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Range of cell-wall alterations enhance saccharification in *Brachypodium distachyon* mutants

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Lignocellulosic plant biomass is an attractive feedstock for the production of sustainable biofuels, but the commercialization of such products is hampered by the high costs of processing this material into fermentable sugars (saccharification). One approach to lowering these costs is to produce crops with cell walls that are more susceptible to hydrolysis to reduce preprocessing and enzyme inputs. To deepen our understanding of the molecular genetic basis of lignocellulose recalcitrance, we have screened a mutagenized population of the model grass *Brachypodium distachyon* for improved saccharification with an industrial polysaccharide-degrading enzyme mixture. From an initial screen of 2,400 M₂ plants, we selected 12 lines that showed heritable improvements in saccharification, mostly with no significant reduction in plant size or stem strength. Characterization of these putative mutants revealed a variety of alterations in cell-wall components. We have mapped the underlying genetic lesions responsible for increased saccharification using a deep sequencing approach, and here we report the mapping of one of the causal mutations to a narrow region in chromosome 2. The most likely candidate gene in this region encodes a GT61 glycosyltransferase, which has been implicated in arabinoxylan substitution. Our work shows that forward genetic screening provides a powerful route to identify factors that impact on lignocellulose digestibility, with implications for improving feedstock for cellulosic biofuel production.

lignocellulosic biofuel | matrix polysaccharides | lignin | feruloylation

Concerns over greenhouse-gas emissions and the sustainability of liquid transportation fuel supplies have led to a rapid expansion of global biofuel production in recent years. Current biofuel production is dominated by bioethanol produced by fermentation of starch or sucrose from food crops and by biodiesel produced by transesterification of plant or animal oils. Although the production of such “first-generation” biofuels can be efficient, there is widespread concern that further expansion will exacerbate anticipated problems with global food security through direct competition for resources. In addition, these crops often require high inputs and consequently have a relatively high carbon footprint (1). A promising alternative to first-generation biofuel is the use of nonfood lignocellulosic plant biomass, available as agricultural waste from food crops or produced from low input, non-food plants such as perennial grasses (2).

Lignocellulosic biomass is principally composed of plant secondary cell walls and comprises ~70% polysaccharides, which can be converted into simple sugars for fermentation (3). The main challenge in producing bioethanol from lignocellulosic biomass is that these polysaccharides occur as part of a complex and indigestible macromolecular material that contains high amounts of lignin. The saccharification (conversion into simple sugars) of lignocellulose therefore requires energy demanding pretreatments and high enzyme inputs, making bioethanol production a costly process (4). Improving the ease and yield of cell-wall saccharification could provide a way of reducing these costs.

One approach to increasing saccharification is to produce crops with cell walls that are more susceptible to hydrolysis, and a range of studies have been carried out to investigate the impact of altering cell-wall components on saccharification. Most of these studies have focused on lignin, which is generally considered to be a major determinant of cell-wall digestibility due to the coating of cell polysaccharides with this complex and insoluble polymer (5). It has been shown that altering both lignin content (6–11) and lignin structure (12, 13) in the cell wall can affect saccharification. However, it has also been shown that alterations in cell-wall components other than lignin can affect biomass digestibility. For example, Penning et al. have recently shown that quantitative trait loci (QTLs) for lignin abundance are independent of those for saccharification in a maize recombinant inbred population (14). In particular, altering cellulose production, deposition, or crystallinity can affect saccharification (15–17) because cellulose typically constitutes around one third of the total mass of plants and the insoluble crystalline cellulose fibers are hard to digest (18). Alterations in matrix polysaccharide content and composition can also affect saccharification (19, 20) by changing the extractability and/or architecture of the cell wall. Furthermore, in grasses, matrix polysaccharides are able to cross-link to lignin and each other via ferulic acid esters (21), and a reduction in these linkages can increase saccharification (22, 23).

The majority of studies investigating the determinants of lignocellulose recalcitrance have taken a reverse genetics approach, disrupting genes that play key roles in cell-wall biosynthesis or expressing genes that encode wall-modifying enzymes. However, the complexity of plant cell-wall biosynthesis and the large number of genes involved, many of which are unknown, mean it is

Significance

Bioethanol produced from waste biomass from crops has the potential to provide a sustainable alternative to petroleum-based transportation fuel that does not compete with human food supply. The main obstacle to this approach is the resistance of this biomass to digestion. Thus, expensive energetic pretreatment and high enzyme inputs are needed to increase digestion. In this study, we screened a population of randomly mutated plants for digestibility with the aim of identifying novel factors that impact on this trait. We found a number of mutants with high digestibility and no impairments in growth or fitness. These mutants show a range of alterations in cell-wall composition, and we have mapped and characterized the mutant with the highest increase in digestibility.

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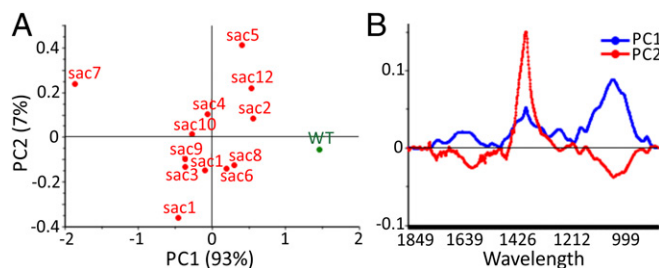


Fig. 2. FTIR analysis of WT and *sac* stems. (A) Principal component analysis (PCA) of baseline corrected and peak normalized FTIR spectra in the range 1,850–850 cm^{-1} , $n = 10$. (B) Loading plots of PC1 and PC2 from the PCA in A.

Mapping the Causal Mutations. Because such screening for saccharification mutants has not previously been reported, it is important to assess whether the approach is sufficiently robust to allow the responsible gene loci to be identified. We used bulk segregant analysis approach to examine the cosegregation of phenotype and genetic lesions in segregating F_2 backcrossed populations (BCF_2) of the putative mutants (36, 37). For the purpose of this study, we report mapping of the *sac1* mutant because it showed the largest increase in saccharification. Saccharification analysis of the BCF_2 population, along with WT, *sac1*, and BCF_1 plants, revealed that the BCF_1 plants had an intermediate level of saccharification between WT and *sac1* (Fig. 4), suggesting incomplete dominance of the WT allele at the position of the causal mutation. Using the lowest saccharification value of the *sac1* mutant plants and the highest saccharification value of the WT plants as threshold values, we calculated the observed percentages of BCF_2 plants with saccharification values resembling plants homozygous WT, heterozygous and homozygous mutant for the causal mutation. This analysis showed that 25.31% of the BCF_2 plants showed a saccharification value comparable with homozygous WT plants whereas 54.77% showed values comparable with heterozygous plants and 19.92% showed values comparable with homozygous *sac1* plants (Fig. 4). These values were not significantly different from the 1:2:1 ratio expected for a single segregating locus (χ^2 , $P = 0.47$).

DNA from the top 40 high-saccharification BCF_2 plants was extracted and pooled for whole-genome sequencing. After alignment of the sequence data to the reference Brachypodium genome, SNPs were called and filtered to retain only mutations that did not occur in the WT, mutations with high quality scores, and mutations resembling those induced by sodium azide (38). The allele frequency of each SNP (i.e., the percentage of reads for the alternative allele) was plotted for each chromosome (Fig. 5), revealing a single peak of high allele frequencies on chromosome 2, with a cluster of 14 SNPs, each with an allele frequency of ≥ 0.9 . The peak spanned 2.6 Mbp and covered 362 annotated genes. Within the 14-SNP cluster, 10 SNPs were in noncoding regions and, of the 4 remaining, 3 were in exons and only 2 would cause a change in the encoded amino acid sequence (Table S3). One SNP caused a glycine-to-serine substitution in Bradi2g01480, predicted to encode a glycosyltransferase family 61 enzyme. The other SNP caused a change from proline to leucine in Bradi2g02520, which encodes a protein of unknown function. Sorts intolerant from tolerant (SIFT) analysis, which provides a score for how likely a mutation is to affect the function of a gene based on alignment of the protein to similar proteins (39), was performed for these two mutations. This analysis suggested that the SNP in the GT61 protein would disrupt structure and function drastically whereas the SNP in Bradi2g02520 would have little impact. It is possible that the whole-genome sequencing has missed SNPs within this region; however, given the high coverage of the sequencing, this seems unlikely. The predicted effect of the GT61 mutation on amino acid sequence and structure/function of the protein compared with the other SNPs identified within the region, makes the GT61 mutation the most likely candidate to be

responsible for the observed phenotype, and we decided to explore this hypothesis further.

Phylogenetic analysis of the GT61 family in various monocot and dicot plants has shown the Bradi2g01480 protein to be part of grass-specific clade C.III (40). Clade C is highly expanded in grasses and contains rice and wheat xylan arabinosyltransferases (XATs) and XYLOSYL ARABINOSYL SUBSTITUTION OF XYLAN (XAX) in rice (40, 41), both involved in arabinosyl substitution. Chiniquy et al. (40) recently showed that a *xax1* mutant in rice exhibits a large reduction in ferulic acid content and an increase in saccharification. Interestingly, the *sac1* mutant shows a very similar phenotype to that of the rice *xax1* mutant, making the SNP in Bradi2g01480 a likely candidate for the causal mutation. The rice *xax1* mutant is reported to show a minor 2.6% reduction in matrix polysaccharide xylose content when assessed by 2 M TFA hydrolysis of alcohol insoluble residue (AIR). In our analysis using this method, there was no significant difference in xylose content of *sac1* AIR (Table S2). However, serial extractions of *sac1* AIR using 1,2-cyclohexanedinitrilotetraacetic acid (CDTA) and Na_2CO_4 to remove pectinaceous fractions, followed by extraction with 4 M KOH, were used to produce a xylan-enriched fraction. Compositional analysis of this fraction revealed a significantly lower level of xylose in *sac1* compared with WT ($P = 0.03$, t test) (Fig. 6), lending further support to the hypothesis that the *sac1* phenotype results from the mutation in a XAX1 ortholog in Brachypodium.

Discussion

Brachypodium was chosen for this study because its small genome size, short life cycle, and simple diploid genetics make it practical for carrying out mutant screens (42). In addition, Brachypodium's fully sequenced genome has significant colinearity with those of major cereal crops, and the species is also seen as a good model for biomass grasses (43). The notable differences in wall composition between monocots and dicots (3) make *A. thaliana* a less suitable model for the cereal straw and grasses that will form much of the biomass for biofuel applications. In this study, we screened a population of chemically mutagenized Brachypodium plants and identified 12 putative mutant lines that exhibited heritable improvements in saccharification with industrial polysaccharide-degrading enzyme mixtures. We analyzed a range of cell-wall and growth characteristics of these lines to get a better understanding of the mutant phenotypes. It is apparent from the cell-wall composition analysis (Fig. 3) that the increased saccharification phenotype is accompanied by a range of different cell-wall modifications among the *sac* lines.

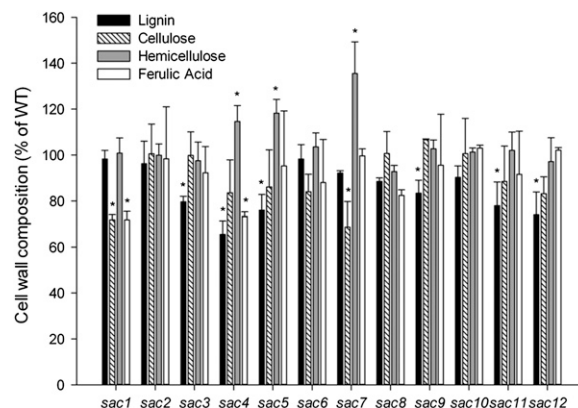


Fig. 3. Lignin, cellulose, matrix polysaccharide, and ferulic acid content of stem AIR of the 12 *sac* lines, expressed as percentage of WT. Data represent mean \pm SD and $n = 3$. Asterisk indicates a significant difference ($P \leq 0.05$) compared with WT.

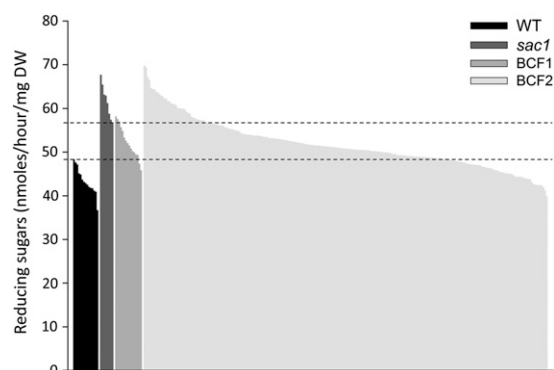


Fig. 4. Saccharification analysis to determine homozygous mutants of a BCF₂ population from a backcross between WT and *sac1*. Level of saccharification of WT and *sac1* plants, BCF₁ plants, and the BCF₂ population resulting from a self of one of the BCF₁ plants is presented. Dotted lines show thresholds for the lowest *sac1* saccharification and highest WT saccharification.

Lines with Altered Lignin Content. It is widely accepted that lignin plays a major role in the recalcitrance of lignocellulose to digestion, and many studies have shown that a reduction in lignin content results in increased saccharification [reviewed in Li et al. (44)]. In line with these studies, six of the *sac* lines (*sac3*, -4, -5, -9, -11, and -12) showed significantly lower levels of lignin than WT. In three lines (*sac3*, *sac11*, and *sac12*), lignin content was the only cell-wall component to be altered significantly from WT. Although the work presented here confirms a major role for lignin in determining saccharification, it is notable that a number of the *sac* lines, including the two with highest saccharification (*sac1* and *sac2*), showed no significant differences in lignin content compared with WT, suggesting that additional factors can be important for saccharification efficiency.

Lines with Reduced Lignin and Alterations in Other Cell-Wall Components. In the *sac4*, -5, and -9 lines, the reduced lignin content was accompanied by alterations in other cell-wall components. Both *sac4* and *sac5* showed significant increases in matrix polysaccharides. Such increases in these polysaccharides have been reported in other plants with altered lignin content (7, 45) and could be a compensatory effect to make up for any loss in cell-wall strength caused by the reduction in lignin. The increase in matrix polysaccharides might also contribute to the increased saccharification of these lines because our saccharification assay does not distinguish between the breakdown of cellulose and matrix polysaccharides.

Lines with Alterations in Cell-Wall Components Other than Lignin. A number of the *sac* lines showed little or no change in lignin content but exhibited alterations in other wall components. Among these lines, *sac1* showed the largest increase in saccharification. One of the features of *sac1* cell walls was a significant decrease in crystalline cellulose content compared with WT, which was also observed in *sac7*. Highly crystalline cellulose is recalcitrant to digestion whereas the amorphous regions and matrix polysaccharides released by TFA treatment as “matrix polysaccharides” are easier to digest. The majority of glucose released by the TFA treatment in *Brachypodium* will be derived from amorphous cellulose because grass secondary cell walls contain only minor amounts of xyloglucan and mixed linkage glucan (MLG) (3). The *sac7* plants showed a significant increase in glucose released by TFA treatment (Table S2), which may indicate an increase in the ratio of amorphous to crystalline cellulose in this line and could contribute to the high saccharification. The reduction in crystalline cellulose in *sac7* was accompanied by a significant increase in matrix polysaccharides, characterized by significant increases in arabinose, mannose, and especially xylose, which suggests an increase in arabinoxylan and mannan content. Interestingly, the differences in matrix polysaccharide composition revealed by TFA extraction followed

by HPAEC were not apparent in the immunocarbohydrate microarray analysis (Fig. S1). The antibodies used for this analysis recognize relatively specific epitopes, and the binding efficiency of the LM11 antibody, which recognizes arabinoxylans, is affected by the amount of arabinose substitution and does not bind well to sorghum or maize arabinoxylan (46). The differences in matrix polysaccharide composition detected by HPAEC were not severe and so may have been too subtle for detection by antibodies.

No difference was seen in glucose content of the TFA extract between *sac1* and WT plants, which suggests a reduction in total cellulose content in this line due to the reduction in crystalline cellulose content. The *sac1* cell walls also showed a significant reduction in the amount of ester-bound ferulic acid, a feature also seen in *sac4*. In cell walls of grasses, ferulic acid esters are abundant on arabinosyl side chains of arabinoxylans (21). These feruloyl esters may be oxidatively dimerized to form cross-links between or within arabinoxylan chains and also between arabinoxylan and lignin (21). It is therefore thought that ferulic esters could play a role in the recalcitrance of plant cell walls to digestion, and a reduction in these residues has been shown to result in increased saccharification in a number of plant species (22, 23, 47). The alkaline pretreatment used in this study is likely to result in partial hydrolysis of the ferulate ester linkages within the cell wall and may therefore be more effective for the *sac1* and *sac4* lines, which have lower ferulic acid content.

Lines with Altered Lignin Structure. A number of the *sac* lines had alterations in lignin structure. In most cases, this alteration consisted of a reduction in thioacidolysis S:G ratio accompanied by an increase in % H units (*sac1*, -4, -6, and -7) whereas *sac2* and *sac9* showed just an increase in % H units. A number of studies have shown transgenic and natural accessions with reduced S:G ratio, no reduction in lignin content, and an increase in digestibility [switchgrass (13), ryegrass (48), maize (49), tall fescue (50), and *A. thaliana* (7)]. The most straightforward hypothesis for the improved saccharification of alkaline-pretreated *sac1*, -2, -4, -6, -7, and -9 lines is their higher frequency in G and/or H units. Studies have shown that about 50% of native grass lignins can be solubilized in alkali due to their high frequency in terminal units (51, 52). These terminal units are prominently H and G units whereas S units are essentially internal. Accordingly,

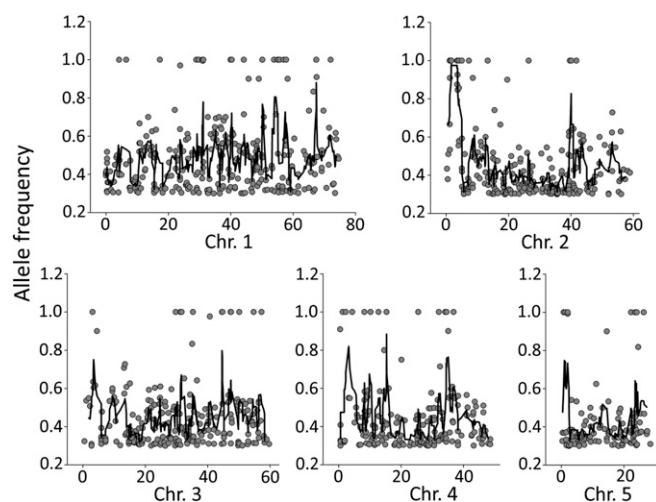


Fig. 5. Allele frequency plots for the five chromosomes of the *Brachypodium sac1* mutant. A *sac1* plant was backcrossed to WT, and DNA from the homozygous mutants of the resulting BCF₂ population was pooled and whole genome-sequenced. SNPs with an allele frequency above 0.3 were called and plotted against position in the chromosome. Gray circles indicate SNP positions on the chromosome and their allele frequencies. The black line shows a five point moving average of the SNP allele frequencies.

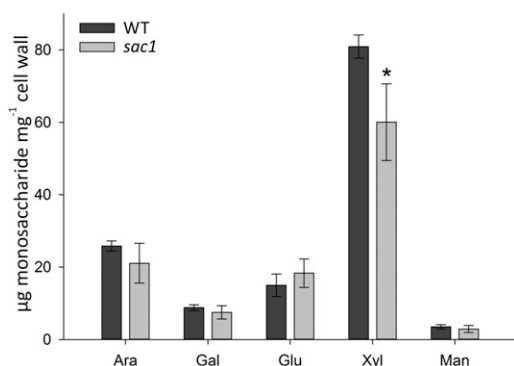


Fig. 6. Monosaccharide composition of a xylan-enriched fraction from WT and *sac1* plants. A xylan-enriched fraction was extracted from AIR using 4 M KOH after pectin removal. Monosaccharides were quantified by hydrolyzing with 2 M TFA, followed by separation and quantification by HPAEC. Data represent mean \pm SD and $n = 3$. Asterisk indicates a significant difference ($P \leq 0.05$) compared with WT. Ara, arabinose; Gal, galactose; Glu, glucose; Man, mannose; Xyl, xylose.

increasing the frequency of H and G units in grass lignin makes the lignin polymers more susceptible to alkaline pretreatment.

Mapping One of the Responsible Mutations. To verify that our screening approach can provide information on genes involved in the digestibility of plant biomass, we have mapped the responsible mutation of the *sac1* line to a narrow genomic region. The most likely responsible mutation within this region is an SNP in a gene encoding a glycosyltransferase, family 61 (Bradi2g01480). GT61 proteins have been implicated previously in arabinoxylan substitution due to genes encoding this family of proteins being more highly expressed and numerous in grasses than dicots (53). Furthermore, members of GT61 clade C have been shown to introduce the arabinosyl side chains onto arabinoxylans (AX) (41). The *sac1* mutant shows a reduction in ferulic acid content and reduced xylose in its xylan-enriched cell-wall fraction, characteristics also reported for the *xax1* mutant in rice (40). XAX1 is a GT61 family clade C member that has been proposed to catalyze the transfer of xylosyl residues on to arabinose side chains of AX (40). The authors hypothesized that the observed reduction in ferulic acid content in the *xax1* mutant is due to loss of xylose substitutions that are required for the feruloylation of neighboring arabinosyl residues, resulting in decreased activity of the feruloyltransferase enzyme(s) (40). This hypothesis remains to be experimentally validated, and the authors (40) alternatively suggested that the xylosyl residues might render the arabinose-bound ferulic acid less susceptible to feruloyl esterases; reductions in xylosyl substitution may therefore lead to increased rates of loss of ferulic acid esters in the *xax1* plants. We propose that the Brachypodium Bradi2g01480 gene encodes an enzyme with a similar function to XAX1 in rice, due to the similar reductions in xylose and ferulic acid content in the mutants, and the grouping of these enzymes together in clade C of the GT61 family by phylogenetic analysis. We believe that the increase in saccharification in this mutant is primarily due to the reduction in ferulic acid content.

The work described in this study has implications for breeding strategies of biofuel crops because it indicates that a number of different routes can be adopted to increase saccharification and potentially thereby lower processing costs. This finding raises the possibility of combining more than one such modification in a plant to obtain even higher saccharification. Importantly, our screen has enabled the identification of plants with increased digestibility but with no major impairment in growth, development, or stem strength. Indeed, a number of the *sac* lines showed increased height, biomass, and/or seed production under glasshouse conditions, raising the possibility of eventually producing crops with both improved yield and processability. Although future work will target the identification and confirmation of responsible genes and translational studies in

crops grown under field conditions, this study gives a positive indication of what could be achieved in this area to improve the sustainability and efficiency of lignocellulosic biofuel production.

Materials and Methods

Plant Growth and Material. The mutant *B. distachyon* population consisted of 2,400 M₂ plants in the Bd21 background that had been chemically mutagenized with sodium azide according to the protocol described in ref. 54. Details of plant growth are given in *SI Materials and Methods*. Internodes from the primary and secondary stems of mature and dried plants were used for all experiments, except where stated otherwise. All internodes excluding the first were cut into 4-mm lengths and ground using a custom-made robotic platform (Labman Automation) [see Gomez et al. (27)].

Saccharification Analysis. Ground stem material from above was weighed into four replicate 4-mg samples per plant in a 96-deep-well plate using the robotic platform mentioned above. Saccharification analysis of this plant material was performed using a liquid handling robotic platform (Tecan Evo 200; Tecan Group Ltd.) (27), as described in *SI Materials and Methods*.

Selection of High-Saccharification Putative Mutants. Plants were selected from the first-round screen if saccharification was 20% higher than WT and significant at $P \leq 0.01$ using a *t* test. To examine heritability, 12 seeds were sown from each putative mutant selected in the first screen and the plant material tested for saccharification as described above. The saccharification trait was considered to be heritable if all of the offspring showed a similar saccharification trait as observed in the first screen.

Fourier Transform Infrared Spectroscopy. Fourier transform infrared (FTIR) spectra were obtained for WT and the 12 *sac* lines for the wavelength range 850–1,850 cm^{-1} using a Spectrum One (Perkin-Elmer). Three spectra were collected for each sample, and the triplicate-averaged spectrum was used for principal component analysis (PCA). Further details are described in *SI Materials and Methods*.

Cell-Wall Composition Analysis. All cell-wall composition analyses were carried out on alcohol insoluble residue (AIR) and in biological triplicates. For details on how AIR was obtained, see *SI Materials and Methods*. Lignin content was quantified using Foster et al.'s (33) acetyl bromide method. Crystalline cellulose content was analyzed using Foster et al.'s (34) anthrone-sulfuric acid method. Matrix polysaccharide compositional analysis of AIR and a xylan-enriched fraction extracted from AIR was performed by hydrolyzing matrix polysaccharides into component monosaccharides and analyzing using HPAEC. The xylan-enriched fraction was extracted from AIR as described in ref. 40. Ferulic acid was extracted using 1 M NaOH and detected and quantified using HPLC. Details of all four analyses are described in *SI Materials and Methods*. Lignin composition was measured by thioacidolysis followed by analysis of the H, G, and S monomer products by GC-MS of their trimethylsilylated derivatives, as described by Lapierre et al. (55). Immunocarbohydrate microarray analysis was performed as described in Alonso-Simón et al. (56).

Stem Mechanical Properties. The strength and stiffness of *sac* and WT stems was assessed using three-point bending tests. Stem lengths of 5 cm were cut from the third internode of dried plant material, and bending tests were performed on this material using a universal testing machine. For further details, see *SI Materials and Methods*.

Mapping. The *sac1* line was backcrossed to WT, the resulting BCF₁ progeny allowed to self and 250 of the BCF₂ seeds sown. The BCF₂ plants were subject to saccharification analysis as described in *SI Materials and Methods*. DNA from the top 40 BCF₂ plants with the highest saccharification was extracted, pooled, and sent for whole-genome sequencing by The Genome Analysis Centre (TGAC). Homo- and heterozygous SNPs were called and filtered to exclude low-quality SNPs. For further details on the mapping process, see *SI Materials and Methods*.

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