6C45	L6C47		2
				WT	L10C30	L13C26		3
				WT	L4C18	L4C19		4
				WT	L10C5	L12C2		5

For the Belgian trial, fully red tree samples were selected from over the entire field to minimize environmental (position) effects. For WT, one tree per block was randomly chosen. This selection resulted in six biological repeats for FS3 and WT and seven for FS40. Because a larger amount of material is required for SSF than for saccharification, pooling was necessary from two trees for both transgenic lines, resulting in five biological repeats for FS3 and six for FS40 and WT. The individual trees with the same letter in the column "pooled for SSF" were combined to obtain enough material for SSF analyses. For the French field trial, five fully red trees were selected from each block and saccharified individually, whereas for SSF and wood compositional analysis, they were pooled from two (occasionally three) trees each (ID1, ID2, and ID3), resulting in 10 biological repeats for FS3, FAS13, and WT. Block numbers refer to the tree position in the field (Fig. S2).

Table S2. Broad-sense heritabilities as a measure of repeatability

Country	Trait	H^2
Belgium	Height	0.82
Belgium	Diameter	0.77
Belgium	Arabinose	0.78
Belgium	Saccharification stem	0.81
Belgium	Saccharification bark	0.67
Belgium	Saccharification mix	0.84
Belgium	FA	0.90
Belgium	CCR marker (A_G)	0.72
Belgium	H + G + S	0.64
Belgium	Klason lignin	0.51
France	AcBr lignin	0.95
France	G	0.93
France	S	0.94
France	Hemicellulose	0.86
France	Galactose	0.92
France	Mannose	0.88
France	Glucose	0.84
France	FA	0.99
France	CCR marker (A_G)	0.97
France	H + G +S	0.99
France	Klason lignin	0.97

As a measure of repeatability, broad-sense heritabilities on an entry-mean basis were calculated with the ad hoc approach described (1) for those traits that showed a significant line effect. For the Klason data, H^2 is the equivalent to the coefficient of determination of the linear regression of the line on the observed phenotype.

1. Piepho HP, Möhring J (2007) Computing heritability and selection response from unbalanced plant breeding trials. *Genetics* 177(3):1881–1888.