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Designing the deconstruction of plant cell walls

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Cell wall architecture plays a key role in the regulation of plant cell growth and differentiation into specific cell types. Gaining genetic control of the amount, composition, and structure of cell walls in different cell types will impact both the quantity and yield of fermentable sugars from biomass for biofuels production. The recalcitrance of plant biomass to degradation is a function of how polymers crosslink and aggregate within walls. Novel imaging technologies provide an opportunity to probe these higher order structures in their native state. If cell walls are to be efficiently deconstructed enzymatically to release fermentable sugars, then we require a detailed understanding of their structural organization in future bioenergy crops.

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Introduction

Grasses, such as maize, sorghum, switchgrass, and *Miscanthus*, and fast-growing trees, such as poplar and willow, can be sustainable and renewable feedstocks for biofuels production. Biorefinery feedstocks are pretreated with acid, alkali, or high temperature/pressure treatments before enzyme hydrolysis to yield glucose and other useful sugars for fermentation to ethanol, or other fuels, and other products. Although optimizing microbial fermentation and the efficient bioconversion to biofuels require significant improvements, attaining current bioenergy target goals requires enormous increases in plant productivity, both in biomass quantity and yield of fermentable sugars. Plant cell walls have evolved to resist breakdown from microbial and mechanical forces — unlocking fermentable sugars from these biomaterials represents a formidable scientific challenge.

The efficiency of bioconversion to fermentable sugars depends upon macroscopic and molecular features of the feedstocks. Macroscopic features include the spatial organization of different cell types, the strength and extent of cell–cell adhesion, and the spatial distribution of lignin. At the molecular level, the composition and architecture of cell walls of different cell types will impact the local migration of enzymes through cell wall pores to cellulose, xylans, and other glycans. Specific architectural features include the nature and extent of the crosslinks between different polysaccharides, the interactions between lignin and carbohydrates, the nature and extent of protein crosslinking, cellulose crystallinity and microfibril size. In this review, we summarize recent key areas of progress toward defining crosslinks and structures that may contribute to recalcitrance to deconstruction, improving the imaging technologies to visualize wall polymer interactions, and defining gene networks for bioenergy-crop-specific cell wall architectures.

Energy grasses and forest crops have distinctive wall architectures

The composition of primary (growing) cell walls of grasses differs from those of most other angiosperms [1–3]. Briefly, the Type I cell wall of all dicots and many monocots comprises cellulose microfibrils crosslinked with xyloglucans to form a network, and embedded in a complex matrix of pectins and proteins [1]. In the Type II cell wall of grasses and related monocots, glucuronoarabinoxylans (GAXs) are the major crosslinking glycans, hydrogen bond to the surface of cellulose microfibrils and crosslink them [2]. Other crosslinking glycans include a grass-specific xyloglucan and glucomannan and, during cell expansion, a (1 → 3),(1 → 4)-β-D-glucan. By sequential chemical extraction and enzymatic digestion of specific polysaccharides, and imaging of the resulting cell wall architectural modifications in maize coleoptile cells, the GAXs occupy most of the volume between cellulose microfibrils, whereas the β-glucans tightly coat the microfibrils [4]. Another distinction of grass walls is the presence of crosslinking phenylpropanoid networks in the primary walls that are deposited as cells mature during development. These phenylpropanoid networks include several patterns of ester, ether, and phenyl–phenyl linkages initiated from arabinosyl residues of the GAX. Chlorite oxidation of the aromatic residues extracts very little material from the grass cell wall but renders the GAXs of the interstitial space between β-glucan-coated microfibrils easily extracted by as little as 0.1 M NaOH [5,6]. Although we have general inventories of the types of carbohydrate and aromatic substances in grass walls, our knowledge of the fine structure of their polymeric components, how they are assembled into a functional architecture, and the dynamics of their interactions

during growth and biomass deposition, are very limited for the perennial grasses.

Only a handful of genes have been identified in grass species that impact cell wall architecture. The four *brown mid-rib* mutants of maize and sorghum are best studied, particularly with respect to the effects of reduced lignin and altered structure on ruminant nutrition [7,8]. Maize *brittle stalk* encodes a COBRA-like protein that impacts cellulose content [9] and subsequent deposition of lignin as the plant transitions from juvenile to adult stage [10]. Researchers are beginning to tap existing diverse germplasm materials to select quantitative trait loci and the underlying genes related to improved wall digestibility [11,12]. Oxidative coupling of esterified and/or etherified ferulic acid residues are among the factors most inhibitory to ruminant digestion of cell walls [13]. As the ease of digestion in ruminants is correlated with performance in saccharification schemes [14,15], the crosslinks of the phenylpropanoid network are important future targets for genetic modification.

Forest crops are enriched in cells with secondary walls. Primary cell walls surround most plant cells, but specialized secondary walls, deposited during differentiation of xylem, phloem, and transfer cells, have distinctive compositions. Cellulose constitutes up to 30% of the mass of primary walls and 60–98% of the mass of secondary walls. The composition of poplar secondary walls has been analyzed and contrasted with primary wall composition: secondary walls are relatively enriched in cellulose and xylans, lignin, some structural proteins, and minor amounts of pectins [16]. The molecular architecture of secondary walls has not been elucidated.

Interacting with these complex extracellular matrices are several hundreds of enzymes. Plant enzymes that remodel cell wall architecture, such as expansins and xyloglucan *endo*-transglycosylases (XETs), could be used to facilitate wall deconstruction. Expansins act to reduce hydrogen bonding by an unknown mechanism. A maize pollen allergen belonging to the expansin B family (EXP B1) binds strongly to xylans, causing swelling of the maize cell wall, perhaps by disrupting GAX–cellulose interactions [17•]. XET cleaves and ligates xyloglucans with new xyloglucan partners such that wall architecture may be modified without detectable changes in composition. A barley XET can link xyloglucans with mixed-linkage glucans or cellulosic substrates, raising the possibility that XETs might link different polysaccharides *in vivo* [18]. However, the relative activity is 500-fold lower than xyloglucan to xyloglucan crosslinking. These transglycosylation activities have the potential to generate novel heteropolymeric networks that impact architecture. *Endo*-β-mannanase has been renamed mannan transglycosylase/hydrolase because it can carry out a transglycosylation reaction in the presence of mannan-derived oligosaccharides [19].

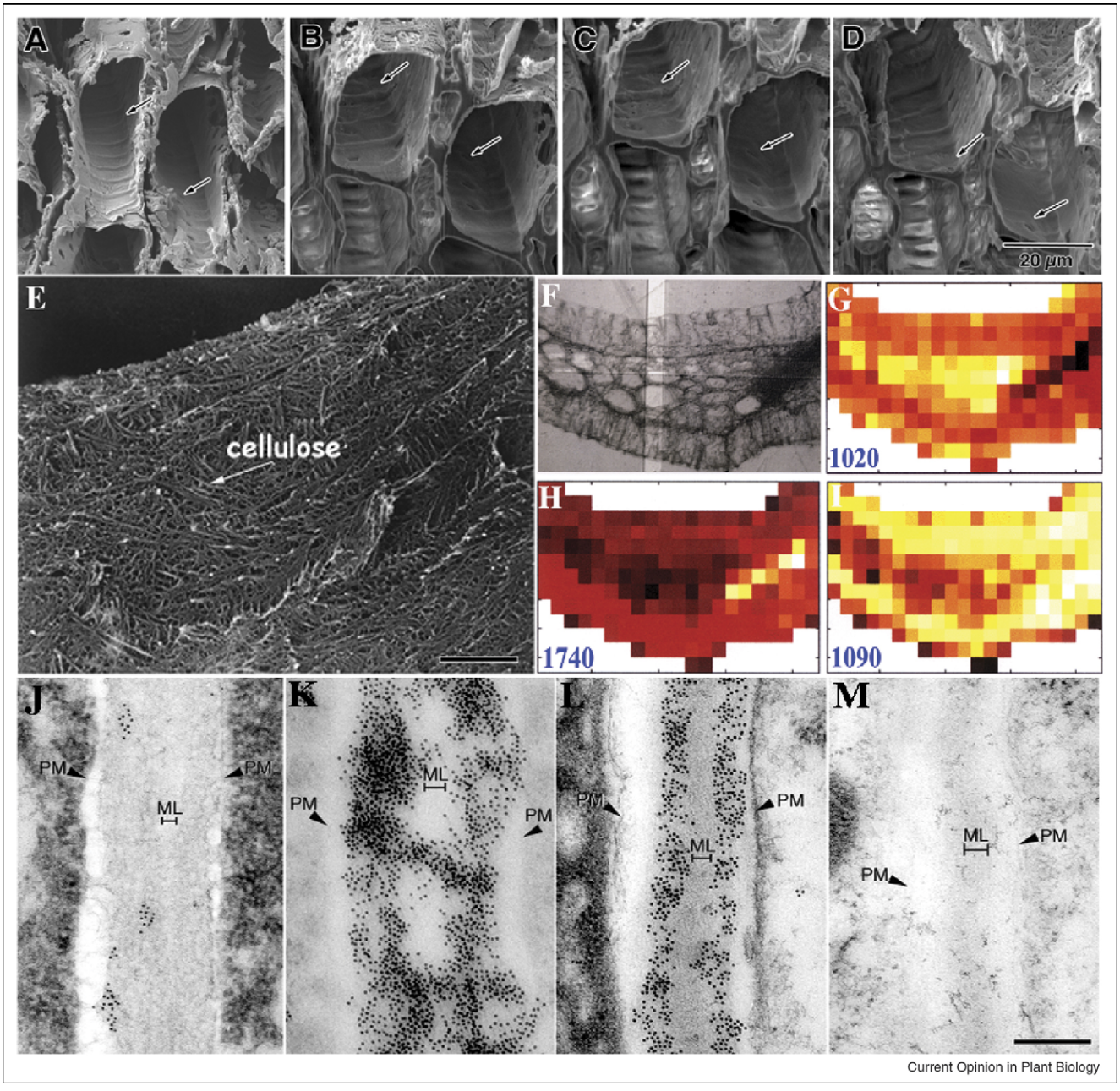
The biomechanical properties of walls are functions of the nature and extent of polymer crosslinking. Analog composites of cellulose with xylogucans or other glycans have been made *in vitro* to simplify the complexity of wall composition and deduce the architectural functions of individual components. For example, cellulosic pellicles of *Gluconacetobacter xylinus* spontaneously form composites with other polysaccharides present in the culture medium that can be sampled for biomechanical tests. Enzymic treatment of cellulose/xyloglucan composites with a xyloglucan *endo*-hydrolase increased stiffness of the pellicle while treatment with an XET enhanced viscoelasticity [20]. Xyloglucans with galactose reduced by 60% produced pellicles with low stress and strain to break compared with cellulose alone, probably as an effect of xyloglucan aggregation introducing weak spots [21]. The mechanical behavior of the composite is consistent with observations of the reduced tensile strength of *Arabidopsis* mutant seedlings compromised in xyloglucan galactosylation [22,23]. Dimerization of rhamnogalacturonan II, a pectic polysaccharide, with boron is also essential to maintain the tensile strength of seedlings [22].

Imaging features of molecular architectures

At cellular resolution, field-emission dual-beam imaging provides the opportunity for the accurate measurement of wall thicknesses and 3D reconstructions of ablated volumes (Figure 1A–D). Direct visualization of cell walls by fast-freeze, deep-etch, rotary-shadowed replicas gave images of walls that showed the architecture of the cellulose/crosslinking glycan network after the extraction of pectins [24] — an example is shown below of a maize primary wall (Figure 1E). However, making replicas is technically difficult and other imaging technologies provide an easier route to imaging at least microfibrils within walls. Field-emission scanning electron microscopy (FESEM) now provides the necessary resolution in scanning rather than transmission EM [25] and has been used to measure microfibril angles in a dwarfed and cellulose-deficient *Arabidopsis* mutant [26]. The reduction in cellulose content in the *procuste* mutant does not account for the extent of dwarfing, but the increased anisotropy in newly deposited microfibrils can be modeled to explain the large effect on organ growth. High-resolution atomic force microscopy (AFM) has the versatility to make measurements in air or under fluid. Sample topography (height data) and elasticity (phase image) can be acquired simultaneously while new probes with sharper tips (1 nm) have reduced problems associated with image broadening. In dried maize stover, internal faces revealed microfibrils of minimum cross-section 5 nm × 10 nm [27]. Dispersing cotton microfibrils in cupri-ethylenediamine solution has provided exceptionally clear AFM images of

single cellulose chains [28]. In force spectroscopy, the binding forces between xylogucan molecules and a cellulose substrate and the AFM tip have been measured [29]. The xylogucan end was covalently linked to the AFM tip and forces to remove the hydrogen-bonded tamarind xyloglucan were of the order of tens of pico-Newtons, approximating to one hydrogen bond per backbone glucose residue.

Figure 1



The focused ion beam of a field-emission dual-beam electron microscope is used to smooth the rough surface of a fractured woody stem (**A**). 'Slice and view' imaging results in ablating 1 μm slices and then imaging the revealed surface. Images (**B–D**) show progression through ~20 μm of the sample. The badly damaged areas of the specimen in (A) are ablated and features (hoops of secondary wall) can be seen to move closer to the surface as material is being removed (arrows) (D Sherman, MC McCann, unpublished results). (**E**) Epidermal cell wall of a maize coleoptile imaged by the fast-freeze, deep-etch, rotary-shadowed replica technique, arrow indicates a single cellulose microfibril [24]; scale bar represents 200 nm. (**F**) Transverse section of a maize coleoptile imaged using an infrared microscope with transmitted visible light [4]. (**G–I**) Chemical images at the indicated wave numbers showing tissue-specific distribution of carbohydrate absorbances at 1020 and 1090 cm^{-1}, and ester absorbances at 1740 cm^{-1} (color scale with white as high abundance to dark red as low abundance). (**J–M**) (1 → 3),(1 → 4)-β-D-glucan epitopes in coleoptile cell walls show dynamic changes in abundance from day 2 (J), to day 3 (K), day 4 (L) and day 5 (M) of elongation [56]. All transmission electron micrographs are at the same magnification (C, J–M); scale bar represents 250 nm.

Tomography reconstructs the interior structure of an object from its projections collected from different directions by tilting the specimen grid in the electron beam. Dual-axis electron tomography has been used to investigate cellulose microfibrils in transmission EM [30••], to a resolution of about 2 nm, resolving individual microfibrils and their 3D organization in the S2 layer of the walls of wood fibers. Individual microfibrils measure about 3.2 nm with an unstained core of about 2.2 nm. Clustering of microfibrils was observed in some regions, but this may be an artefact induced by a delignification step necessary for sample preparation.

Raman and mid-infrared microscopies provide complementary techniques for chemical imaging of plant cell walls. Raman scattering depends on changes in the polarizability of functional groups because of molecular vibration, while infrared absorption depends on changes in intrinsic dipole moments. Infrared chemical imaging provides cellular resolution of cell wall compositions in tissue sections ([31], Figure 1F–I) using a step-scanning spectrometer equipped with a camera and a focal plane array detector. The spatial resolution is limited to 10 μm by diffraction effects. Improved resolution, to 1 μm, is obtained with Raman imaging though sample fluorescence can be a problem. Laser-based confocal Raman microscopy has been used to generate intensity maps of lignin and cellulose distribution in cell wall layers of black spruce wood, resolving signals from cell corners, the middle lamella, and different S layers within the secondary wall [32•]. The Raman effect is inherently weak. Wood tissues have sufficient density to give enough Raman scattering to obtain a spectrum but this is not true of primary walls. However, recent developments in surface-enhanced Raman spectroscopy, with application of small silver or gold colloids to samples, or to the tip of a probe in contact with the sample, provides the opportunity for local signal amplification and much improved Raman signal detection [33]. Combinations of visible and Raman imaging with AFM in a single instrument have also been advantageous because of not having to displace the sample [34].

Molecular probes for epitopes of polysaccharides within wall structures continue to be a major goal, and the specificity of carbohydrate-binding modules (CBMs) for particular features is providing a new range of probes with specificity for native structures [35•,36]. CBMs constitute domains of microbial hydrolytic enzymes, usually linked to domains with catalytic function. High-resolution images of their binding [37••] have been achieved by using semiconductor quantum dots incorporated into a double His-tagged recombinant CBM for direct imaging. Water-soluble and highly luminescent quantum dots of (CdSe)ZnSe bind five histidines at the zinc surface while retaining desirable electronic properties for imaging at the EM level.

Gaining genetic control of cell wall architecture

Gaining genetic control of cell wall composition is a key goal for the improvement of bioenergy crops, but must be achieved without compromising plant performance. Although lignin content can be reduced by downregulation of lignin biosynthetic enzymes, an undesirable consequence is the reduction of plant stature. In the phenylpropanoid pathway, *p*-coumaroyl CoA is at a junction of pathways leading to flavonoids or cell wall phenylpropanoid compounds. Hydroxycinnamoyl transferase (HCT) catalyzes the synthesis of shikimate and quinate esters of *p*-coumaric acid. Using *Arabidopsis*, HCT-silenced plants are dramatically dwarfed but this is because of altered flavonoid accumulation not lignin synthesis: suppressing flavonoid accumulation by the repression of chalcone synthase restores wild-type growth and normal auxin transport [38••]. Thus, the phenylpropanoid pathway can be genetically modified without deleterious consequences to plant growth.

We have estimated that plants devote about 10% of their genome, about 2500 genes in *Arabidopsis*, to construction and dynamic rearrangement of their cell walls during growth [39]. Over 1600 genes encode annotated carbohydrate-active enzymes in the poplar genome [40]. The expression of subsets of gene family members are spatially and temporally regulated such that gene networks acting in particular cell types may be defined together with master regulatory genes controlling these gene networks.

The gene networks that encode the machinery to build and modify walls in bioenergy species are crucial to identify, but progress has been most rapid in *Arabidopsis*. The expression patterns of individual gene family members are being measured using whole or custom microarrays in order to define sets of genes that are coregulated [41]. A custom chip of 765 cell wall-related genes with sequence-specific 70mer oligonucleotides from *Arabidopsis* has been used to examine expression patterns in different organs and growth stages [42]. Earlier sets of global transcriptional analyses to identify secondary-wall-related gene networks [43,44,45••,46] are complemented by two recent studies. Fifty-two genes representing a 'core xylem gene set' with a novel *cis*-regulatory element in 13 of their promoter sequences, ACAAAGAA, were identified based on *in silico* analyses of Affymetrix GeneChip datasets [47]. Coexpression analyses using phenylpropanoid genes as anchors allowed identification of candidate genes for lignin biosynthesis [48] using near full-genome 70mer oligoarrays.

Identification of key regulators of cell wall synthesis in specific cell types is an important means of modifying biomass composition. Gain-of-function experiments of NAC-domain-containing and MYB proteins show

increased and ectopic secondary wall formation. Overexpression of vascular-related NAC domain (VND) 6 and VND7 induced transdifferentiation of various cell types in *Arabidopsis* into metaxylem-like and protoxylem-like vessel elements, respectively [45••]. NST (NAC secondary wall thickening promoting factor) 1, 2, and 3 regulate secondary wall formation in cells other than vessels [49••]. NST3 was previously identified as SND1 [50,51]. NST1 and NST2 act redundantly in secondary wall formation in anther endothecium [52] and NST1 and NST3 act redundantly in interfascicular fibers and secondary xylem [49••,50]. Another member of the NAC transcription family ANAC012 suppresses wall formation in xylary fibers when overexpressed, acting as a negative regulator [53]. MYB26 appears to regulate NST1 and NST2 expression and might act to specify endothecial cell differentiation before secondary wall formation is initiated [54••]. MicroRNA sequences have also been implicated in regulatory control of transcription factors in *Populus* [55], and upregulated during the formation of tension wood.

Conclusions and future directions

A logical path to the coexistence of food crops and bioenergy crops is to reduce the agronomic footprint of future energy plants. The productivity of bioenergy plants can be improved by redesigning the form and stature of the plant in the field and by maximizing sugar yield per plant. Recently, there has been an emphasis on modifying lignin as the reasonable path to improving sugar yield. However, given that 5-fold to 10-fold increases in biomass per acre are required to meet current bioenergy targets, we believe that a basic strategy should be the opposite of a popular low-cal beer commercial, which asks 'more taste or less filling'. Modifying lignin content for 'less taste' can increase sugar yield per plant, but modulating carbohydrate content, 'more filling', will be necessary to achieve order of magnitude increases. Uncoupling cellulose and cell wall polysaccharide synthesis from lignification offers great promise to optimize energy plant for their end use, yet maintain wall structural integrity so as not to compromise the functions of the wall in protecting the protoplast within.

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