Supporting Information

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SI Materials and Methods

Plant Growth. All plants used in experimentation were sown in low nitrate soil (John Innes Seed Compost). Seeds were vernalized in the dark at 4–6 °C for 21 d before being transferred to the greenhouse at 20 °C with a 16-h light photoperiod. Watering was stopped ∼10.5 wk after sowing, when plants had reached full maturity, and plants were harvested once they had completely dried.

Saccharification Analysis. Plant material was tested for saccharification using a liquid handling robotic platform (Tecan Evo 200; Tecan Group Ltd.) [see Gomez et al. (1)]. Plant material was pretreated with 0.5 M NaOH at 90 °C for 20 min, followed by enzymatic digestion performed at 50 °C for 8 h with shaking with a 4:1 mixture of Celluclast to Novozyme 188 (Novozymes). The key enzyme activity of Celluclast is cellulase that hydrolyzes (1,4)-β-glucosidic linkages in cellulose and other beta-D-glucans into glucose, cellobiose, and higher glucose polymers. It is produced by submerged fermentation of the fungus *Trichoderma reesei* and has an activity of ≥700 endoglucanase units (EGU)/g (μmol reducing sugars released per gram of enzyme per minute). The key enzyme activity of Novozyme 188 is cellobiase that hydrolyzes cellobiose to glucose. It is obtained by submerged fermentation of an *Aspergillus niger* microorganism and has an activity of ≥250 cellobiase units (CBU)/g (μmol of glucose released per gram of enzyme per minute). The mixture of enzymes is diluted such that 7 filter paper units of enzyme is added per 1 mg of biomass. Saccharification potential was determined by measuring the amount of reducing sugars using a colorimetric assay using 3-methyl-2-benzothiazolinone hydrozone (MTBH) as described in ref. 1. Each plate contained standard reactions of 50 nmol, 100 nmol, and 150 nmol of glucose. Change in color was read with a Tecan Sunrise microplate absorbance reader at 620 nm.

Fourier Transform Infrared Spectroscopy. Fourier transform infrared (FTIR) spectra were measured using a Spectrum One (Perkin-Elmer) equipped with a diamond that allows collection of spectra directly on the sample without any sample preparation. Ground stem material from WT and *sac* plants was applied to the diamond, and a pressure arm was used to apply a constant pressure on the samples to ensure good contact between the sample and the IR beam. Spectra were acquired for the wavelength range 850–1,850 cm^{-1} at a spectral resolution of 4 cm^{-1}, and 256 scans were taken for each spectrum. Three spectra were collected for each sample, and the triplicate-averaged spectrum was used for principal component analysis (PCA). PCA was carried out using The Unscrambler software (CAMO). Spectra were normalized using peak normalization and were linear baseline corrected before performing PCA.

Cell-Wall Composition Analysis. To obtain alcohol insoluble residue (AIR), 100 mg of ground stem material was subject to 30 min incubation with shaking at room temperature, followed by centrifugation at 3,000 × *g* at 4 °C for 10 min and removal of the supernatant. The pellet was washed with the following solutions in this order: twice with chloroform:methanol (1:1, vol/vol), twice with 80% (vol/vol) methanol, and once with 100% methanol, after which samples were left to dry overnight at room temperature.

Lignin content was quantified using Foster et al.'s (2) acetyl bromide method, based on the method reported by Fukushima and Hatfield (3). Briefly, 3 mg of AIR was weighed into a 5-mL volumetric flask, and 250 μL of freshly prepared acetyl bromide solution (25% vol/vol acetyl bromide in glacial acetic acid) was added. Samples were incubated at 50 °C for 2 h, followed by a further 1 h with mixing every 15 min. Samples were cooled to room temperature, 1 mL of 2 M NaOH and 175 μL of freshly prepared 0.5 M hydroxylamine hydrochloride were added, samples were taken to 5 mL with glacial acetic acid and mixed, and the absorption was read using a Shimadzu UV-1800 spectrophotometer at 280 nm. Lignin content (μg·mg^{-1} cell wall) was determined using the following formula:

$$(absorbance \div (coefficient \times path\ \ length)) \times ((total\ \ volume \times 100\%) \div biomass\ \ weight).$$

The coefficient is specific to the type of plant that is being analyzed, and, for grasses, a coefficient of 17.75 is used (3).

Crystalline cellulose content was analyzed using Foster et al.'s (4) method, based on the method reported by Updegraff (5). Briefly, 1 mL of Updegraff reagent [acetic acid:nitric acid:water (8:1:2, vol/vol/vol)] was added to 4 mg of AIR, heated at 100 °C for 30 min, cooled to room temperature, and centrifuged at 10,000 × *g* for 15 min. The pellet was washed four times with 1.5 mL of water, air dried, and incubated with 175 μL of 72% (vol/vol) H_2SO_4 for 45 min at room temperature. Samples were centrifuged at 10,000 × *g* for 15 min after adding 825 μL of water. Finally, the glucose content of the supernatant was quantified using the colorimetric anthrone assay as follows: 10 μL of each sample was added to a 96-well polystyrene microtiter plate with 90 μL of water and 200 μL of anthrone reagent (2 mg anthrone mL^{-1} concentrated H_2SO_4). A standard curve for glucose (0 μg, 2 μg, 4 μg, 6 μg, 8 μg, and 10 μg) was also added to each plate. The plate was heated at 80 °C for 30 min and allowed to cool, and the absorption was read at 620 nm using a Tecan Sunrise microplate.

Noncellulosic monosaccharide analysis was performed using high-performance anion exchange chromatography (HPAEC) (Carbopac PA-10; Dionex). AIR samples of 3 mg were hydrolyzed with 0.5 mL of 2 M trifluoroacetic acid (TFA) for 4 h at 100 °C, cooled to room temperature, and evaporated completely. The pellet was rinsed twice with 200 μL of isopropanol, once with 500 μL of water, and finally resuspended in 100 μL of deionized water. Samples were filtered with 0.45-μm polytetrafluoroethylene (PTFE) filters and separated by HPAEC as described in ref. 6. The separated monosaccharides were quantified by using external calibration with a mixture of seven monosaccharide standards at 100 μM (arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose) that were subjected to acid hydrolysis in parallel with the samples.

Ester-bound ferulic acid in the cell was quantified using a protocol based on Fry's method (7). To release the polysaccharide-bound ferulic acid, 1 mL of 1 M NaOH was added to 10 mg of AIR and incubated under argon at 25 °C in the dark for 24 h. The pH was brought to ∼1 by the addition of 2 M TFA and partitioning of the ferulate into the organic phase was achieved by addition of 1 mL of butan-1-ol, vigorous shaking, and removal of the upper organic phase for analysis. This partitioning was repeated twice. Finally, the organic phases were combined, the butan-1-ol was evaporated, and the residue was redissolved in 200 μL of 50% (vol/vol) methanol. The extracted ferulic acid was analyzed using HPLC on an activated reverse-phase C18 5-μm (4.6 × 250 mm) XBridge column (Waters Inc.) in methanol:acetic acid (19:1, vol/vol), with a 20–70% (vol/vol) methanol gradient over 25 min at a flow rate of 2 mL·min^{-1}. Ferulic acid was detected and quantified with a SpectraSYSTEM UV6000LP photo-diode array detector (Thermo Scientific), with UV–visible spectra collected at 240–400 nm, and analyzed against a ferulic acid standard.

Stem Mechanical Properties. The strength and stiffness of mutant and WT stems were assessed using three-point bending tests, using a universal testing machine (model 3367; Instron). Stem segments were placed on two supports, separated by 2 cm, and a 2-mm pushing probe was manually lowered until just in contact with the stem. The pushing probe was set to automatically lower at a rate of 10 mm·min^{-1}. Stem strength was measured by the maximum bending stress, λ_{max} [$\lambda_{max} = F_{max}Lr/4I$, where F_{max} is the maximum force the sample can withstand before failure, L is the distance between supports, r is the radius of the stem, and I is the second moment of the cross-sectional area of the stem ($\pi r^4/4$)]. Stem diameter was measured using a digital caliper. Stem stiffness was measured by the bending modulus (MPa), which is calculated by R/I, where R is the resistance of the stem to curvature [$R = L^3$ (dF/dY)/48, where dF/dY is the initial slope of the force displacement curve acquired from the bending test].

Mapping. DNA from the top 40 BCF_2 plants with the highest saccharification was obtained by grinding ≤100 mg of leaf material in liquid nitrogen (LN), followed by extraction using a Biosprint 15 with a Biosprint 15 DNA Plant Kit, and quantified using a Qubit Fluorometer (Life Technologies). DNA from these 40 plants was pooled with equal concentrations and sent for Illumina paired-end whole-genome sequencing by The Genome Analysis Centre (TGAC). Twenty-one Gbp of data of high quality were achieved, giving an average coverage of 78.7×. Assembly of the sequence data to the reference genome and identification of SNPs in the F_2 pools that did not occur in the WT dataset were performed by TGAC. SNPs were removed if they did not resemble a mutation caused by sodium azide mutagenesis (i.e., G to A, C to T, or A to T, and vice versa), if the phred quality score was <20, if the read depth was below five, and if the strand bias had a score of >1.

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2. Foster CE, Martin TM, Pauly M (2010) Comprehensive compositional analysis of plant cell walls (Lignocellulosic biomass). Part I: Lignin. *J Vis Exp* (37):e1745.
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		HG (Low DE) (mAb JIM5)	HG (High DE) (mAb JIM7)	HG new spec. (mAb LM18)	HG (mAb LM19)	HG (mAb LM20)	(1→4)-b-D-galactan (mAb LM5)	(1→5)-a-L-arabinan (mAb LM6)	Xylogalacturonan (mAb LM8)	Xyloglucan (mAb LM15)	Terminal xylose (mAb LM23)	(1→4)-b-D-xylan (mAb LM10)	(1→4)-b-D-xylan/arabinoxylan (mAb LM11)	Arabinan (mAb LM13)	Feruloylated arabinan (mAb LM12)	Feruloylated (1→4)-b-D-galactan (mAb LM9)	(1→3)b-D-glucan (mAb Anti-cal)	(1→3)(1→4)b-D-glucan (mAb Anti-MLG)	mannan (mAb LM21)	mannan/ galactomannan (mAb LM22)	(mAb UX1)	Cellulose (mAb CBM3a)	Cellulose (mAb CBM4-1)	Cellulose (mAb CBM9-2)	Extensin (mAb JIM19)	Extensin (mAb LM1)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP (mAb LM16)
CDTA	WT	5	2	5	3	1	3	17	0	1	0	1	5	0	38	0	2	11	1	3	0	0	0	0	0	9	19	2	0
	Sac5	6	2	7	3	1	0	11	0	1	0	0	1	0	23	0	1	5	1	1	0	0	0	0	0	6	11	1	0
	Sac9	6	4	10	6	3	1	14	0	0	0	0	1	0	8	0	2	1	0	1	1	0	0	0	0	10	17	1	0
	Sac8	3	2	2	1	0	2	17	0	1	0	0	2	1	29	0	1	6	0	1	0	0	0	0	0	9	16	2	0
	Sac4	7	4	10	6	2	0	12	0	0	0	0	2	0	8	0	1	2	0	0	1	0	0	0	0	9	14	0	0
	Sac12	6	4	8	4	1	0	12	0	0	0	0	2	0	24	0	1	7	0	1	1	1	0	0	0	8	11	1	0
	Sac2	6	5	9	5	2	1	12	0	0	0	1	3	0	24	0	1	6	0	1	0	0	0	0	0	10	12	1	0
	Sac10	6	8	10	6	5	2	14	0	1	0	0	2	0	23	0	2	10	0	1	0	0	0	0	0	9	14	1	0
	Sac11	5	4	8	4	1	1	10	0	1	0	0	2	0	26	0	1	11	1	1	0	0	0	0	0	8	11	1	0
	Sac6	4	3	6	3	1	3	16	0	1	0	0	3	0	37	0	2	11	1	1	0	1	0	0	0	12	16	2	0
	Sac1	6	5	9	5	2	0	12	0	0	0	0	1	0	12	0	1	4	1	1	0	0	0	0	0	9	12	0	0
	sac3	4	3	7	3	1	1	12	0	1	0	0	4	0	35	0	1	11	0	1	0	0	0	0	0	7	11	1	0
	sac7	4	3	7	2	3	1	14	0	1	0	0	3	1	31	0	1	8	0	1	0	0	0	0	0	8	12	1	0
NaOH	WT	1	1	3	9	0	45	40	0	19	20	89	58	8	6	0	30	88	3	11	13	4	0	0	0	4	38	5	1
	Sac5	0	1	2	7	0	43	37	0	17	17	84	55	5	6	0	28	88	2	8	12	6	0	0	0	4	37	5	1
	Sac9	1	1	2	7	0	49	37	0	17	11	70	47	4	5	0	28	85	1	7	11	4	1	0	0	3	38	5	1
	Sac8	0	1	2	8	0	41	38	0	18	22	85	55	8	6	0	30	88	1	8	12	4	0	0	0	2	35	6	2
	Sac4	0	1	1	6	0	41	35	0	14	13	66	44	3	4	0	26	85	2	7	9	5	0	0	0	2	37	5	1
	Sac12	0	1	1	7	0	40	38	0	16	18	79	55	6	5	0	28	89	2	8	12	5	0	0	0	2	37	5	1
	Sac2	0	1	1	6	0	41	36	0	16	18	77	51	5	5	0	27	87	2	7	10	5	0	0	0	1	37	5	1
	Sac10	0	1	2	9	0	47	40	0	20	25	87	58	10	6	0	30	95	1	8	12	4	1	0	0	1	39	6	2
	Sac11	0	1	2	7	0	40	39	0	16	17	84	56	7	5	0	26	90	1	7	12	5	0	0	0	1	39	5	1
	Sac6	0	1	2	8	0	43	41	0	18	18	88	57	8	6	0	29	96	2	7	12	4	1	0	0	1	38	5	2
	Sac1	0	1	2	7	0	43	41	0	17	13	82	51	6	5	0	28	99	1	7	11	4	0	0	0	1	41	6	1
	sac3	0	1	2	6	0	37	36	0	16	19	89	56	7	5	0	26	94	1	6	9	4	0	0	0	1	34	4	1
	sac7	0	1	2	8	0	43	40	0	17	19	93	57	8	5	0	29	95	2	6	12	4	0	0	0	1	40	5	2

Fig. S1. Immunocarbohydrate microarray analysis of WT and *sac* AIR. The heatmap represents relative mean signal produced by the binding of each antibody to each sample. Samples were extracted with 1,2-cyclohexanedinitrilotetraacetic acid (CDTA) and 4 M NaOH. The maximal signal for each antibody was set to 100, and all other values were adjusted accordingly. $n = 3$.

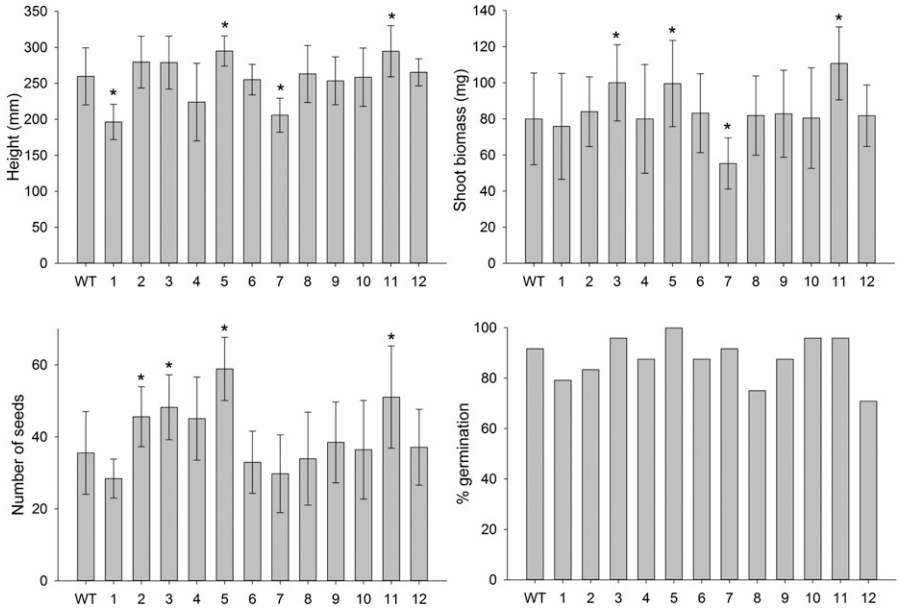

Fig. S2. Phenotyping (height, biomass, number of seeds, and percentage germination) of WT and the 12 *sac* lines. Asterisks indicate significant difference ($P \leq 0.05$) compared with WT.

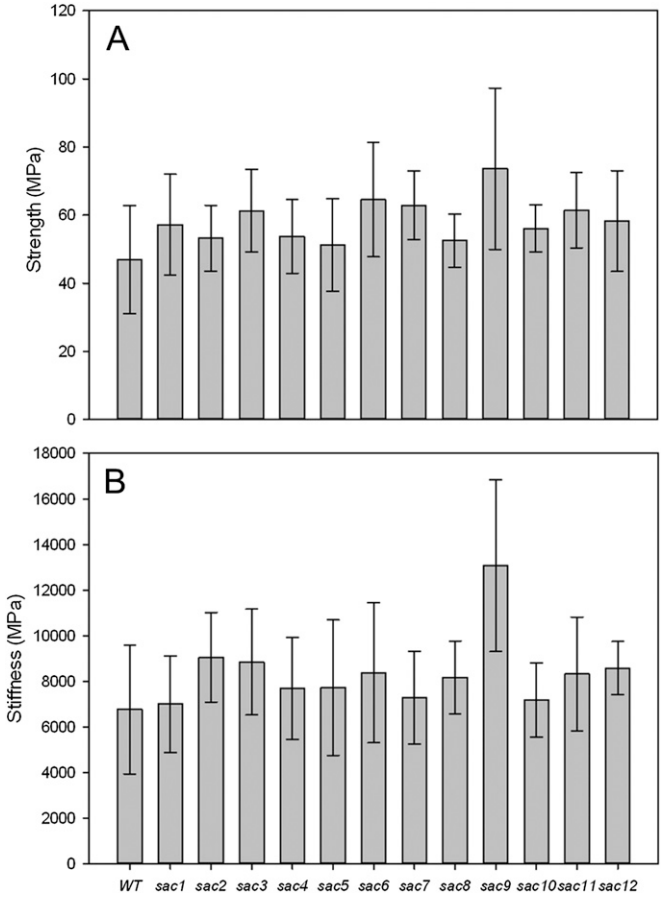

Fig. S3. Stem physical properties of *sac* and WT plants. (*A*) Stem strength measured by the maximum bending stress (MPa). (*B*) Stem stiffness measured by the initial force required to bend the stem (MPa). Data represent mean ± SD and $n = 8$.

Table S1. Total thioacidolysis yield and lignin monomer relative mol % of WT and *sac* AIR

Line	Thioacidolysis yield, μmol·g^{-1} AIR	% H	% G	% S	S:G ratio
WT	142.0 ± 5.4	3.2 ± 0.1	26.7 ± 0.7	70.1 ± 0.7	2.63
sac1	96.3 ± 3.6*	5.2 ± 0.1*	34.3 ± 1.3	60.6 ± 1.2*	1.78*
sac2	97.8 ± 1.8*	4.9 ± 0.2*	28.3 ± 0.4	66.8 ± 0.5	2.36
sac3	116.5 ± 8.3	4.17 ± 0.2	27.5 ± 0.3	68.3 ± 0.4	2.49
sac4	79.4 ± 3.4*	5.1 ± 0.1*	34.4 ± 0.1*	60.5 ± 0.0*	1.76*
sac5	114.7 ± 0.9*	4.1 ± 0.3	27.3 ± 0.2	68.6 ± 0.3	2.51
sac6	97.9 ± 7.6	5.1 ± 0.2*	34.8 ± 0.6*	60.1 ± 0.8*	1.73*
sac7	78.4 ± 9.7	5.6 ± 0.2*	35.8 ± 0.6*	58.6 ± 0.4*	1.64*
sac8	146.2 ± 3.9	3.4 ± 0.2	25.8 ± 0.3	70.9 ± 0.5	2.75
sac9	93.0 ± 2.2*	5.4 ± 0.1*	33.3 ± 1.2	61.2 ± 1.2*	1.84
sac10	155.1 ± 9.1	3.1 ± 0.2	28.0 ± 0.9	68.9 ± 1.1	2.47
sac11	119.8 ± 6.9	4.0 ± 0.1	28.7 ± 0.5	67.3 ± 0.6	2.34
sac12	139.2 ± 2.2	3.7 ± 0.1	26.8 ± 0.7	69.5 ± 0.6	2.59

Data show mean ± SD, $n = 3$. Asterisks indicate significant difference ($P \leq 0.01$) compared with WT.

Table S2. Monosaccharide composition of matrix polysaccharides in WT and *sac* plants (μg·g^{-1} AIR)

Line	Fucose	Arabinose	Galactose	Glucose	Xylose	Mannose
WT	2.09 ± 0.99	82.98 ± 3.95	49.72 ± 3.76	53.33 ± 2.76	118.22 ± 5.80	18.98 ± 2.18
sac1	3.15 ± 0.45	86.56 ± 4.04	51.17 ± 3.57	51.43 ± 2.46	123.32 ± 7.99	17.23 ± 2.20
sac2	2.40 ± 0.78	87.99 ± 7.44	50.18 ± 2.64	54.25 ± 4.09	123.32 ± 7.47	16.83 ± 2.82
sac3	1.47 ± 0.80	79.32 ± 4.26	44.42 ± 3.52	61.89 ± 20.57	107.85 ± 5.91	27.48 ± 3.83*
sac4	4.53 ± 0.35*	95.74 ± 5.83*	57.54 ± 5.14	55.98 ± 4.39	133.30 ± 7.28*	28.73 ± 0.31*
sac5	3.03 ± 1.32	94.70 ± 4.04*	55.67 ± 1.41	58.40 ± 0.31	135.18 ± 2.45*	28.49 ± 6.46*
sac6	2.66 ± 0.58	87.20 ± 6.72	51.71 ± 4.56	58.85 ± 2.97	123.30 ± 8.17	17.34 ± 0.92
sac7	2.77 ± 0.86	99.33 ± 6.90*	58.96 ± 8.37	68.05 ± 2.75*	159.02 ± 14.40*	38.64 ± 4.76*
sac8	1.76 ± 0.38	71.46 ± 4.90*	45.72 ± 4.88	50.10 ± 1.94	100.54 ± 4.09*	30.73 ± 4.57*
sac9	3.45 ± 0.18	92.25 ± 3.19*	56.36 ± 1.40*	52.73 ± 1.08	125.42 ± 7.14	22.65 ± 1.17*
sac10	1.69 ± 0.83	80.49 ± 3.26	49.06 ± 2.38	60.79 ± 6.90	118.94 ± 1.84	18.56 ± 1.24
sac11	2.12 ± 0.51	86.67 ± 10.64	47.69 ± 4.08	63.47 ± 14.75	124.06 ± 12.41	16.31 ± 1.99
sac12	2.26 ± 1.23	82.26 ± 12.97	46.86 ± 6.40	53.55 ± 3.34	116.63 ± 14.39	26.03 ± 1.43*

Data show mean ± SD, $n = 3$. Asterisks indicate significant difference ($P \leq 0.05$) compared with WT.

Table S3. SNPs in the 14-SNP cluster with allele frequencies ≥ 0.9 in chromosome 2 of the *sac1* mutant

SNP position	Ref base	Alt base	Region	Gene	Annotation	Ref aa	Alt aa
1022576	G	A	Exon	2g01480	Glycosyltransferase family GT61	Glycine	Serine
1116925	C	T	Noncoding				
1116938	A	G	Noncoding				
1206457	T	C	Noncoding				
1577928	G	A	Noncoding				
1666374	G	A	Exon	2g02510	Protein kinase family	Threonine	Threonine
1675680	C	T	Exon	2g02520	Putative protein	Proline	Leucine
1692493	T	C	Intron				
3523570	T	C	Noncoding				
3523571	G	A	Noncoding				
3527190	T	C	Noncoding				
3644990	C	T	Noncoding				
3648056	T	C	Noncoding				
3663412	T	C	Noncoding				