Enhancing cellulose utilization for fuels and chemicals by genetic modification of plant cell wall architecture

Wilfred Vermerris^{1,2,3,*} and Alejandra Abril^{2,3}

¹Department of Microbiology & Cell Science

²University of Florida Genetics Institute

³Graduate Program in Plant Molecular & Cellular Biology

* Corresponding author:

Dr. Wilfred Vermerris
University of Florida
Cancer / Genetics Research Complex
2033 Mowry Road
Gainesville, FL 32610
USA
T: +1 352-273-8162
E: wev@ufl.edu

Abstract

Cellulose from plant biomass can serve as a sustainable feedstock for fuels, chemicals and polymers that are currently produced from petroleum. In order to enhance economic feasibility, the efficiency of cell wall deconstruction needs to be enhanced. With the use of genetic and biotechnological approaches cell wall composition can be modified in such a way that interactions between the major cell wall polymers – cellulose, hemicellulosic polysaccharides and lignin – are altered. Some of the resulting plants are compromised in their growth and development, but this may be caused in part by the plant's overcompensation for metabolic perturbances. In other cases novel structures have been introduced in the cell wall without negative effects. The first field studies with engineered bioenergy crops look promising, while detailed structural analyses of cellulose synthase offer new opportunities to modify cellulose itself.

1. Cellulose utilization

Cellulose is a linear polymer of β -1,4-linked D-glucopyranose molecules and the major structural component in the cell walls of terrestrial plants. At an estimated annual global production of 180 Gtons, cellulose is considered the most abundant biological polymer on Earth [1]. Cellulose has been used for millenia as a fiber for textiles (flax, cotton, jute) and paper (cotton, trees). More recent uses of cellulose include absorbent materials, insulation, fillers and membranes (reviewed by [2]). In the past decade, cellulose has also gained considerable interest as a source of glucose for the microbial production of biofuels and bio-based chemicals. To date the latter products have been produced primarily via fermentation of more readily obtainable sugars from corn starch and sugarcane, but the relatively high inputs to produce these crops, and the 'food versus fuel' debate [3,4] has shifted the focus to cellulose from the vegetative parts of crops as an abundant source of fermentable sugars.

A major challenge with the use of cellulose as a source of biofuels and bio-based chemicals is that the cell walls of plants have evolved to withstand attempts of microbes and insects to degrade them. This recalcitrance is the result of the intricate cell wall architecture, in which different polymers – cellulose, hemicellulosic polysaccharides, pectin, and lignin – interact. In order to utilize cellulose efficiently, this complex matrix needs to be disrupted so that cellulolytic enzymes can access and degrade the cellulose. A common approach is to use a thermochemical pretreatment of the biomass that disrupts the structure of the cell wall, and breaks chemical bonds between and within the constituent cell wall polymers (reviewed by [5–7]). This process is energy intensive and therefore costly, while the cellulolytic enzymes themselves also contribute substantially to the cost of the end products [8].

Modifying cell wall architecture to reduce recalcitrance is justified by the fact that current agricultural production practice limits the exposure to adverse environmental conditions. For example, agrichemicals and biotechnology can help reduce the impact of pests and pathogens. It should be noted, however, that when modified plants do not grow and

develop normally under controlled conditions, there is reason for concern over their performance in the field.

This article provides an overview of the recent developments to enhance enzymatic access to cellulose through genetic modification of cellulose itself, as well as the other major cell wall polymers.

2. Improved understanding of cellulose structure and synthesis

Cellulose is synthesized by cellulose synthases (CesA's), membrane-bound enzyme complexes that synthesize D-glucan chains from UDP-D-glucose. Cellulose synthases form rosette structures consisting of six cellulose synthase complexes (CSCs) each consisting of six catalytic CesA subunits. One rosette can thus synthesize 36 glucan chains, which aggregate in a cellulose microfibril. The individual members within a group of six CesA molecules are not identical and vary in make up between primary and secondary cell walls (reviewed in [9–11]).

Detailed structural information on CesA can clarify the role of the various domains in catalysis, and can ultimately guide attempts to engineer novel CesA units that synthesize cellulose with tailored properties. Major progress has been made in the understanding of cellulose synthase structure through X-ray diffraction studies of *Rhodobacter sphaeroides* BcsA and BcsB, that form a membrane-integrated complex responsible for cellulose synthesis and transport in this bacterium [12]. Structural analysis indicated that a glucan chain grows by a single glucose residue at a time. It was further proposed that the 180°-rotation between adjacent residues likely occurs following the addition of a new UDP-D-glucose residue to the growing glucan strand (**Figure 1**). Two studies on the structure of plant CesAs built on this model. Sethaphong et al. [13] modeled the cotton CesA protein on a BscA template, whereas Olek et al. [14] performed solution X-ray scattering on a soluble catalytic domain of rice CesA8. Due to the difference in approach, two structural models differed substantially in the relative positions of the plant-conserved region (P-CR) [15] and class-specific region (CSR) unique to plant CesAs [16].

These structural models are helpful in explaining the impact of known mutations and in determining which sites to target for mutagenesis or other modifications. A key aim is to reduce crystallinity of cellulose to improve hydrolysis, as shown for the *Arabidopsis aegaeus* and *ixr1-2* mutants [17]. Sethaphong et al. [13] described two additional *Arabidopsis* mutants that produced less crystalline cellulose, although the impact on enzymatic saccharification was not documented. Unfortunately, since all mutants with less crystalline cellulose identified so far also display reduced hypocotyl and root length, biomass production in crop plants may be compromised, as evidenced by the aberrant growth of transgenic tobacco expressing the *ixr1-2* mutant allele [18]. There is, however, evidence that cellulose crystallinity is influenced by genetic factors that do not compromise plant growth [19], so this offers an alternative route to enhancing saccharification. In the meantime, novel spectroscopic methods are expected to contribute to more accurate determination of cellulose structure *in planta* [20,21].

3. Modifying lignin

Lignin is deposited in the cell wall to create a water-impermeable layer around the cell, which provides a physical barrier to pests and pathogens, contributes to structural support, and facilitates water transport [22]. Lignin is formed from the oxidative coupling of hydroxycinnamyl alcohols and related compounds [23], which are synthesized via from phenylalanine (**Figure 2**; also explains abbreviations). Lignin fills the voids in between the cellulose and hemicellulosic polysaccharides, and this presents a major complication when the goal is to gain access to cellulose. Modifying the degree of lignification is therefore a logical approach to improving access to cellulose. Reduced lignification also enhances the efficiency of cellulolytic enzymes (reviewed by [24]).

3.1 Reduction of lignin content

Reduction in lignin content can be accomplished by the down-regulation of monolignol biosynthetic genes to limit the supply of monolignols, or by down-regulation of the genes encoding the oxidative enzymes involved in the polymerization of lignin. Van Acker et al. [25]. performed a comprehensive analysis of the impact of mutations in the genes encoding the monolignol enzymes on lignin content and lignin subunit composition in Arabidopsis, and how these changes influenced biomass conversion efficiency. They then used these data to show that lignin content was the key factor influencing saccharification yield, with or without pretreatment, consistent with earlier results obtained from transgenic alfalfa [26]. In Arabidopsis, the laccase-encoding genes *LAC4* and *LAC17* were shown to be highly expressed in stems. Analysis of mutants *lac4* and *lac17* showed a reduction in lignin content of 20% [27], confirming the long-suspected role of laccases in lignification [28]. The *lac4/lac17* double mutant contained 40% less lignin. The single and double mutations did not affect plant size and growth under non-stress conditions, but the yield of glucose from the double mutant was increased approximately three-fold compared to wild type after treatment with a commercial cellulase without pretreatment [27]. Silencing of another oxidative enzyme, a cationic cell wall peroxidase expressed during secondary growth in tobacco stems, resulted in 40-50% reduction of lignin content [29], enhancing the enzymatic saccharification of the transgenics by 50% [30]. These transgenic tobacco plants displayed morphological differences, but biomass yield was not affected.

3.2 Changes in lignin subunit composition

Lignin subunit composition, *i.e.* the proportion of H-, G-, and S-residues, can be modified by varying the supply of hydroxycinnamyl alcohol monomers through decreasing or increasing the metabolic flux through the monolignol biosynthetic pathway with the use of natural genetic variants, mutations or transgenic approaches [22,31]. While in the angiosperm dicots Arabidopsis and alfalfa lignin content appears to be the main determinant of biomass conversion efficiency [25,26], in grasses lignin subunit composition appears to be much more important, with a low S/G ratio generally most beneficial. This is evident from comparisons of an allelic series of sorghum *brown*

midrib12 mutants [32], novel sorghum *brown midrib* mutants [33], and the brachypodium *cad1* and *spaghetti1* mutants [34,35]. The same observations had been made in maize [36], transgenic switchgrass [37] and transgenic sugarcane [38]. In contrast, saccharification yields of poplar were shown to benefit from a high S/G ratio [39]. Taken together, these observations indicate that different species require different strategies to enhance biomass conversion efficiency.

The shift from S- and G- residues to H-residues can be accomplished through down-regulation of C3'H or HCT, and this improves saccharification yields in transgenic alfalfa [26]. The proportion of H-residues in the *Arabidopsis cse* mutant [40] was 30-fold higher than in the wild type. The mutant contained up to 36% less lignin and produced 40% less biomass, but the glucose yield following enzymatic saccharification of unpretreated biomass was four-fold higher than the wild type. The *Arabidopsis ref8-1* mutant lacks C3'H activity [41], resulting in lignin consisting almost exclusively of H-residues. This mutant is severely dwarfed, originally assumed to be due to the inability of pure H-lignin to support normal growth and development. The discovery of the involvement of the Mediator complex, a multi-subunit transcriptional co-regulator, in lignin biosynthesis provided evidence that global transcriptional regulation played an important role in phenylpropanoid metabolism [41]. A triple mutant *med5a/5b ref8-1* did not display the severe growth defects of the *ref8-1* mutant, contained normal amounts of lignin comprised almost entirely of H-residues, and enzymatic saccharification of its cell walls yielded twice as much glucose [43]. These observations led to the proposed involvement of the functional mediator complex in (over)reacting to metabolic perturbances by modulating gene expression, and may provide a key to understanding observed changes in gene expression in other plants in which phenylpropanoid metabolism was perturbed [27,30,44,45] (**Figure 3**).

3.3 Enzyme engineering

An alternative to modifying the flux through the monolignol biosynthetic pathway is the engineering of enzymes with altered substrate specificities. This has the potential of generating compounds not normally made by the plant, thus limiting the potential for feedback inhibition or accumulation of toxic intermediates. Mutations in the gene encoding isoeugenol *O*-methyltransferase resulted in monolignol 4-*O*-methyltransferase (MOMT) activity, and biomass from transgenic *Arabidopsis* plants expressing this MOMT transgene contained less lignin, had higher cellulose digestion efficiency, without negative effects on growth and development [46]. The basis for a similar approach in sorghum was laid by detailed structural and biochemical analyses of the catalytic mechanisms of HCT and COMT [47,48].

3.4 Modification of lignin structure

The apparent flexibility in lignin composition led to the idea of designing lignin more amenable to degradation, either through the introduction of subunits that reduce the degree of polymerization or through the incorporation of novel monomers via relatively labile bonds. *Arabidopsis* expressing a bacterial hydroxycinnamoyl CoA hydratase lyase

(HCHL) that converts hydroxycinnamoyl CoA esters to their corresponding hydroxybenzaldehydes, contained lignin with reduced molecular weight, and up to 78% higher yields of fermentable sugars following pretreatment and enzymatic saccharification, although senesced plants were shorter and produced less biomass [49]. These changes were attributed to the limited ability of hydroxybenzaldehydes (or derived acids or alcohols) to undergo oxidative coupling to monolignols, since they lack a β -carbon. Hence, the hydroxybenzaldehydes exist as terminal residues, and limit the degree of polymerization.

Evaluation of the degradability of maize primary walls that had been lignified *in vitro* with various monolignol substitutes showed that some of these substitutes were readily incorporated in the lignin and enhanced degradability [50–52]. The first successful *in planta* application of this approach was recently accomplished by the introduction of a feruloyl-CoA monolignol transferase (FMT) from *Angelica sinensis* in transgenic poplar [53]. The resulting coniferyl ferulate was incorporated in the lignin and resulted in twice the yield of glucose following enzymatic saccharification, without apparent negative impacts on the transgenic trees grown under greenhouse conditions. A compendium of possible monolignol substitutes is available in [54].

3.5 Field studies

Many of the reports describing transgenic approaches to improving biomass conversion were based on model species or crop species grown in greenhouses or growth chambers. This left unanswered the question whether these transgenic plants would perform well under natural conditions, being subjected to pathogens, insects, and drought stress. Several recent reports suggest that modified lignin composition does not automatically compromise agronomic performance. Yields of fermentable sugars in field-grown transgenic sugarcane with reduced COMT activity were up to 72% higher in the transgenics compared to controls, at the expense of a small (15%) reduction in biomass yield [55]. A 34% increase in fermentable sugars with no reduction in biomass yield was reported based on a two-year field study with transgenic switchgrass with reduced COMT activity [56].

4. Modifying hemicellulosic polysaccharides

Hemicellulosic polysaccharides represent a group of heterogeneous, amorphous, non-cellulosic polymers. The most abundant classes of hemicellulosic polysaccharides in the secondary walls of grasses, angiosperm dicots, and softwood trees (conifers) are glucuronoarabinoxylans (GAX), glucuronoxylans, and galactoglucomannans, respectively [57,58]. Hemicellulosic polysaccharides interact directly with the cellulose microfibrils and therefore affect access to cellulose by cellulolytic enzymes, as demonstrated by the effect of xylan removal on enzymatic saccharification yields in a variety of species [7,59–63]. The influence of the amount of hemicellulose on sugar release has also been documented in a genetic study using a maize population varying in cell wall composition [64,65]. In addition, the presence of *O*-acetyl groups on

hemicellulosic polysaccharides has been shown to impair enzymatic hydrolysis of cellulose as well as fermentation [61,66].

A 59% reduction in the substitution of rice glucuronoarabinoxylan (GAX) with feruloyl esters resulted in a 62% increase in the yield of glucose from enzymatic saccharification of rice biomass [67]. The change in GAX was the result of the *xax1* mutation, which prevents the substitution of xylan with xylosyl-arabinosyl residues, and consequently the substitution of arabinosyl residues with feruloyl residues. The latter are implicated in the crosslinking of neighboring GAX chains, and the phenolic moiety of ferulate can serve as a nucleation site for lignin [68]. The brachypodium *sac1* mutant has a similar cell wall and saccharification phenotype, likely because *Sac1* is the ortholog of *XAX1* [69]. Similarly, transgenic rice overexpressing the BAHD acyltransferase *OsAt10* gene, which caused a reduction in the ratio of feruloyl/*p*-coumaroyl substitution of GAX, led to enhanced saccharification yields [70].

Petersen et al. [71] engineered xylan distribution in Arabidopsis as a proof of concept that overall levels of xylans can be reduced, while maintaining structural properties of xylem vessels. This elegant strategy relied on the transformation of *irregular xylem* mutants with the respective functional copies under control of xylem-specific promoters. The resulting transgenics displayed improved growth and development relative to the mutants, and yielded up to 42% more sugars following enzymatic saccharification.

O-acetylation of hemicellulosic polysaccharides is carried out by proteins with a trichome birefringence-like domain (TBL), encoded by a large gene family (reviewed by [72]) The Arabidopsis *eskimo1* mutant [73] carries a mutation in a member of the *TBL29* gene family, which reduced the level of xylan *O*-acetylation by 60% [74], and yet caused an 8% reduction in the yield of glucose (on a mass basis). What seems to be an inconsistency in the relationship between *O*-acetylation and saccharification [61] needs to be viewed, however, in the context of a 25% reduction in crystalline cellulose content in the *eskimo1* mutant, along with other cell wall-related changes.

Conclusions

The biochemical study of CesA's has been challenging due to their membrane-bound localization, multimeric organization, and their insoluble product, but this also means that there is still ample room to explore manipulation of cellulose synthesis itself as a way to enhance cellulose production and utilization, with novel crystallographic and spectroscopic approaches offering new opportunities. Substantial progress has been made in documenting how different modifications to cell wall composition and architecture affect plant growth and development as well as cell wall recalcitrance. As these studies continue, it will be important to extend the research to actual crop plants. This is challenging due to the more limited genetic resources, restrictions on the use of GMO-technology, longer establishment and generation times, and the need for multi-year, multi-location field evaluations as is standard in the evaluation of commercial germplasm, while the long-term financial support necessary to conduct these trials is often limited. Results from recent field studies with transgenic biomass crops are

encouraging. However, since the kinds of changes necessary to enable bioenergy crops to perform at their full potential will be difficult to achieve without biotechnology, there is much work to be done to convince the general public of both the necessity to divest from fossil fuels and the need to invest in the science and technology that will make that a reality.

Acknowledgements

The authors gratefully acknowledge financial support for research on genetic improvement of bioenergy grasses from Biomass Research and Development Initiative competitive grant No. 2011–10006–30358 from the USDA National Institute of Food and Agriculture; from the U.S. Department of Energy’s Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office and sponsored by the U.S. Department of Energy’s International Affairs under award number DE-PI0000031; and from the Southeastern SunGrant Center and USDA National Institute of Food and Agriculture award No. 2010-38502-21854.

References

1. Festucci-Buselli R: **Structure, organization, and functions of cellulose synthase complexes in higher plants.** *Brazilian J Plant Physiol* 2007, **19**:1–13.
2. Ten E, Vermerris W: **Functionalized polymers from lignocellulosic biomass: state of the art.** *Polymers* 2013, **5**:600–642.
3. Chakravorty U, Hubert M-H, Nøstbakken L: **Fuel versus food.** *Annu Rev Res Econ* 2009, **1**:645–663.
4. Rosegrant MW, Msangi S: **Consensus and contention in the food-versus-fuel debate.** *Annu Rev Environ Res* 2014, **39**:1–18,24.
5. Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB: **Biomass pretreatment: fundamentals toward application.** *Biotechnol Adv* 2011, **29**:675–85.
6. Hu F, Ragauskas A: **Pretreatment and lignocellulosic chemistry.** *Bioenerg Res* 2012, **5**:1043–1066.
7. Leu S-Y, Zhu JY: **Substrate-related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding.** *Bioenerg Res* 2012, **6**:405–415.
8. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW: **The challenge of enzyme cost in the production of lignocellulosic biofuels.** *Biotechnol Bioeng* 2012, **109**:1083–7.

9. Crowell EF, Gonneau M, Stierhof Y-D, Höfte H, Vernhettes S: **Regulated trafficking of cellulose synthases.** *Curr Opin Plant Biol* 2010, **13**:700–5.
10. McFarlane HE, Döring A, Persson S: **The cell biology of cellulose synthesis.** *Annu Rev Plant Biol* 2014, **65**:69–94.
11. Kumar M, Turner S: **Plant cellulose synthesis: CESA proteins crossing kingdoms.** *Phytochemistry* 2014, doi:10.1016/j.phytochem.2014.07.009.
12. Morgan JLW, Strumillo J, Zimmer J: **Crystallographic snapshot of cellulose synthesis and membrane translocation.** *Nature* 2013, **493**:181–6.
 - The first detailed crystallographic analysis of a bacterial cellulose synthase provided novel insights in how glycosyl transferase activity and the translocation of the nascent cellulose are coupled. The 3D model that was generated formed the basis for structural models of plant Cesa's.
13. Sethaphong L, Haigler CH, Kubicki JD, Zimmer J, Bonetta D, DeBolt S, Yingling YG: **Tertiary model of a plant cellulose synthase.** *Proc Natl Acad Sci USA* 2013, **110**:7512–7.
 - First detailed 3D-model of a plant CesaA, which enabled mapping of amino acids changed by mutations that alter cellulose crystallinity.
14. Olek AT, Rayon C, Makowski L, Kim HR, Ciesielski P, Badger J, Paul LN, Ghosh S, Kihara D, Crowley M, et al.: **The structure of the catalytic domain of a plant cellulose synthase and its assembly into dimers.** *Plant Cell* 2014, **26**:2996-3009.
 - Solution X-ray scattering was used to obtain detailed structural information on the structure of a recombinant rice CesaA, providing evidence that CesaA dimers form the structural basis of rosettes.
15. Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM: **Higher plants contain homologs of the bacterial *celA* genes encoding the catalytic subunit of cellulose synthase.** *Proc Natl Acad Sci USA* 1996, **93**:12637–42.
16. Vergara CE, Carpita NC: **Beta-D-glycan synthases and the *CESA* gene family: lessons to be learned from the mixed-linkage (1→3),(1→4) beta-D-glucan synthase.** *Plant Mol Biol* 2001, **47**:145–60.
17. Harris DM, Corbin K, Wang T, Gutierrez R, Bertolo AL, Petti C, Smilgies D-M, Estevez JM, Bonetta D, Urbanowicz BR, et al.: **Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CESA1^{A903V} and CESA3^{T942I} of cellulose synthase.** *Proc Natl Acad Sci USA* 2012, **109**:4098–103.

18. Sahoo DK, Stork J, DeBolt S, Maiti IB: **Manipulating cellulose biosynthesis by expression of mutant *Arabidopsis* *prom24::CESA3*^(*ixr1-2*) gene in transgenic tobacco.** *Plant Biotechnol J* 2013, **11**:362–72.
19. Vandenbrink JP, Hilten RN, Das KC, Paterson AH, Feltus FA: **Analysis of crystallinity index and hydrolysis rates in the bioenergy crop *Sorghum bicolor*.** *Bioenerg Res* 2011, **5**:387–397.
20. Barnette AL, Lee C, Bradley LC, Schreiner EP, Park YB, Shin H, Cosgrove DJ, Park S, Kim SH: **Quantification of crystalline cellulose in lignocellulosic biomass using sum frequency generation (SFG) vibration spectroscopy and comparison with other analytical methods.** *Carbohydr Polym* 2012, **89**:802–9.
21. Park YB, Lee CM, Koo B-W, Park S, Cosgrove DJ, Kim SH: **Monitoring meso-scale ordering of cellulose in intact plant cell walls using sum frequency generation spectroscopy.** *Plant Physiol* 2013, **163**:907–13.
22. Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W: **Lignin biosynthesis and structure.** *Plant Physiol* 2010, **153**:895–905.
23. Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph S a., Christensen JH, et al.: **Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids.** *Phytochem Rev* 2004, **3**:29–60.
24. Zeng Y, Zhao S, Yang S, Ding S-Y: **Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels.** *Curr Opin Biotechnol* 2014, **27**:38–45.
25. Van Acker R, Vanholme R, Storme V, Mortimer JC, Dupree P, Boerjan W: **Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in *Arabidopsis thaliana*.** *Biotechnol Biofuels* 2013, **6**:46.
 - A comprehensive analysis of the impact of cell wall composition on biomass conversion properties based on a collection of arabidopsis mutants in which lignin biosynthesis is perturbed
26. Chen F, Dixon RA: **Lignin modification improves fermentable sugar yields for biofuel production.** *Nat Biotechnol* 2007, **25**:759–61.
27. Berthet S, Demont-Caulet N, Pollet B, Bidzinski P, Cézard L, Le Bris P, Borrega N, Hervé J, Blondet E, Balzergue S, et al.: **Disruption of *LACCASE4* and *17* results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems.** *Plant Cell* 2011, **23**:1124–37.
28. Whetten R, Sederoff R: **Lignin biosynthesis.** *Plant Cell* 1995, **7**:1001–1013.

29. Blee KA, Choi JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP: **A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification.** *Phytochemistry* 2003, **64**:163–176.
30. Kavousi B, Daudi A, Cook CM, Joseleau J-P, Ruel K, Devoto A, Bolwell GP, Blee KA: **Consequences of antisense down-regulation of a lignification-specific peroxidase on leaf and vascular tissue in tobacco lines demonstrating enhanced enzymic saccharification.** *Phytochemistry* 2010, **71**:531–42.
31. Vanholme R, Storme V, Vanholme B, Sundin L, Christensen JH, Goeminne G, Halpin C, Rohde A, Morreel K, Boerjan W: **A systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*.** *Plant Cell* 2012, **24**:3506–29.
32. Sattler SE, Palmer NA, Saballos A, Greene AM, Xin Z, Sarath G, Vermerris W, Pedersen JF: **Identification and characterization of four missense mutations in *Brown midrib 12 (Bmr12)*, the caffeic acid *O*-methyltransferase (COMT) of sorghum.** *Bioenerg Res* 2012, **5**:855–865.
33. Sattler SE, Saballos A, Xin Z, Funnell-Harris DL, Vermerris W, Pedersen JF: **Characterization of novel sorghum *brown midrib* mutants from an EMS-mutagenized population.** *G3* 2014, doi:10.1534/g3.114.014001.
34. Timpano H, Sibout R, Devaux M-F, Alvarado C, Looten R, Falourd X, Pontoire B, Martin M, Legée F, Cézard L, et al.: ***Brachypodium* cell wall mutant with enhanced saccharification potential despite increased lignin content.** *Bioenerg Res* 2014, doi:10.1007/s12155-014-9501-1.
35. Bouvier d'Yvoire M, Bouchabke-Coussa O, Voorend W, Antelme S, Cézard L, Legée F, Lebris P, Legay S, Whitehead C, McQueen-Mason SJ, et al.: **Disrupting the cinnamyl alcohol dehydrogenase 1 gene (*BdCAD1*) leads to altered lignification and improved saccharification in *Brachypodium distachyon*.** *Plant J* 2013, **73**:496–508.
36. Vermerris W, Saballos A, Ejeta G, Mosier NS, Ladisch MR, Carpita NC: **Molecular breeding to enhance ethanol production from corn and sorghum stover.** *Crop Sci* 2007, **47**:S142–153.
37. Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Chen F, Foston M, Ragauskas A, Bouton J, et al.: **Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass.** *Proc Natl Acad Sci USA* 2011, **108**:3803–8.
38. Jung JH, Fouad WM, Vermerris W, Gallo M, Altpeter F: **RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass.** *Plant Biotechnol J* 2012, **10**:1067–76.

39. Studer M, DeMartini J: **Lignin content in natural populus variants affects sugar release.** *Proc Natl Acad Sci USA* 2011, **108**:6300–5.
40. Vanholme R, Cesarino I, Rataj K, Xiao Y, Sundin L, Goeminne G, Kim H, Cross J, Morreel K, Araujo P, et al.: **Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in *Arabidopsis*.** *Science* 2013, **341**:1103–6.
 - The most recent addition to enzymes involved in phenylpropanoid metabolism. The discovery of this enzyme led to a revision of the monolignol biosynthetic pathway and explained several inconsistencies between experimental observations and the prior version of the pathway.
41. Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C: **The *Arabidopsis* REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism.** *Plant J* 2002, **30**:33–45.
42. Bonawitz ND, Soltau WL, Blatchley MR, Powers BL, Hurlock AK, Seals LA, Weng J-K, Stout J, Chapple C: **REF4 and RFR1, subunits of the transcriptional coregulatory complex mediator, are required for phenylpropanoid homeostasis in *Arabidopsis*.** *J Biol Chem* 2012, **287**:5434–45.
43. Bonawitz ND, Kim JI, Tobimatsu Y, Ciesielski PN, Anderson NA, Ximenes E, Maeda J, Ralph J, Donohoe BS, Ladisch M, et al.: **Disruption of mediator rescues the stunted growth of a lignin-deficient *Arabidopsis* mutant.** *Nature* 2014, **509**:376–80.
 - This study provided clear evidence for the involvement of transcriptional regulation in phenylpropanoid metabolism in *Arabidopsis*, and opens new perspectives on how the biosynthesis of cell wall polymers is monitored and adjusted.
44. Gallego-Giraldo L, Escamilla-Trevino L, Jackson LA, Dixon RA: **Salicylic acid mediates the reduced growth of lignin down-regulated plants.** *Proc Natl Acad Sci USA* 2011, **108**:20814–9.
45. Saballos A, Sattler SE, Sanchez E, Foster TP, Xin Z, Kang C, Pedersen JF, Vermerris W: **Brown midrib2 (*Bmr2*) encodes the major 4-coumarate:Coenzyme A ligase involved in lignin biosynthesis in sorghum (*Sorghum bicolor* (L.) moench).** *Plant J* 2012, **70**:818–30.
 - The first report on a *4CL* mutant, which enabled analysis of transcript levels of *Bmr2* itself as well as several paralogs, and which provided evidence for a sensing mechanism.
46. Zhang K, Bhuiya M-W, Pazo JR, Miao Y, Kim H, Ralph J, Liu C-J: **An engineered monolignol 4-*O*-methyltransferase depresses lignin biosynthesis**

and confers novel metabolic capability in *Arabidopsis*. *Plant Cell* 2012, **24**:3135–52.

- This study provided proof of concept that novel monolignols can be synthesized in planta via protein engineering, and that the resulting cell wall composition is more amenable to enzymatic saccharification.

47. Walker AM, Hayes RP, Youn B, Vermerris W, Sattler SE, Kang C: **Elucidation of the structure and reaction mechanism of sorghum hydroxycinnamoyltransferase and its structural relationship to other Coenzyme A-dependent transferases and synthases.** *Plant Physiol* 2013, **162**:640–51.
48. Green AR, Lewis KM, Barr JT, Jones JP, Lu F, Ralph J, Vermerris W, Sattler SE, Kang C: **Determination of the structure and catalytic mechanism of sorghum bicolor caffeic acid *O*-methyltransferase and the structural impact of three *brown midrib12* mutations.** *Plant Physiol* 2014, **165**:1440–1456.
49. Eudes A, George A, Mukerjee P, Kim JS, Pollet B, Benke PI, Yang F, Mitra P, Sun L, Cetinkol OP, et al.: **Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification.** *Plant Biotechnol J* 2012, **10**:609–20.
50. Grabber JH, Schatz PF, Kim H, Lu F, Ralph J: **Identifying new lignin bioengineering targets: 1. monolignol-substitute impacts on lignin formation and cell wall fermentability.** *BMC Plant Biol* 2010, **10**:114.
51. Tobimatsu Y, Elumalai S, Grabber JH, Davidson CL, Pan X, Ralph J: **Hydroxycinnamate conjugates as potential monolignol replacements: In Vitro lignification and cell wall studies with rosmarinic acid.** *ChemSusChem* 2012, **5**:676–86.
52. Elumalai S, Tobimatsu Y, Grabber J: **Epigallocatechin gallate incorporation into lignin enhances the alkaline delignification and enzymatic saccharification of cell walls.** *Biotechnol Biofuels* 2012, **5**:1–13.
53. Wilkerson CG, Mansfield SD, Lu F, Withers S, Park J-Y, Karlen SD, Gonzales-Vigil E, Padmakshan D, Unda F, Rencoret J, et al.: **Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone.** *Science* 2014, **344**:90–3.

- Labile interunit linkages in were engineered in poplar lignin through the expression of a transgene identified in a Chinese medicinal species. This study demonstrated that novel lignin precursors could be synthesized and transported to the cell wall. The proof that monolignol ferulates were incorporated in lignin was particularly elegant.

54. Vanholme R, Morreel K, Darrah C: **Metabolic engineering of novel lignin in biomass crops.** *New Phytol* 2012, **196**:978–1000.
55. Jung JH, Vermerris W, Gallo M, Fedenko JR, Erickson JE, Altpeter F: **RNA interference suppression of lignin biosynthesis increases fermentable sugar yields for biofuel production from field-grown sugarcane.** *Plant Biotechnol J* 2013, **11**:709–16.
 - The first field study with transgenic sugarcane in which COMT activity was reduced showed that gains in saccharification yield can be maintained under field conditions using a commercial bioenergy grass.
56. Baxter HL, Mazarei M, Labbe N, Kline LM, Cheng Q, Windham MT, Mann DGJ, Fu C, Ziebell A, Sykes RW, et al.: **Two-year field analysis of reduced recalcitrance transgenic switchgrass.** *Plant Biotechnol J* 2014, **12**:914–924.
57. Scheller HV, Ulvskov P: **Hemicelluloses.** *Annu Rev Plant Biol* 2010, **61**:263–89.
58. Pauly M, Gille S, Liu L, Mansoori N, de Souza A, Schultink A, Xiong G: **Hemicellulose biosynthesis.** *Planta* 2013, **238**:627–42.
59. Selig MJ, Knoshaug EP, Adney WS, Himmel ME, Decker SR: **Synergistic enhancement of cellobiohydrolase performance on pretreated corn stover by addition of xylanase and esterase activities.** *Bioresour Technol* 2008, **99**:4997–5005.
60. Kumar R, Wyman CE: **Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies.** *Biores Technol* 2009, **100**:4203–13.
61. Selig MJ, Adney WS, Himmel ME, Decker SR: **The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes.** *Cellulose* 2009, **16**:711–722.
62. DeMartini JD, Pattathil S, Miller JS, Li H, Hahn MG, Wyman CE: **Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass.** *Energy Environ Sci* 2013, **6**:898.
63. Zhang J, Tang M, Viikari L: **Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases.** *Biores Technol* 2012, **121**:8–12.
64. Torres AF, Van der Weijde T, Dolstra O, Visser RGF, Trindade LM: **Effect of maize biomass composition on the optimization of dilute-acid pretreatments and enzymatic saccharification.** *Bioenerg Res* 2013, **6**:1038–1051.

65. Torres AF, Noordam-Boot CMM, Dolstra O, van der Weijde T, Combes E, Dufour P, Vlaswinkel L, Visser RGF, Trindade LM: **Cell wall diversity in forage maize: genetic complexity and bioenergy potential**. *Bioenerg Res* 2014, doi:10.1007/s12155-014-9507-8.
66. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW: **Technoeconomic analysis of biofuels: a wiki-based platform for lignocellulosic biorefineries**. *Biomass Bioenergy* 2010, **34**:1914–1921.
67. Chiniquy D, Sharma V, Schultink A, Baidoo EE, Rautengarten C, Cheng K, Carroll A, Ulvskov P, Harholt J, Keasling JD, et al.: **XAX1 from glycosyltransferase family 61 mediates xylosyltransfer to rice xylan**. *Proc Natl Acad Sci USA* 2012, **109**:17117–22.
 - A rice mutant with modified xylan that reduces crosslinking to lignin yields more glucose following enzymatic saccharification.
68. Ralph J: **Hydroxycinnamates in lignification**. *Phytochem Rev* 2009, **9**:65–83.
69. Marriott PE, Sibout R, Lapierre C, Fangel JU, Willats WGT, Höfte H, Gómez LD, McQueen-Mason SJ: **Range of cell-wall alterations enhance saccharification in *Brachypodium distachyon* mutants**. *Proc Natl Acad Sci USA* 2014, doi:10.1073/pnas.1414020111.
70. Bartley LE, Peck ML, Kim S-R, Ebert B, Manisseri C, Chiniquy DM, Sykes R, Gao L, Rautengarten C, Vega-Sánchez ME, et al.: **Overexpression of a BAHD acyltransferase, *Osat10*, alters rice cell wall hydroxycinnamic acid content and saccharification**. *Plant Physiol* 2013, **161**:1615–33.
71. Petersen PD, Lau J, Ebert B, Yang F, Verhertbruggen Y, Kim JS, Varanasi P, Suttangkakul A, Auer M, Loqué D, et al.: **Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants**. *Biotechnol Biofuels* 2012, **5**:84.
 - This study provided proof of concept that xylan levels in biomass can be reduced to enhance biomass conversion properties, without compromising xylem vessel integrity
72. Gille S, Pauly M: **O-acetylation of plant cell wall polysaccharides**. *Front Plant Sci* 2012, **3**:1–7.
73. Bouchabke-Coussa O, Quashie M-L, Seoane-Redondo J, Fortabat M-N, Gery C, Yu A, Linderme D, Trouverie J, Granier F, Téoulé E, et al.: **Eskimo1 is a key gene involved in water economy as well as cold acclimation and salt tolerance**. *BMC Plant Biol* 2008, **8**:125.

74. Xiong G, Cheng K, Pauly M: **Xylan *O*-acetylation impacts xylem development and enzymatic recalcitrance as indicated by the *Arabidopsis* mutant *tbl29***. *Mol Plant* 2013, **6**:1373–5.

Figure legends

Figure 1 **A.** Cellulose is linear polymer of β -1,4-linked D-glucopyranose residues with cellobiose as the repeating unit. **B.** Cellulose synthase (CesA) synthesizes cellulose from UDP-glucose. Based on crystallographic studies of the membrane-localized BcsA and BcsB proteins in the cellulose-synthesizing bacterium *Rhodobacter sphaeroides*, Morgan et al. [12] proposed a model in which a single UDP-glucose residue, bound in the active site (represented by the bowl shape), is added at a time, with rotation along the newly formed acetal bond enabling a spatially constrained oligomer able to move in the direction of the red arrow into the tunnel formed by BcsB.

Figure 2. Schematic representation of lignin biosynthesis in cell walls of vegetative tissues. Compound names: **1p.** L-phenylalanine **1t.** L-tyrosine **2.** cinnamic acid **3.** *p*-coumaric acid **4.** *p*-coumaroyl-CoA **5.** *p*-coumaraldehyde **6.** *p*-coumaryl alcohol **7.** *p*-coumaroyl shikimate (R = shikimate) **8.** caffeoyl shikimate (R = shikimate) **9.** caffeic acid **10.** caffeoyl-CoA **11.** feruloyl-CoA **12.** coniferaldehyde **13.** coniferyl alcohol **14.** 5-hydroxyconiferaldehyde **15.** 5-hydroxyconiferyl alcohol **16.** sinapaldehyde **17.** sinapyl alcohol **18.** ferulic acid. Enzyme abbreviations: **PAL:** phenylalanine ammonia lyase; **TAL:** tyrosine ammonia lyase; **C4H:** cinnamic acid 4-hydroxylase; **4CL:** 4-coumarate CoA ligase; **HCT:** hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase; **C3'H:** *p*-coumarate 3-hydroxylase (*p*-coumaroyl shikimate 3' hydroxylase) **CSE:** caffeoyl shikimate esterase; **CCoAOMT:** caffeoyl-CoA *O*-methyltransferase; **CCR:** cinnamoyl-CoA reductase; **CAD:** cinnamyl alcohol dehydrogenase; **F5H:** ferulic acid 5-hydroxylase (coniferyl alcohol/coniferaldehyde 5-hydroxylase); **COMT:** caffeic acid *O*-methyltransferase (5-hydroxyconiferyl alcohol/5-hydroxyconiferaldehyde *O*-methyltransferase); **LAC:** laccase; **POX:** peroxidase; **CALDH:** coniferaldehyde dehydrogenase. Activities of some enzymes are different than their names imply; the main activity is indicated in parentheses. Light-grey structure **1t** and enzyme TAL represent a grass-specific route to **3**. HCT can convert **8** to **10**, but not very efficiently. This figure is based on [22, 40].

Figure 3. One or more sensors that detect metabolites, enzyme activity or transcript levels appear to be involved in the coordination of cell wall biosynthesis, and enabling compensatory mechanisms based on transcriptional and hormonal responses to react to perturbances. This hypothetical model is based on [43, 44].

Figure 4. A representation of a lignin fragment from an angiosperm displaying a subset of interunit linkages. The structure on the left represents native lignin with G- and S-residues connected via β -*O*-4 linkages (blue), β - β linkages (resinol; purple) and 4-*O*-5' ether linkages (biphenylether; yellow). A coniferaldehyde end group (magenta) is also present. Possible branchpoints are indicated by dashed lines. The structure in the center represents ZipLigninTM containing a coniferyl-ferulate unit (red). Under mild alkaline conditions, the ester linkage in this unit is hydrolyzed, and the lignin breaks down in fragments with low molecular weight that can be much removed more easily than the large domains in the native lignin. This figure is based on [22, 53].

Figure 2
[Click here to download high resolution image](#)

Figure 3
[Click here to download high resolution image](#)

Figure 4

***Graphical abstract (for review)**