

Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CESA1^{A903V} and CESA3^{T942I} of cellulose synthase

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The mechanisms underlying the biosynthesis of cellulose in plants are complex and still poorly understood. A central question concerns the mechanism of microfibril structure and how this is linked to the catalytic polymerization action of cellulose synthase (CESA). Furthermore, it remains unclear whether modification of cellulose microfibril structure can be achieved genetically, which could be transformative in a bio-based economy. To explore these processes *in planta*, we developed a chemical genetic toolbox of pharmacological inhibitors and corresponding resistance-conferring point mutations in the C-terminal transmembrane domain region of CESA1^{A903V} and CESA3^{T942I} in *Arabidopsis thaliana*. Using ¹³C solid-state nuclear magnetic resonance spectroscopy and X-ray diffraction, we show that the cellulose microfibrils displayed reduced width and an additional cellulose C4 peak indicative of a degree of crystallinity that is intermediate between the surface and interior glucans of wild type, suggesting a difference in glucan chain association during microfibril formation. Consistent with measurements of lower microfibril crystallinity, cellulose extracts from mutated CESA1^{A903V} and CESA3^{T942I} displayed greater saccharification efficiency than wild type. Using live-cell imaging to track fluorescently labeled CESA, we found that these mutants show increased CESA velocities in the plasma membrane, an indication of increased polymerization rate. Collectively, these data suggest that CESA1^{A903V} and CESA3^{T942I} have modified microfibril structure in terms of crystallinity and suggest that in plants, as in bacteria, crystallization biophysically limits polymerization.

cell wall | polysaccharide | quinoxiphen

The fundamental unit comprising naturally produced cellulose is the *para*-crystalline microfibril, which consists of multiple parallel chains of unbranched β -1,4-glucan that aggregate via hydrogen bonds and van der Waals forces. Cellulose-synthesizing organisms typically produce microfibrils from large plasma membrane (PM)-localized cellulose-synthesizing complexes (CSCs) (1). The geometry of CSCs range from either linear arrays of particles with single rows, as seen in the bacterium *Gluconacetobacter xylinus* [formerly *Acetobacter xylinus*(*m*)], to multiple rows, as found in the green alga *Oocystis apiculata*, to hexagonal arrays of particles termed rosettes that occur in Charophycean algae and all land plants (2). The arrangement of particles within the CSCs of these and other organisms has been correlated with the size and shape of the cellulose microfibrils that they produce (2). A detailed analysis of microfibril structure has revealed the existence of two crystalline allomorphs of naturally occurring cellulose, cellulose I α (triclinic unit cell) and I β (monoclinic unit cell) (3). The elucidation of the crystal structure of both forms has helped determine the hydrogen-bonding network within and between glucan chains as well as between sheets of chains so

formed (4, 5). Analysis of higher plant cellulose suggests not only the presence of large numbers of ordered and disordered crystal-surface chains (6), but that the inner chains of the microfibril core are more crystalline, while more amorphous forms of cellulose compose the chains in the surrounding *para*-crystalline sheath (7). These analyses have raised the question that if the geometry of the CSC determines the lateral dimensions of the microfibril (8), then does it also determine microfibril structure or is this feature instead dictated by glucan chain self-assembly?

Pioneering studies with the bacterium *A. xylinum* have shown that the fluorescent dye Calcofluor disrupts the crystallization of cellulose by hydrogen bonding to individual glucan chains immediately upon their emergence from the cell, thereby preventing their assembly into a microfibril (9). The disruption of microfibril assembly caused an increase in the rate of glucan chain polymerization, suggesting that the two processes are biophysically coupled and that crystallization is rate limiting (10). However, during plant cellulose biosynthesis, it remains unclear whether a link exists between cellulose crystallization and the CSC function in terms of polymerization. This is in part due to a lack of low-crystallinity cellulose mutants available to explore this postulate. Moreover, in plant cells as opposed to bacteria, the requirement for cellulose production is not dispensable and is linked to the anisotropic expansion of the cell and assaying polymerization after pharmacological decrystallization is complicated by the inherent fluorescence of the β -glucan binding dye Calcofluor (11).

In *Arabidopsis*, 10 *CESA* genes have been identified (12). A combination of biochemical and genetic analyses has shown that at least three cellulose synthase (*CESA*) subunits directly interact to form a CSC during primary (13, 14) and secondary (15) cell wall cellulose formation. The temperature-sensitive mutation (or homozygous allele) *rsw1-1* (*CESA1*) results in primary wall deficiencies accompanied by a disappearance of rosettes from the membrane at the nonpermissive temperature (16), and strong homozygous alleles are either male gametophyte or embryo lethal (14, 17). *CESA3* is coexpressed with *CESA1*, and null homozygous *cesa3* alleles are also male gametophyte lethal (14). The remaining

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primary cell wall *CESA* genes display a degree of redundancy (13, 14). In addition to *CESA*, several other genes have been linked to a role in cellulose biosynthesis by null alleles resulting in cellulose deficiency (18). Recent studies with *CESA* fused to fluorescent reporter proteins suggest that CSCs move at relatively stable trajectories at the PM via a microtubule (MT) guidance mechanism (19). However, when MTs are completely depolymerized, relative *CESA* velocity remains unchanged in otherwise wild-type (WT) plants, suggesting that energy provided by glucan chain polymerization is primarily responsible for movement rather than MT motor proteins (19, 20). The effect that genetic mutations have on cellulose polymerization rates can therefore be explored by examining the displacement velocity of fluorescently labeled *CESA* at the PM. Although previous analyses of the *CESA* velocity in cellulose deficient or MT mutants have suggested that polymerization rate can be altered, these studies have not addressed the influence that microfibril structure could impart on *CESA* velocity (21–24).

Here, we identified a mutant, *CESA1*^{A903V} (*cesa1*^{aegeus}) by forward chemical genetics and created a double mutant with the previously identified *CESA3*^{T942I} (*cesa3*^{ixr1-2}) (25). X-ray diffraction (XRD) and ¹³C solid-state nuclear magnetic resonance (SSNMR) spectroscopy are employed to show that *cesa1*^{aegeus} and *cesa3*^{ixr1-2} confer changes to the ordered crystallization of glucan chains in the interior of cellulose microfibrils. Lower crystallinity cellulose displays greater digestibility characteristics relative to WT and reveals a consistent coupled association between crystallization and polymerization that is conserved between bacterial (10) and plant cellulose biosynthetic processes.

Results

Identification and Characterization of the Cellulose Biosynthesis Inhibitor Quinoxiphen. The herbicide quinoxiphen (Fig. 1A; Fig. S1) was identified in a screen for compounds that inhibit cell growth anisotropy. Germination on quinoxiphen above the IC₅₀ value of 1.0 μM caused severe cell and tissue swelling of the hypocotyl and upper root in *Arabidopsis* seedlings (Fig. 1B). In addition, there was a 50% inhibition of ¹⁴C-glucose incorporation into crystalline cellulose in plants germinated and grown on a 5.0 μM concentration of quinoxiphen (Fig. 1C). Differences in cell wall composition based on FTIR spectra were detected in quinoxiphen treated seedlings (Fig. S2A and B), as well as significant increases in the abundance of cell wall neutral sugars (Fig. S2C). Additional analysis of seedlings exposed to quinoxiphen showed other cell wall compositional alterations, including hyper-accumulation of callose and ectopic lignin production; both common phenotypes observed following chemical inhibition of cellulose biosynthesis (25, 26) (Fig. S3A–D).

In expanding *Arabidopsis* hypocotyl tissue, cellulose biosynthesis can be visualized in living cells by labeling the CSC with fluorescently tagged *CESA* proteins. To establish a possible cause of the anisotropic growth disruption and cellulose content reduction, we used spinning disk confocal microscopy to examine the effect of quinoxiphen on the behavior of YFP-*CESA6* particles in the focal plane of the PM. Consistent with previous observations, following treatment with the herbicide isoxaben (19), CSCs were depleted from the PM, a change visualized in time-projected images by the loss of tracks created by particle translocation (Fig. 1D). Concurrently, label accumulated in trafficking compartments at the cell cortex, most of which were immobilized (Fig. 1D, see ref. 27 for further analysis of this redistribution syndrome). In contrast, no marked differences in morphology or general dynamics were observed in actin or MT cytoskeletons after quinoxiphen treatment (Fig. S4). Hence, we hypothesized that quinoxiphen targets cellulose biosynthesis, rather than causing broad cell toxicity, and that its mechanism of action is similar to that of isoxaben.

Resistance to Quinoxiphen Is Conferred by a Semidominant *CESA1* Allele. To identify possible targets of quinoxiphen, we performed

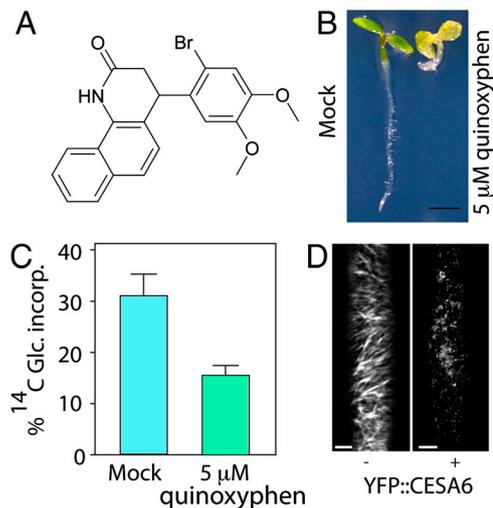


Fig. 1. Identification of quinoxiphen as a cellulose biosynthesis inhibitor. (A) Structure of 4-(2-bromo-4,5-dimethoxy-phenyl)-3,4-dihydro-1H-benzoquinolin-2-one (C₂₁H₁₈BrNO₃ MW 412.28), named herein quinoxiphen. (B) *Arabidopsis* seedlings grown under continuous light for 5 d on MS agar containing 5 μM quinoxiphen (Right) showed reduce growth compared with control plant (Left). Scale bar, 10 mm. (C) Incorporation of ¹⁴C-glucose in the acid-insoluble fraction in WT treated and untreated with 5.0 μM quinoxiphen. (**P* < 0.001, Student's *t* test, *n* = 3 with 200 seedling per replicate). (D) Time-lapse confocal images of YFP::*CESA6* in hypocotyl cells of 3-d-old etiolated plants were compared between mock control treatment and 20 μM quinoxiphen treatment revealing clearance of the PM CSCs after 120 min. Each image in D is an average of 60 frames taken at 5-s intervals on the same Z plane (*n* = 3). Scale bar, 10 μm.

a forward genetic screen and isolated an *Arabidopsis* mutant showing strong resistance to quinoxiphen (>10 mM), which we named *aegeus* (Fig. 2A). Given the similar phenotypes induced by quinoxiphen and isoxaben, as observed with live-cell imaging, we tested whether the two drugs shared the same resistance locus. In an allelism test between *aegeus* and the isoxaben resistant mutant *ixr1-2*, the F1 progeny from crosses between the two homozygotes showed sensitivity when germinated and grown in media containing 5 μM of quinoxiphen or 10 nM of isoxaben (both lethal doses to WT), indicating that the two resistance loci were not allelic. In addition, homozygous *aegeus* plants displayed sensitivity to isoxaben, and homozygous *ixr1-2* plants were sensitive to quinoxiphen. Using a positional cloning approach to identify the gene conferring resistance to quinoxiphen, we found that the *aegeus* mutation corresponds to the replacement of an alanine with a valine in the fourth transmembrane spanning domain at amino acid position 903 in the C-terminal end of the *CESA1* protein (Fig. 2B and C). The mutated alanine is not highly conserved among primary cell wall *CESAs*, but is flanked by several highly conserved residues (Fig. 2C). The *aegeus* mutant displayed semidominant inheritance similar to that observed for isoxaben resistance in the *ixr* mutants (25, 26). Both *cesa1*^{aegeus} and *cesa3*^{ixr1-2} occur within the C-terminal region of *CESA* proteins comprising six transmembrane domains, and both occur in essential genes for cellulose biosynthesis in the primary wall (Fig. 2B (14, 16). Complementation of *cesa1*^{aegeus} by transformation with WT *CESA1* restored sensitivity to quinoxiphen.

Generation of Double Drug-Resistant Mutants. We generated the double mutant *cesa1*^{aegeus}/*cesa3*^{ixr1-2}, thereby creating a primary cell wall CSC containing two essential *CESA* subunits with mutations conferring drug resistance. The double mutant showed resistance to both quinoxiphen and isoxaben, but also a far more pronounced dwarf phenotype than either of the single mutants (Fig. S2D–F). Significant differences were also apparent during

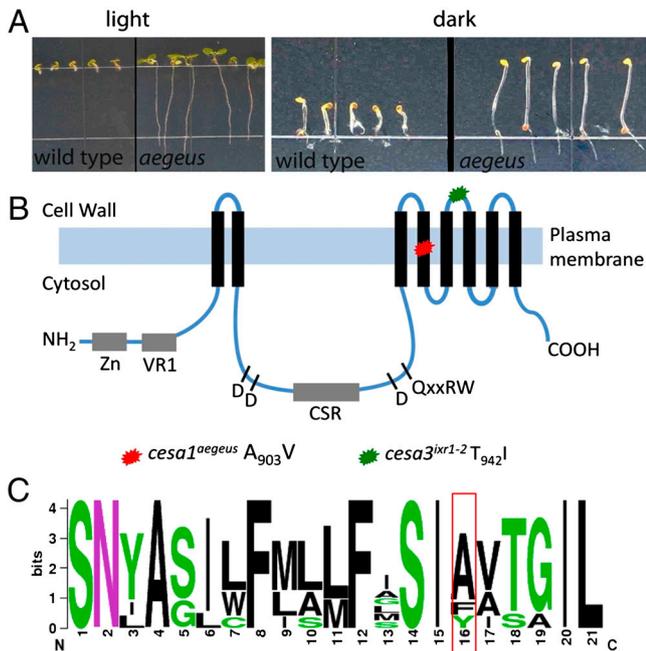


Fig. 2. The *aegeus* mutant is resistant to quinoxiphen. (A) The phenotype of the *aegeus* mutant compared to WT when grown for 7 d in the light (Left) or the dark (Right) on media containing 5 μ M quinoxiphen ($n = 5$). (B) The gene corresponding to the *aegeus* mutation was cloned using a map-based approach (Fig. S3 and Table S2) and revealed an alanine to valine change at position 903 in the AtCESA1 protein (At4g3241). (C) Sequence logo assessment of residues in the fourth transmembrane domain of primary cell wall CESA proteins illustrates the location and conservation of the mutated alanine residue in CESA1 (red box). Amino acids are colored according to their chemical properties: Polar amino acids are green, basic are blue, acidic are red, and hydrophobic are black.

the first 7 d of dark-grown hypocotyl elongation (Fig. S2F and Fig. S5b) and in light-grown root growth (Fig. S5A and C). Examination of crystalline cellulose content of mature stem and dark-grown hypocotyls revealed a significant reduction in all mutants compared with WT (Table 1).

To dissect the structure of the cell wall in these mutants, we combined neutral sugar analysis, glycosidic linkage analysis (Tables S1 and S2) and 13 C SSNMR (Fig. 3). Neutral sugar composition analysis revealed significant differences in neutral sugars as a proportion of total cell wall (Table S1). In addition, although there was little to no hyper-accumulation of callose in the mutant hypocotyls (Fig. S3E–H), similar to quinoxiphen treatment, the double mutant *cesa1^{aegeus}/cesa3^{ixr1-2}* showed increased ectopic lignin production (Fig. S3I–L). Quantitative 13 C SSNMR spectra of uniformly 13 C labeled cell walls indicated increased intensities

Table 1. Effect of mutation on cellulose content and crystallite size

Genotype	Cellulose content, μ g/mg AIR			Crystallite size, $\text{\AA} \pm$ SE
	Hypocotyls	Stems		
WT	415 \pm 20.8	217 \pm 6.5		23.4 \pm 0.26
<i>cesa1^{aegeus}</i>	249 \pm 13.4*	146 \pm 7.2*		21.9 \pm 0.35*
<i>cesa3^{ixr1-2}</i>	297 \pm 6.8*	150 \pm 13.7*		22.0 \pm 0.48*
<i>cesa1^{aegeus}/cesa3^{ixr1-2}</i>	161 \pm 6.3*	140 \pm 12.0*		22.5* \pm 0.34*

Asterisks in cellulose measurements of dark-grown hypocotyls and mature stems indicate $P < 0.001$ Student's t test, $n = 3$. Cellulose content presented as μ g cellulose per mg acetone insoluble residue (AIR). Mutant and WT plants were grown to maturity, and the first internode of the stem was exposed to synchrotron X-ray analysis to measure crystallite size (lateral dimensionality). Asterisks indicate $P < 0.01$ Student's t test, $n = 4$.

of pectin and hemicellulose peaks relative to the interior cellulose C4 peak at 89 ppm for all mutants (Fig. 3A and B). For example, the arabinan (Ara) C1 signal at 108 ppm, the galacturonic acid (GalA) and rhamnose (Rha) C1 signal at 101 ppm, and the C2/C4 signals of xyloglucan (XG) Glc, GalA, Rha, and the Ara signals at 80–83 ppm, increased markedly, consistent with linkage analysis (Fig. S6A and B and Table S2). The relative amounts of glycoprotein to interior cellulose also increased in the mutants by about twofold compared to WT. Hence, the *cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and *cesa1^{aegeus}/cesa3^{ixr1-2}* display quantitatively less cellulose and relative increases in pectin, hemicellulose and glycoprotein as a proportion of the cell wall.

***cesa1^{aegeus}* and *cesa3^{ixr1-2}* Mutations Cause Increased Cellulose Synthase Movement in the PM.** We examined the in vivo behavior of the CSC complexes in the *aegeus* mutant, with and without the *ixr1-2* mutation, by creating double and triple mutants in stable transgenic plants expressing fluorescent reporters, and using spinning disk laser confocal microscopy to visualize the movement of the CSCs in living cells. Bidirectional trajectories of fluorescent CSCs were visualized at the PM focal plane of dark-grown transgenic seedlings in all mutant combinations (epidermal cells in the upper hypocotyl; Fig. 4A and Fig. S4). The relative mean velocity of labeled CESA complexes was significantly greater in *cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and the double mutant than in transgenic lines that carried WT alleles of *CESA1* and *CESA3* ($P < 0.0001$, ANOVA with Bonferroni tests) (Fig. 4A). Previously, increased polymerization rates were observed in studies of bacterial cellulose synthesis, where the pharmacological disruption of crystallization prevented microfibril assembly and caused an increased polymerization rate (9, 10). Based on the difference in CESA behavior (Fig. 4A), and prior evidence from bacteria, we postulated that cellulose microfibril structure may be aberrant in *cesa1^{aegeus}* and *cesa3^{ixr1-2}* mutants, and may reflect differences in cellulose crystallization.

***cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and *cesa1^{aegeus}/cesa3^{ixr1-2}* Mutants Produce Structurally Aberrant Cellulose Microfibrils.** As an initial experiment, we measured the relative crystallinity index (RCI) for each sample using XRD based on the Segal method (28). This assay is incapable of determining any accurate structural information for the cellulose crystallites and instead reflects the proportion of crystalline to amorphous cell wall components (29, 30). *cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and *cesa1^{aegeus}/cesa3^{ixr1-2}* all show significantly reduced RCI values compared to WT (Fig. 4B). However, as indicated by the 13 C SSNMR analysis, the hemicellulose fraction relative to interior cellulose also increased in the mutants, which will contribute to the amorphous peak present in the XRD data and skew the RCI determination toward lower crystallinity. Therefore, to obtain a more accurate estimation of the microfibril structure, we examined the first internode of mature stem using high-energy synchrotron radiation XRD and obtained an estimation of the crystallite width using the Scherrer equation (31). This method allows the examination of intact plant samples, and so disruptive grinding or chemical treatments are unnecessary. Synchrotron XRD data indicated a significant reduction in the crystallite width for *cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and *cesa1^{aegeus}/cesa3^{ixr1-2}* compared to WT (Table 1). However, within biomass samples the use of 13 C SSNMR is required to delineate accurate structural information about cellulose (32, 33).

Further analysis of the actual crystallite structure was obtained from quantitative 13 C SSNMR spectra of uniformly 13 C-labeled cell wall (Fig. 3A). Based on the intensity ratio of interior C4 (iC4) (88.5–91.5 ppm) to surface C4 (sC4) (84–86 ppm) peak intensities, we found that the double mutant had 8% lower crystallinity relative to WT (Table S3), while the single mutants had smaller differences from the WT. The mutants also displayed additional intensities in the regions of the iC4 peak, with a new

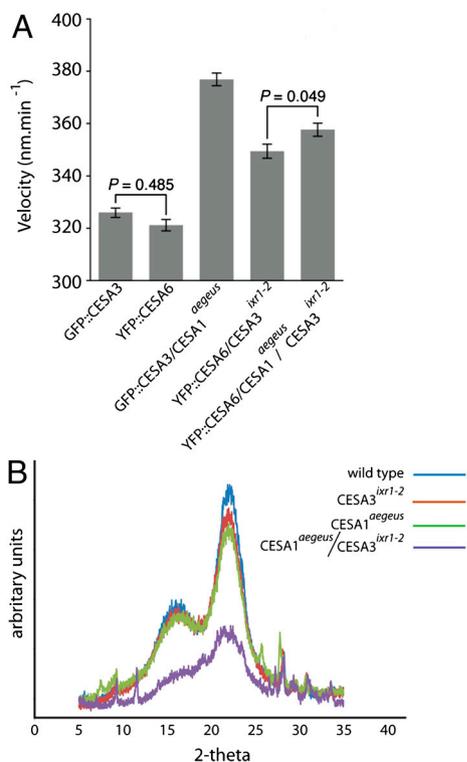


Fig. 4. CESA1^{aegeus} and CESA3^{ixr1-2} mutants display increased CESA particle velocity at the PM focal plane and altered cellulose structure. (A) Epidermal cells of 3-d-old seedling expressing translational fusion reporters were imaged in the upper hypocotyl with spinning disk confocal microscopy (72 frames). CESA complex velocities from the indicated genotypes were compared using ANOVA with Bonferroni tests and significant difference indicated at $P < 0.0001$. Each result represents ≥ 720 CESA complexes from ≥ 10 cells from unique plants. Error bars represent SEM. The mean velocities, from left to right, were 377 ± 2.4 , 349 ± 2.7 , 358 ± 2.5 , 326 ± 1.8 , and 321 ± 2.2 . (B) Analysis of *cesa1^{aegeus}*, *cesa3^{ixr1-2}*, and *cesa1^{aegeus}/cesa3^{ixr1-2}* structural changes in the cellulose fingerprint compared between mutant plants and WT were initially screened by wide-angle XRD using Bragg-Brentano reflective geometries to obtain an RCI: WT = 73.1 ± 1.1 , **cesA3^{ixr1-2}* = 68.7 ± 1.4 , **cesA1^{aegeus}* = 68.4 ± 2.0 , **cesA1^{aegeus}/cesA3^{ixr1-2}* = 65.4 ± 1.8 (* $P < 0.01$ Student's *t* test, $n = 3$).

the indirect (via enhanced rate of polymerization) stimulatory effect of reduced crystallization. Collectively, data from plants appear consistent with bacterial cellulose synthesis (9, 10) and illustrate a biophysical link between crystallinity and polymerization.

The identification of quinoxphen represents a unique cellulose biosynthesis inhibitor (CBI) (Fig. 1 B and C) that induces rapid clearance of YFP::CESA6 from the PM (Fig. 1D), reminiscent of isoxaben (19). While the identification of quinoxphen represents a useful research tool, the resistance loci provide intriguing similarities and differences to isoxaben. Resistance to isoxaben was conferred by mutations in CESA3^{T942I} and CESA6^{R1064W} (26). By contrast, CESA3^{T942I} and CESA6^{R1064W} do not confer quinoxphen resistance, which was mapped to CESA1^{A903V}. Despite subunit specificity, quinoxphen and isoxaben resistance was similarly conferred by mutations in the C-terminal transmembrane spanning region. It is possible that for both CBIs the site of inhibition occurs in this region. The C-terminal transmembrane spanning region has been proposed to mediate subunit association in the CSC (38), and therefore a CBI targeting this region could inhibit CSC formation. Supporting this postulate, live-cell imaging studies revealed clearance of YFP::CESA6 from the PM after quinoxphen treatment, although resistance was conferred by CESA1^{A903V}. Precedence for the observed resistance mechanism whereby drug-binding affinity is

displaced via mutation of residues in a transmembrane spanning region has been observed in the case of hydrophobic sliding for drug resistance in HIV-type 1 protease (39).

In terms of application, if cellulose crystallinity influences the rate of enzymatic hydrolysis (34), cellulose from CESA1^{aegeus} and CESA3^{ixr1-2} (Fig. 3) should be more digestible by a cellulase cocktail. Our results supported this postulate and revealed that mutant cellulose was more readily enzymatically cleaved to individual glucan components. Hence, mutations identified herein could provide insights about how to modify cellulose synthesis *in planta* for improved bioconversion.

Materials and Methods

Chemical libraries used and screening conditions have been described in ref. 20 and supplemental on-line material. Transgenic plants expressing various fluorescent protein tagged genes or gene knockouts were developed by reciprocal crossing and have been described elsewhere (13, 19, 20).

Seedlings expressing GFP::CESA3 or YFP::CESA6 were grown in the dark for 3 d and imaged using spinning disk confocal microscopy, as described previously (26) except a DMI6000 B inverted microscope with Adaptive Focus Control (Leica) was used.

Cell Wall Structural Analysis by XRD. *Arabidopsis* material was prepared and relative crystallinity index (RCI) determined as previously described (35) with a control of synthetic crystalline cellulose (Avicel®, FMC-Biopolymer). Three experimental replicates for mutant and WT biomass samples were performed. Synchrotron X-ray scattering experiments were performed at the macromolecular crystallography beamline F1 of the Cornell High Energy Synchrotron Source. A high-flux wiggler X-ray beam with a photon energy of 13 keV was focused to a spot size of 20 microns using an X-ray focusing capillary with 4 mrad acceptance. Plant samples were mounted in a helium-filled enclosure to reduce air scattering. Due to the high intensity of the microbeam, no further processing was necessary. A Quantum 210 area detector was placed at a distance of 120 mm from the sample, and the full 2D cellulose fiber diffraction image was recorded. Data were integrated using the Fit2D software. Scherrer equation analysis was performed by fitting a Gauss peak to the 200 reflection.

Cell Wall Structural Analysis by ¹³C Magic Angle Spinning SSNMR. For ¹³C solid-state magic angle NMR plants were grown in sterile media supplemented with U-¹³C glucose and ¹⁵N NH₄Cl (100 mM), and cell walls were extracted, desalted, and examined by NMR as previously described (40). ¹³C SSNMR spectra were measured on a Bruker Avance 600 (14.1 T) spectrometer operating at resonance frequencies of 600.13 MHz for ¹H and 150.9 MHz for ¹³C. A double-resonance 4-mm magic-angle-spinning (MAS) probe was used to measure all spectra. Typical radio-frequency field strengths were 62–70 kHz for ¹H decoupling and 50 kHz for ¹³C pulses. The samples were spun under 7- to 12-kHz MAS. ¹³C chemical shifts were referenced to the ¹³CO signal of α-glycine at 176.49 ppm on the TMS scale. For quantitative ¹³C 1D experiments, the ¹³C magnetization was created by direct excitation using a 90° pulse and using a long recycle delay of 20 s. To preferentially detect mobile polysaccharides, a short recycle delay of 2 s was used in the ¹³C direct polarization experiment. ¹³C chemical shifts were assigned by reference with the recently published result (40) and were confirmed by 2D ¹³C-¹³C spin diffusion correlation experiments with a 40-ms mixing time and by 2D J-INADEQUATE experiments (40). The latter correlates ¹³C double-quantum chemical shifts with single-quantum chemical shifts, and gives relatively high spectral resolution.

Additional Details. See *SI Materials and Methods* for additional details.

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