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Roles of Cellulose and Xyloglucan in Determining the Mechanical Properties of Primary Plant Cell Walls[1]

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The primary cell walls of growing and fleshy plant tissue mostly share a common set of molecular components, cellulose, xyloglucan (XyG), and pectin, that are required for both inherent strength and the ability to respond to cell expansion during growth. To probe molecular mechanisms underlying material properties, cell walls and analog composites from *Acetobacter xylinus* have been measured under small deformation and uniaxial extension conditions as a function of molecular composition. Small deformation oscillatory rheology shows a common frequency response for homogenized native cell walls, their sequential extraction residues, and bacterial cellulose alone. This behavior is characteristic of structuring via entanglement of cellulosic rods and is more important than cross-linking with XyG in determining shear moduli. Compared with cellulose alone, composites with XyG have lower stiffness and greater extensibility in uniaxial tension, despite being highly cross-linked at the molecular level. It is proposed that this is due to domains of cross-linked cellulose behaving as mechanical elements, whereas cellulose alone behaves as a mat of individual fibrils. The implication from this work is that XyG/cellulose networks provide a balance of extensibility and strength required by primary cell walls, which is not achievable with cellulose alone.

Plant cell walls are the dominant determinant of tissue mechanical properties and play major roles in the development of cellular structure during growth. Primary walls of growing and fleshy tissues have a conserved general composition of cellulose, hemicellulose and pectin. Outside of the *Gramineae*, the major hemicellulose in primary walls is XyG, which occurs in a limited range of molecular variants (Vincken et al., 1997).

Molecular architecture within primary cell walls has been observed by microscopy (McCann et al., 1990) and inferred from studies of sequential chemical or enzymatic deconstruction, resulting in a number of proposed models (McCann and Roberts, 1991; Talbott and Ray, 1992; Carpita and Gibeaut, 1993) that emphasize two co-extensive networks based on cellulose/hemicellulose and pectin. A degree of anisotropy is also inferred from microscopic studies, with cellulose fibrils exhibiting lateral organization within layers, as well as the potential for inter-layer register (McCann et al., 1990; Reis et al., 1994). Most studies of molecular composition and architecture use cell wall material (CWM) isolated from homogenized tissue and containing submillimeter pieces. Mechanical measurements on these materials are therefore difficult to extrapolate directly to native cell walls. Conversely, mechanical tests are easy to perform on whole plant tissues, but the underlying cellular structure precludes direct interpretation in terms of CWM properties.

One way to circumvent these problems would be to synthesize the polymer network structure(s) characteristic of primary cell walls without the constraints of a cellular framework. This is not yet possible either biochemically or from a plant source, but can be achieved using the cellulose-producing bacterium *Acetobacter xylinus* ATCC 53524 (formerly known as *Acetobacter aceti* subsp. *xylinum*). Fermentation in the presence of a XyG mimics (Whitney et al., 1995) the characteristic network architecture previously observed for de-pectinated primary walls (McCann et al., 1990) and results in cellulose crystallinity levels and polymorph contents typical of native walls (Whitney et al., 1995; Foster et al., 1996; Yamamoto et al., 1996). In this report we use *A. xylinus*-derived composites as models for cell wall mechanical properties.

The two principal material requirements for primary walls are a high intrinsic strength and the ability to accommodate cellular expansion during active growth (McQueen-Mason, 1997). As signatures for these two features, we have used small deformation oscillatory rheology and large deformation uniaxial tensile testing, respectively. In small deformation oscillatory rheology, the response of materials to applied sinusoidal strains is assessed as a function of strain amplitude and frequency of oscillation under conditions that average to zero strain. Provided the strain (amplitude of oscillation) applied is within the linear viscoelastic region, this provides a probe of essentially unperturbed rheological behavior. Key parameters obtained by this method include the in-phase (elastic or storage) component of shear modulus G′, the out-of-phase (viscous or loss) component of shear modulus G″, and the dynamic viscosity, which is the ratio of shear modulus to frequency (Ross-Murphy, 1995). Molecular origins for small deformation behavior are addressed through comparison between sequential chemical extraction residues from homogenized primary CWMs and both homogenized and nonhomogenized *A. xylinus*-derived samples. Large deformation tensile testing requires centimeter-sized pieces and cannot be carried out on homogeneous CWM. However, *A. xylinus*-derived composites are amenable to study in this test, which serves to mimic in vivo elongation processes. Results suggest that cellulose and XyG contribute different features toward the characteristic observed material prop-

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erties of primary plant cell walls, and that supramolecular organization or microstructure plays a key role in determining wall mechanical properties.

MATERIALS AND METHODS

CWMs

Tomato (*Lycopersicon esculentum* cv FM 6203) pericarp material was prepared from mature green and red fruit by boiling freshly peeled, de-seeded, and diced flesh (1.3 kg) in 4 L of 96% (w/v) ethanol for 15 min. The product was homogenized (Silverson mixer, fine setting) for 3 min and filtered (62-μm stainless steel sieve). The retained CWM was washed extensively with ethanol until colorless, rehydrated with deionized water (to approximately 10% dry weight), and stored at 4°C.

Sequential extraction of CWM was performed essentially as described by Selvendran and O'Neill (1987) and modified according to Mort et al. (1991). CWM (20 g wet weight) was dispersed in 300 mL of 500 mM imidazole/HCl buffer, pH 7.0, and stirred for 6 h at ambient temperature (AT) to give the I1 extract. The residue was recovered by centrifugation (20,000*g*, 20 min, AT). After sampling, the remaining CWM was re-extracted with a further 300 mL of 500 mM imidazole/HCl, pH 7.0, for 5 h at AT (I2 extract). Further sequential extractions were with 200 mL of 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ at 1°C for 16 h (N1 extract) and then at AT for 5 h (N2 extract), followed by 0.5 M and then 1 M KOH (200 mL) at AT for 16 h (K1 and K2 extracts). Between extractions and prior to sampling, all residues were washed extensively with deionized water. Samples were stored in 0.02% (w/v) NaN_3.

After pre-incubation with cell wall degrading enzymes (McFeeters and Armstrong, 1984; Quemener et al., 1993) Celluclast, Novozyme, and Viscozyme (Novo-Nordisk: 1 mg mL^{-1} each), monosaccharide analysis was performed by methanolysis (87°C for 16 h using 2 M methanolic HCl). Samples were dried under a vacuum prior to silylation (30 min, AT, 200 μL of pyridine:hexamethyl disilazene: trimethylchlorosilane, 5:1:1). Analysis of the silyl derivatives by GC was performed on a Carlo-Erba Mega series GC using a CP SIL 5 column (25-m × 0.32-mm i.d., Chrompack) and a temperature program of 150°C to 210°C at 2°C min^{-1}. Onion CWM and sequential extraction residues were prepared and analyzed as described previously (McCann et al., 1990; Foster et al., 1996).

Acetobacter xylinus-Derived Composites

A. xylinus was grown in the presence and absence of added tamarind XyG as described previously (Whitney et al., 1995), with some fermentations carried out under static rather than agitated conditions. Growth without agitation resulted in essentially identical yields and cellulose-to-XyG ratios. The same general microscopic features as pellicles produced under agitated conditions, i.e. the apparently isotropic arrangement of cellulose fibrils in the absence of XyG, and extensive fibril cross-linking in the presence of XyG were conserved (Fig. 1). Cellulose/XyG composites produced under agitated conditions showed significant

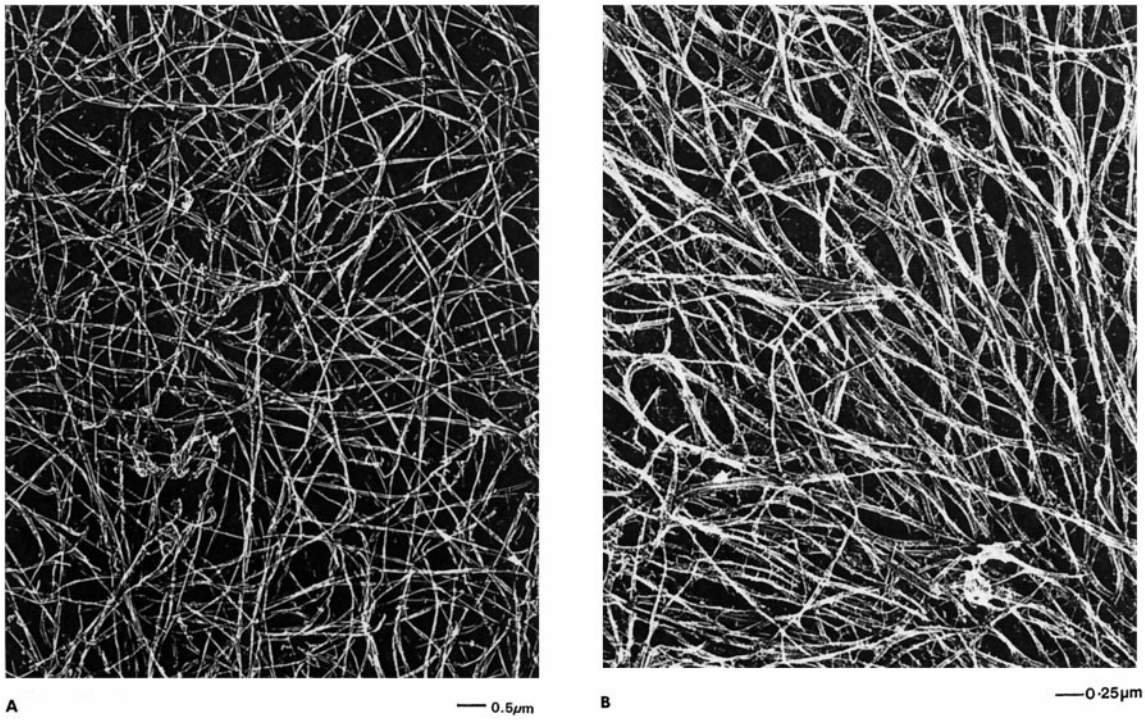

Figure 1. Transmission electron micrographs of tungsten/tantalum/carbon replicas of cellulose (A) and cellulose/XyG ex. *A. xylinus* (B) (representative of structures imaged from several fermentations).

alignment of cellulose fibrils (Whitney et al., 1995); this effect was apparently lessened with static growth conditions. For some rheological tests, *A. xylinus*-derived pellicles were ground with an Ultra-Turrax to give millimeter and submillimeter particles similar in size to CWM.

Small Deformation Oscillatory Rheology

Measurements were made using a Rheometrics RDA II strain-controlled rheometer at 20°C. Parallel plate geometry was used with a 12.5-mm radius top plate, a 25-mm radius bottom plate, and a rim to contain dilute samples. Cellulose-based material from *A. xylinus* fermentation was examined both as single intact pieces, and after pulverization using the same conditions as for isolating CWM. Pulverized samples were analyzed as a particulate dispersion at a sufficiently high concentration to avoid gross syneresis, but not so high as to risk dehydration artifacts. In practice, dry matter contents were in the range 1% to 6% (w/v). To reduce sample slip, plates were roughened by attachment of fine emery paper. Mechanical spectra (frequency sweeps) were recorded over the range 0.5 to 200 rad s^{-1} at 0.5% strain. Strain sweeps were performed at 6.3 rad s^{-1} from 0.05% to 200% strain.

Uniaxial Tensile Testing

Rectangular strips of material (typical geometry 30 × 3 × 1 mm) were cut using a razor blade. The two ends were placed directly between vice grips in a Minimat (Polymer Laboratories, Loughborough, UK) and the grips moved apart at a constant speed of 10 mm min^{-1}. A 20-Newton load beam was used to record the force required for extension as a function of time. From geometrical measurements, force/deformation data could be converted into apparent stress/strain profiles.

RESULTS

CWMs

Carbohydrate compositions obtained for red tomato CWM are shown in Table I. Reduced GalUA content and increased Glc content with successive treatments indicates that extraction of some but not all pectin was achieved. Only KOH extractions were found to release XyG. Data for green tomato CWM was broadly similar with the main difference being the greater level of Gal at the green stage. These data are all consistent with previous reports (Seymour et al., 1990).

Small Deformation Oscillatory Rheology

To determine appropriate experimental conditions for CWM, the strain sensitivity of samples was assessed. In all cases, essential invariance of moduli was observed up to an approximately 1% strain. Above this value, a marked decrease in modulus with additional applied strain was observed (Gidley et al., 1997), indicative of a structural breakdown (yield and flow) within the dispersion of CWM particles. Conversely, the modulus invariance at low strain is indicative of a mechanically stable structure. All subsequent measurements were obtained at 0.1% or 0.5% strain. Figure 2A shows a typical frequency response for storage (G′) and loss (G″) moduli and dynamic viscosity (η^*) for a tomato CWM. Characteristic features are G′ values increasing with frequency and higher than G″ values, which show a marked increase at high frequencies. Dynamic viscosity shows a linear decrease (on the log-log plot) with frequency with a slope (exponent) of about −0.8. Very similar qualitative features were seen for CWM from a nongraminaceous monocot (onion; data not shown), as well as the particulate residues from sequential extractions of all CWMs with 0.5 M imidazole (×2), sodium carbonate (×2), 0.5 M KOH, and 1.0 M KOH. The frequency response of the residue following the second Na_2CO_3 extraction is shown as an example in Figure 2B: All qualitative features are similar to Figure 2A. For some samples, measurements were extended down to 0.02 rad s^{-1}, with no major changes in response.

The continuous nature of the cellulosic pellicles produced by *A. xylinus* means that both intact and pulverized samples can be analyzed by small deformation oscillatory rheology. The same characteristic features observed in CWMs and their chemical extraction residues were seen for both intact and pulverized bacterial cellulose/XyG composites (Fig. 2, C and D). This shows that there is no qualitative difference in small deformation oscillatory properties for these cellulose-based materials between continuous pieces and dispersions of pulverized material produced by homogenization. These features were also seen for both intact and comminuted bacterial cellulose (Fig. 2E), but not for XyG (Fig. 2F) or pectin (data not shown) in the absence of cellulose.

To assess the quantitative contribution of structural features to modulus values, data were obtained for a range of concentrations of pulverized materials (both from plants and *A. xylinus*). Figure 3A shows the concentration depen-

Table I. *Molar percentages of carbohydrate residues in red tomato pericarp CWM and selected sequential extraction residues*[a]

CWM	Ara	Rha	Xyl	GalUA	GlcUA	Man	Gal	Glc
Total CWM	4	3	7	30	1	7	6	42
After I1	4	4	8	26	2	5	5	46
After N1	4	3	9	19	2	6	5	53
After K2	3	2	5	10	1	6	5	66

[a] See "Materials and Methods" for details of I1, N1, and K2 extraction protocols.

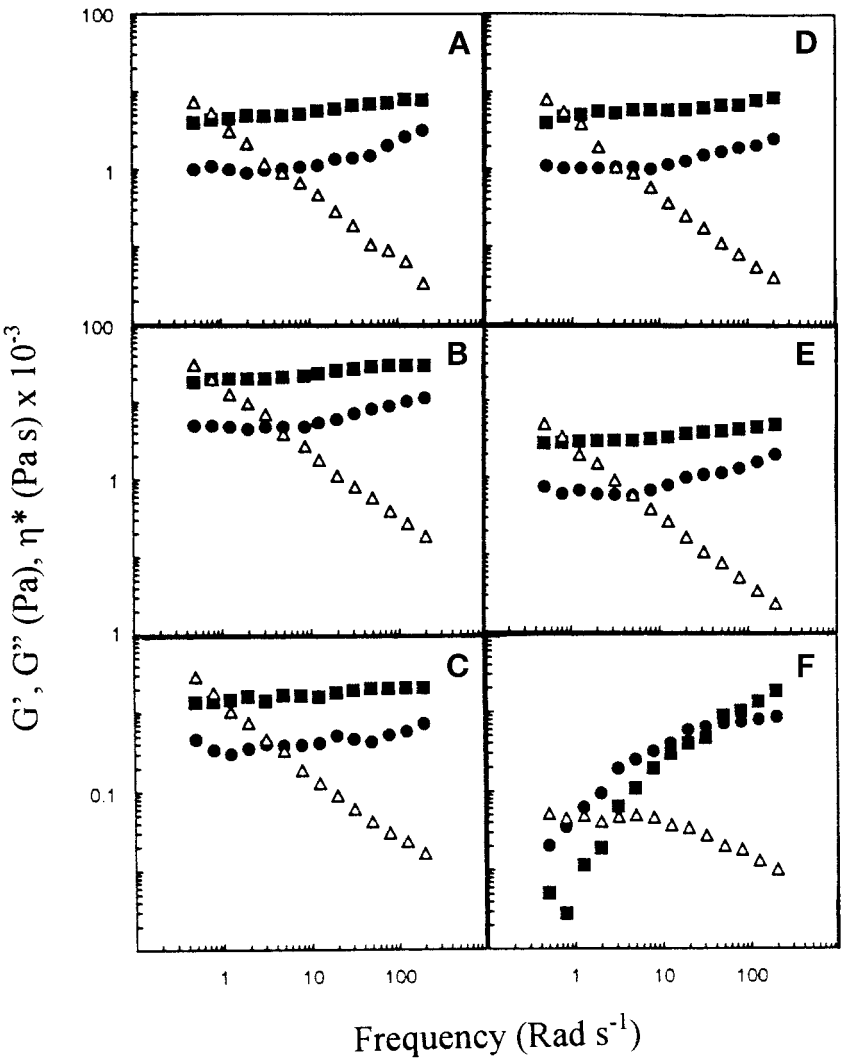

Figure 2. Small deformation rheology of green tomato fruit CWM (3.2% dry weight) (A), residue of tomato CWM after extraction with imidazole and sodium carbonate (N2 residue: 6.3% dry weight) (B), intact cellulose/XyG composite (0.8% dry weight) (C), pulverized cellulose/XyG composite (5.1% dry weight) (D), pulverized *A. xylinus* cellulose (1.5% dry weight) (E), and XyG solution (1.0% [w/v]) (F). Storage (G′, ■) and loss (G″, ●) moduli and dynamic viscosity (η^*, △) values are plotted against oscillatory frequency at 0.5% strain and 20°C.

dence of elastic modulus for CWM isolated from mature green tomatoes and residues from sequential chemical extraction. There was no obvious ordering of moduli on the basis of chemical composition. However, when plotted against cellulose concentration (Fig. 3B), modulus values showed variation with the sequence of extraction steps. Those with the lowest cellulose contents (unextracted CWM, Table I) showed the highest moduli when expressed against cellulose concentration. This behavior indicates that noncellulosic wall components make a (secondary) contribution to modulus values, despite the dominant role of cellulose in determining the response to oscillation frequency (Fig. 2). For all materials, irrespective of concentration, qualitative features of frequency response were similar to those shown in Figure 2, A to E.

Pulverized *A. xylinus* cellulose and cellulose/XyG composites have broadly similar moduli to CWMs. However, when expressed against cellulose concentration, cellulose/XyG composites had lower moduli (Fig. 4). For all concentrations of *A. xylinus*-derived materials, frequency responses were qualitatively similar to those shown in Figure 2.

Uniaxial Tensile Testing

Fermentation of *A. xylinus* for 2 to 3 d results in disc-like pellicles several centimeters in diameter and 1 to 2 mm in thickness in the presence and absence of added XyG. Agitation during fermentation resulted in visually more heterogeneous pellicles than static conditions, probably due to aggregation of bacteria during the early stage of fermentation. Tensile failure properties, but not small deformation oscillatory behavior, were more variable for pellicles produced under agitated conditions. In a few cases failure was observed to occur close to one of the clamps. Data from these experiments were not included in subsequent analyses.

Apparent stress/strain behaviors for replicates of cellulose and cellulose/XyG pellicles produced under static conditions are shown in Figure 5. Pre-failure properties (the slope is the apparent stiffness) were highly reproducible with a range of failure stresses and strains typical of relatively homogeneous materials. In the presence of XyG, apparent stiffness and failure stresses were lower, whereas failure strains were higher. Despite their mechanical vari-

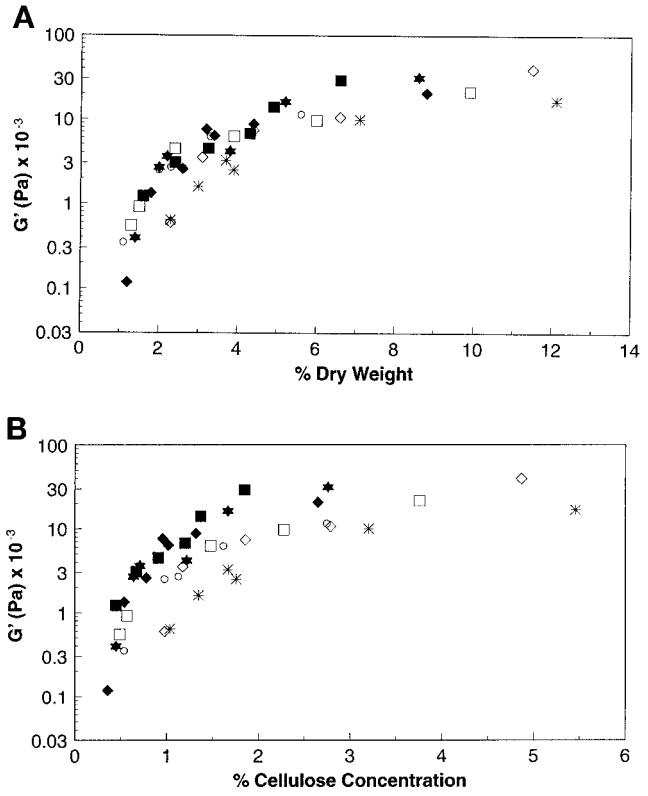

Figure 3. Storage (elastic) shear modulus values for green tomato fruit CWM (0.5% strain, 10 Hz, 20°C) as a function of sequential extraction treatments (see text for details) plotted against dry weight (A) and cellulose (B) concentration calculated from data illustrated in Table I. Native (■), I1 (♦), I2 (★), N1 (□), N2 (◇), K1 (*), and K2 (○) residues are shown.

ability, all traces for cellulose/XyG materials produced under agitated conditions showed lower apparent stiffness than for cellulose alone (data not shown). For all samples, extension resulted in a gradual thinning of the material up to the point of failure. After failure there was only a small elastic recovery, i.e. there was a significant apparent plastic component in the deformation and failure behavior. TEM analysis of strips broken by extension showed no obvious differences in either ribbon isotropy or cross-linking compared with the unstretched material shown in Figure 1 (data not shown).

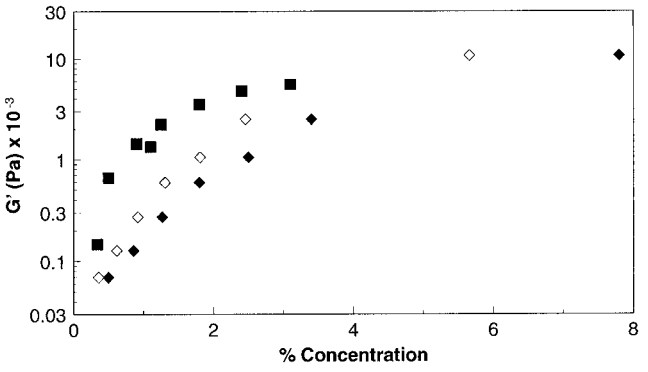

Figure 4. Storage (elastic) shear modulus values for comminuted cellulose/XyG composites (0.5% strain, 10 Hz, 20°C) plotted against both dry weight (♦) and cellulose (◇) concentrations, compared with values for pulverized cellulose alone (■).

DISCUSSION

Cellulose Fibrils Dominate Small Deformation Shear Rheology

A requirement for primary cell walls is to have sufficient strength to withstand cellular turgor pressure. Small deformation oscillatory rheology provides a probe of essentially unperturbed mechanical properties, providing information on viscoelasticity over a range of frequencies. Results may also be capable of interpretation in terms of structural models derived from polymer physics (Ross-Murphy, 1995; Jain and Cohen, 1981).

There is a striking similarity in frequency responses of both moduli and dynamic viscosity for all cellulose-containing systems in small deformation oscillatory tests. The same features, i.e. a small rise in G′ with frequency, a shallow minimum in G″, and a constant slope of about −0.8 for the dependence of dynamic viscosity with frequency, are seen for cellulose alone, cellulose cross-linked with

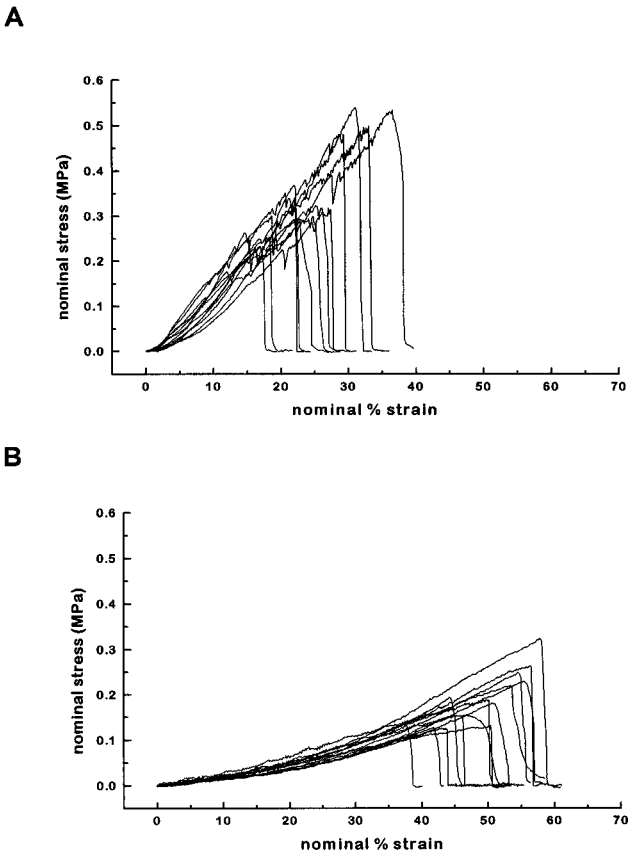

Figure 5. Apparent stress/strain curves for cellulose (A) and cellulose/XyG (B) composites under uniaxial tension. Results shown are for replicates from several independent *A. xylinus* fermentation runs. Cellulose contents of materials tested ranged from 3.3% to 8.3% (w/w) (A) and from 3.1% to 6.6% (w/w) (B).

XyG, and cell wall cellulose in the presence of a co-extensive pectin network. This suggests that the cellulosic fibrils dominate the frequency response signature for small deformation properties. Similar features have previously been observed for suspensions of F-actin filaments (Janmey et al., 1994). The close comparison with the behavior of F-actin filaments is interesting because this is a system thought to be structured purely by entanglements of relatively rigid rods (Janmey et al., 1994). Furthermore, a theoretical basis for this type of small deformation behavior is provided from the predicted rheology of solutions of rod-like entities with no specific interactions between rods (Jain and Cohen, 1981). The effectiveness of this mechanism of structuring increases with the stiffness, length, and concentration of rod-like species. The inference from Figures 2 and 3 is that cell walls are also effectively structured by this mechanism. For bacterial cellulose with apparent rod lengths in excess of several micrometers, a relatively low concentration will result in significant dynamic entanglements and therefore effective "solid-like" ($G' > G''$) structuring. The apparent isotropic arrangement of cellulose fibrils will maximize the number of mechanically significant contacts or entanglements per fibril length. No specific molecular interactions between cellulose fibrils need to be invoked for this structuring mechanism to be effective.

In cellulose/XyG composites, extensive cross-linking between fibrils provides an additional potential structuring mechanism. The observation (Fig. 4) that cross-linking cellulose with XyG serves to reduce modulus values is therefore initially counter-intuitive, but can be seen as a demonstration that for stiff enough rods, entanglement structuring is far more mechanically productive under small deformation test conditions than tethering by thin cross-links. This modulus reduction is suggested to be due to a degree of alignment of cellulose fibrils leading to a reduction in the number of entanglements per fibril.

The inference (Fig. 3) that pectin components can add to shear modulus values in CWMs suggests that the presumed (McCann et al., 1990; Talbott and Ray, 1992; Carpita and Gibeaut, 1993) co-extensive pectin network contributes in a secondary fashion to the behavior dominated by cellulose. This could be due either directly to the mechanical properties of the pectin network or to a reduction in porosity of the system causing a decrease in the distance between effective cellulose entanglements. Small deformation oscillatory behavior is indicative of the responses of hydrated primary cell walls to mechanical forces that do not result in a significant net deformation. The inference from the present data is that cellulose is the key mechanical component contributing to this behavior. Therefore, the primary strategy available to the plant to modulate mechanical strength of primary cell walls under small deformation conditions would be to control the level of cellulose.

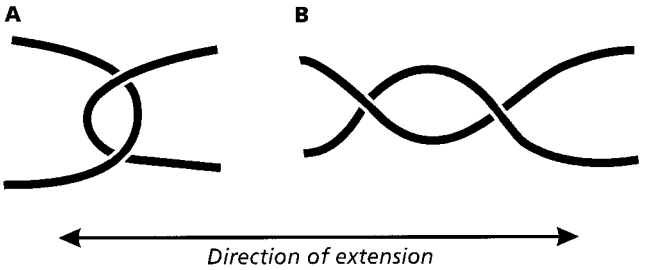

Figure 6. Schematic illustration of conceptual features hypothesized to be involved in the response of cellulose-based composites to tensile extension close to failure. Both forms of knots can serve to restrict extension and, if present in sufficient numbers, to cause bulk failure. Structural elements that contribute to these knots may be individual fibrils or domains of entangled and/or cross-linked fibrils.

Xyloglucan/Cellulose Interactions Affect Extension Properties

Extensive cross-linking by XyG is accompanied by decreased stiffness and increased extensibility compared with cellulose alone (Fig. 5). This is inconsistent with the multiple breakage of XyG cross-links under tensile extension. Decreased stiffness implies, conversely, that the number and/or strength of mechanically effective interactions are reduced in the presence of XyG. We propose that this is a result of a domain structure for the material in which zones of cellulose cross-linked with XyG behave as mechanical elements connected to adjacent elements/domains by physical entanglements. An alternative explanation could be that alteration of cellulose organization by deposition into xyloglucan is responsible for the observed differences compared with cellulose alone. If this were the case, then little mechanical consequence of selective removal of cross-links with endo-glucanase would be predicted. However, preliminary results show that the uniaxial tensile stiffness of cellulose/XyG composites is increased by glucanase treatment to values similar to those obtained for cellulose alone (E. Chanliaud, J. De Silva, and M.J. Gidley, unpublished data), suggesting that the presence of XyG cross-links directly results in reduced composite stiffness. Micrographs (Fig. 1, and Whitney et al., 1995) show a degree of fibril alignment related to tethering by cross-links of similar lengths. In contrast, the cellulose-only system contains entangled single fibrils resulting in a greater number of mechanically relevant interactions per unit volume, which are strained or broken upon elongation of the bulk material. The proposed explanation suggests that XyG is cross-linked to cellulose in actively growing regions in order to facilitate turgor-mediated cell expansion/extension (Cosgrove, 1997).

We propose that failure under tension in these materials occurs when a sufficient number of fibril or domain "knots" are produced under the applied tensile strain, such that further extension can only be achieved by a catastrophic breakage of a series of knots. A conceptual model illustrating two types of knots is shown in Figure 6. One type involves a looped link (Fig. 6A), which is pulled tight under tensile forces, and the other involves a condensation of entanglements (Fig. 6B) aligned with the direction of extension. Either of these conceptual types could produce a macroscopic failure if present at sufficient density. We propose that the apparent isotropy of fibrils in the cellulose-only system produces more looped links upon extension than the larger domains within the cellulose/

XyG composite. This would lead to failure by knot breakage at lower strains for the cellulose-only system. Tensile extension of cellulose/XyG composites is envisaged to occur by alignment of cross-linked domains with the tensile direction eventually resulting in restraining knots at greater strains than for the more intimately entangled cellulose system. As we have no direct microscopic evidence for knots, however, these concepts are purely hypothetical at the moment. Overall, it can be argued that the extensibility of cellulose/XyG composites may allow a more efficient use of cellulose without imparting excessive strength in zones of active growth.

In summary, the present study highlights the mechanical role played by physical entanglement of cellulose rods in primary cell wall homogenates and analogs, and demonstrates that extensive cross-linking of cellulose with XyG results in a weaker, less stiff, and more extensible structure. This suggests that a biological role for cellulose/XyG networks is to provide the appropriate mix of strength at small deformation and extensibility under large deformation required for primary cell wall function in growing tissues.

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