Cellulose-Pectin Spatial Contacts Are Inherent to Never-Dried Arabidopsis Primary Cell Walls: Evidence from Solid-State Nuclear Magnetic Resonance

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Running Head:

Cellulose-Pectin Contacts in Never-Dried Primary Walls

Revised for *Plant Physiology*

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Cellulose-Pectin Spatial Contacts Are Inherent to Never-Dried
Arabidopsis thaliana Primary Cell Walls: Evidence from Solid-State
NMR

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One-sentence summary:

Solid-state NMR of never-dried Arabidopsis primary cell walls at ambient temperature
demonstrate that sub-nanometer pectin - cellulose contacts are an inherent feature of cell walls.
Footnotes

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Daniel J. Cosgrove (dcosgrove@psu.edu).

TW and YBP performed the experiments; all authors contributed to the experimental design and data interpretation; DJC and MH conceived and supervised the project; TW wrote the first article draft; all authors contributed to the writing.
Abstract

The structural role of pectins in plant primary cell walls is not yet well understood due to the complex and disordered nature of the cell wall polymers. We recently introduced multidimensional solid-state nuclear magnetic resonance (SSNMR) spectroscopy to characterize the spatial proximities of wall polysaccharides. The data showed extensive cross peaks between pectins and cellulose in the primary wall of Arabidopsis thaliana, indicating sub-nanometer contacts between the two polysaccharides. This result was unexpected because stable pectin-cellulose interactions are not predicted by in-vitro binding assays and prevailing cell-wall models. To investigate whether the spatial contacts that give rise to the cross peaks are artifacts of sample preparation, we now compare never-dried Arabidopsis primary walls with dehydrated and rehydrated samples. 1D $^{13}$C spectra, 2D $^{13}$C-$^{13}$C correlation spectra, water-polysaccharide correlation spectra, and dynamics data, all indicate that the structure, mobility, and intermolecular contacts of the polysaccharides are indistinguishable between never-dried and rehydrated walls. Moreover, a partially depectinated cell wall in which 40% of homogalacturonan (HG) is extracted retains cellulose-pectin cross peaks, indicating that the cellulose-pectin contacts are not due to molecular crowding. The cross peaks are observed both at -20˚C and at ambient temperature, thus ruling out freezing as a cause of spatial contacts. These results indicate that rhamnogalacturonan I and a portion of HG have significant interactions with cellulose microfibrils in the native primary wall. This pectin-cellulose association may be formed during wall biosynthesis and may involve pectin entrapment in or between cellulose microfibrils, which cannot be mimicked by in-vitro binding assays.
Abbreviations:
Arabinose: Ara, A
Cell wall: CW
Galactose: Gal
Galacturonic acid: GalA, GA
Glucose in Xyloglucan: G
Homogalacturonan: HG, HGA
Interior crystalline cellulose: i
Surface amorphous cellulose: s
Rhamnogalacturonan I: RGI
Rhamnogalacturonan II: RGII
Rhamnose: Rha, R
Xyloglucan: XyG
Xylose: Xyl, x
Cross polarization: CP
Direct polarization: DP
Magic-angle spinning: MAS
solid-state NMR: SSNMR
$^{13}$C–$^1$H dipolar order parameter: $S_{CH}$
Proton-driven spin diffusion: PDSD
Heteronuclear correlation: HETCOR
Dipolar-edited medium- and long-distance: MELODI
Dipolar chemical-shift: DIPSHIFT
**Introduction**

Despite decades of research, many aspects of the three-dimensional structure of the primary cell walls (CW) of eudicotyledons and non-commelinid monocotyledons remain uncertain because of the insoluble, amorphous and complex nature of CW polymers and their tight interactions *in muro* (Jarvis, 1992; Cosgrove, 2005). Unlike massively-crosslinked bacterial cell walls, non-covalent interactions between polysaccharides play major structural roles in plant CWs (Albersheim et al., 2011). Our concepts of the arrangement and interactions of cellulose, hemicellulose and pectins in the CW have largely been shaped by electron microscopy and by chemical or enzymatic dissections, approaches that are limited in their ability to resolve specific noncovalent interactions or that result in considerable perturbation of native CW structures (McCann et al., 1990; Talbott and Ray, 1992; Carpita and Gibeaut, 1993). Recently, 2D and 3D SSNMR techniques have been applied to study the three-dimensional architecture of plant CW polysaccharides (Dick-Perez et al., 2011; Dick-Perez et al., 2012; Wang et al., 2014). The SSNMR experiments detect polysaccharide conformation, mobility and sub-nanometer interatomic contacts, and the measurements were done on CW samples that were minimally perturbed from the native state. Interestingly, the 2D and 3D $^{13}$C correlation spectra showed clear cross peaks between cellulose and rhamnose or galacturonic acid residues of pectic polysaccharides. Together with molecular dynamics simulations (Matthews et al., 2006; Zhao et al., 2014) enzymatic and biomechanical analyses (Park and Cosgrove, 2012a, 2012b), these data led to a revised concept of the CW architecture in which cellulose makes physical contact with both xyloglucan and pectins and in which CW extensibility is controlled at limited biomechanical ‘hotspots’ where multiple microfibrils come into close contact (Cosgrove, 2014). This new model contrasts with the prevailing tethered network model, which depicts a scaffold of cellulose microfibrils tethered by xyloglucans and embedded in a separate but co-extensive gel-like phase made of pectin (Carpita and Gibeaut, 1993; Somerville et al., 2004; Cosgrove, 2005). Additional SSNMR data demonstrated that expansins target limited sites in the CW with altered cellulose conformation and enriched xyloglucan content (Wang et al., 2013), sites that are very similar to the hypothesized biomechanical ‘hotspots’ (Park and Cosgrove, 2012b).

Despite these advances, the structural roles of pectins in the CW remain puzzling and unresolved. The major pectic polysaccharides include homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Caffall and Mohnen, 2009). Neutral pectins, including arabinans and galactans, are found as side chains of RGI and possibly as free polymers. RGII units are cross linked by borate diesters that influence the strength and porosity of primary cell walls (Ishii and Matsunaga, 1996; Fleischer et al., 1999). Some pectins may be covalently linked to arabinogalactan proteins (Tan et al., 2013) or xyloglucan (Popper and Fry, 2008; Cornuault et al., 2014), but the prevalence and structural significance of such hybrid molecules remain to be determined. Early extraction studies of a number of plant cell walls showed that pectic polysaccharides remained with cellulose in the unextracted residue (Ryden and Selvendran, 1990; Goodneratne et al., 1994). But these results were not accounted for in traditional CW structural models (Cosgrove, 2001), which depict pectins as a relatively compliant gel-like network in which the cellulose-xyloglucan network is embedded. In this view, interactions between the two networks are limited to polymer entanglements and do not include extensive non-covalent interactions. Supporting this model are *in-vitro* binding studies that indicate negligible affinity between pectins and cellulose, in contrast to the high affinity between xyloglucan and cellulose (Zykwinska et al., 2005; Zykwinska et al., 2008). The recent 2D and
3D SSNMR spectra showed a significant number of cross peaks between cellulose and pectins (Dick-Perez et al., 2011; Dick-Perez et al., 2012), indicating that some of the pectic sugars come into sub-nanometer contact with cellulose. While NMR cross peaks report inter-atomic distances and not binding energies, the cross peaks nevertheless suggest that significant interactions exist between cellulose and some of the pectins. Such interactions, if true, would disagree with the in-vitro binding assays but would be consistent with the early extraction results.

An unexpected result such as this one naturally meets with skepticism and a search for alternative explanations. One possibility is that the preparation of the first CW samples, which included washing with ethanol and other organic solvents followed by air drying and rehydration, might have caused pectins to precipitate irreversibly onto cellulose and other wall components, thus giving rise to artificial spatial contacts between different polymers. A second possibility is that the intermolecular contacts result from molecular crowding of the wall polymers rather than specific interactions stabilized by hydrogen bonding, van der Waals interactions or other non-covalent interactions (Cosgrove, 2014). Binding assays indicate that neutral arabinan and galactan sidechains of RGI moderately bind cellulose in vitro (Zykwinska et al., 2005; Zykwinska et al., 2008), leading to the proposal that pectic sidechains may function as structural linkers between cellulose microfibrils (Zykwinska et al., 2007; Peaucelle et al., 2012). Although the mechanical significance of pectins was not confirmed by experiments in which wild-type Arabidopsis cell walls were subjected to pectin-loosening treatments, pectins assumed a larger mechanical role in xyloglucan-deficient cell walls (Park and Cosgrove, 2012a).

In this work, we investigate the nature of the pectin-cellulose contacts in greater depth by carrying out SSNMR experiments on a new set of CW samples that allows us to assess the effects of sample preparation, hydration history, temperature, and polysaccharide density on the cellulose-pectin spatial contacts. We prepared Arabidopsis primary cell walls that were never dried throughout the sample preparation. We show that $^{13}$C chemical shifts, intra- and intermolecular cross peaks, segmental order parameters and nuclear spin relaxation times are indistinguishable between the never-dried and rehydrated walls. Difference 2D spectra (Wang et al., 2015) that suppress intramolecular cross peaks are obtained that better reveal the intermolecular cross peaks between cellulose and pectins. Variable-temperature experiments show that cross peaks persist at ambient temperature, thus ruling out artifactual interaction by freezing. Finally, a partially HG-extracted cell wall retains cellulose-pectin correlation peaks, indicating that molecular crowding is not the main cause of the intermolecular contacts. Taken together, these data indicate that cellulose-pectin spatial proximity is an intrinsic feature of the never-dried primary cell wall, and some of the pectins may be entrapped within or between cellulose microfibrils.

Results

We first compared the never-dried CW#1 with a previous sample, CW#4, which was prepared from a different batch of plant material, washed with organic solvents, air-dried and rehydrated (Table 1). CW#4 was used for measuring the previously published 2D and 3D spectra (Dick-Perez et al., 2011; Wang et al., 2012). As we show in Supplemental Fig. S1, the quantitative $^{13}$C direct-polarization (DP) spectra of CW#4 have significantly higher pectin and glycoprotein signals than CW#1, indicating that the different seedlings and wall isolation methods used in the two preparations cause different wall compositions. In 2D $^{13}$C correlation
spectra, both samples show cellulose-pectin correlation peaks (Fig. S2), with CW#4 having moderately higher cross-peak intensities, consistent with its higher pectin content. Given the different wall compositions between CW#1 and CW#4, it is difficult to compare the effects of dehydration on wall structure, thus we took the same batch of plant material as CW#1, dehydrated it to produce sample CW#2, then rehydrated it to give sample CW#3 (Table 1).

1D $^{13}$C and $^{1}$H NMR spectra of never-dried and rehydrated cell walls

We first examined the effects of lyophilization and rehydration on the structure and dynamics of wall polysaccharides by comparing the quantitative $^{13}$C DP spectra of cell walls #1, #2, and #3 (Fig. 1a). The never-dried CW#1 exhibits many sharp pectin peaks such as the 53.7-ppm peak of methyl esters (full width at half maximum = 0.38 ppm), the 21.2-ppm peak of acetyl (0.81 ppm), the 17.6-ppm peak of Rha C6 (0.68 ppm) and the 79.7-ppm peak of GalA C4 and Rha C2 (0.70 ppm). Lyophilization (CW#2) severely broadened all these peaks, giving linewidths of ~8 ppm for the methyl ester peak and ~9 ppm for the acetyl group, indicating that water removal traps a broad distribution of conformations in these pectins. The loss of dynamic bulk water is confirmed by the $^{1}$H spectrum (Fig. 1b). In comparison, the cellulose linewidths are less perturbed by drying: the 89-ppm interior cellulose C4 (iC4) peak increased its linewidth from 2.9 ppm to 3.8 ppm, and the 65-ppm iC6 linewidth is also only moderately broadened by lyophilization. The relative stability of cellulose linewidths under dehydration is analogous to the insignificant broadening of cellulose peaks by freezing to cryogenic temperatures (Wang et al., 2013), and indicates that the conformational rigidity of cellulose microfibrils renders their NMR linewidths significantly independent of environmental changes.

When water is added back (CW#3), the $^{13}$C DP spectrum is fully restored to the never-dried state, with similarly narrow pectin peaks. Thus, the conformations and hydrogen bonding of polysaccharides are reversible after rehydration, suggesting that the average polysaccharide structure in the dried CW is similar to that of the never-dried wall. The resumption of the narrow pectin linewidths also means that the broad conformational distribution of pectins in the dry wall is present in the never-dried wall but they interconvert rapidly to give narrow linewidths at motionally averaged chemical shifts. In other words, polysaccharides with narrow linewidths due to dynamic averaging in the hydrated wall should manifest broad linewidths in the dehydrated wall.

To assess if polysaccharide mobilities are affected by hydration history, we measured $^{13}$C cross-polarization (CP) spectra (Fig. 2a) and DP spectra with short recycle delays (Fig. 2b). The former preferentially enhance the signals of immobile polymers while the latter preferentially detect mobile polysaccharides. The never-dried CW#1 and the rehydrated CW#3 have identical intensity distributions and linewidths in both the CP and DP spectra, indicating that these two samples have identical distributions of mobile and rigid molecules. For example, the short-recycle-delay DP spectra exhibit strong and narrow pectin carbonyl signals (165 - 180 ppm), arabinose C1 peak (108-ppm), GalA and Rha peaks (100, 80, 69 and 17 ppm), the methyl ester peak and the acetyl peak.

**Pectin-cellulose spatial contacts from 2D $^{13}$C-$^{13}$C correlation NMR spectra**

To investigate whether the cellulose-pectin cross peaks reported previously (Wang et al., 2012) were caused by irreversible collapse of the polysaccharides during dehydration, we
measured the 2D $^1$H-driven $^{13}$C-$^{13}$C spin diffusion (PDS) spectrum of the never-dried CW#1 with a long mixing time of 1.5 s and compared it with the spectrum of the rehydrated CW#3 (Fig. 3). The CW#1 2D spectrum is remarkably similar to that of CW#3, with multiple cellulose - pectin cross peaks such as the iC6-HG/RC1 cross peak at (65, 101) ppm and the iC4-HG/RC1 peak at (89, 101 ppm). The 2D spectra have asymmetric cross-peak intensities around the diagonal: in the F1 - F2 frequency dimensions, the cellulose - pectin cross peaks are higher than the pectin - cellulose cross peaks. This results from the higher CP intensities and slower $T_1$ relaxation of cellulose compared to pectins. **Fig. 3c, d** show key cellulose and pectin cross sections. In the 89-ppm and 65-ppm cross sections of interior crystalline cellulose and the 84-ppm cross section of surface cellulose, XyG backbone, and arabinose C4, unambiguous pectin cross peaks such as the 101-ppm peak of Rha and HG C1 and the 80-ppm peak of GalA C4 and Rha C2 are detected at mixing times longer than 500 ms. Cross peaks to mixtures of hemicellulose and pectin signals, such as the 99-ppm peak of Xyl and GalA C1 and the 69-ppm peak of GalA C2, Rha C5 and Xyl C4, are also observed. Conversely, the pectin cross sections exhibit well-resolved cross peaks to cellulose iC4 and iC6. The pectin-cellulose cross peaks have relatively high signal-to-noise ratios of 10-35. The never-dried CW#1 (black) and rehydrated CW#3 (red) show similar intensities for the pectin-cellulose cross peaks within experimental uncertainty, indicating that lyophilization and rehydration have negligible impact on the spatial proximities between the closely packed cellulose and pectins.

The 2D spectra in **Fig. 3** contain both intermolecular and intramolecular correlation signals. To better distinguish these two types of cross peaks, we measured a difference 2D spectrum (Wang et al., 2015) of CW#4 by subtracting two PDS spectra with mixing times of 1.0 s and 0.2 s. In the resulting difference spectrum (**Fig. 4a**), intermolecular cross peaks manifest as positive intensities while intramolecular cross peaks such as iC4-iC6 are removed. Both cellulose-pectin cross peaks and surface-interior cellulose cross peaks expected for the microfibril structure are clearly observed in the difference spectrum. Examples are iC4-R/HGC1 (89, 101 ppm), iC4-RC2/GAC4 (89, 80 ppm) and iC6-R/HGC1 (65, 101 ppm) peaks.

The 2D spectra in **Figs. 3 and 4** were measured at -20°C where pectins were immobilized. To test whether the cross peaks indicative of close spatial contacts are an artifact of sample freezing, we measured 2D PDS spectra at ambient temperature. **Fig. 5** shows that cellulose-pectin cross peaks remain at 20°C, albeit with slightly lower intensities than at low temperature due to the mobility of pectins at high temperature, which weakens the dipolar couplings that drive magnetization transfer. Thus, the close proximity between cellulose and pectins is present at ambient temperature, and the mild freezing in the SSNMR experiments does not perturb the wall structure.

**Polysaccharide mobilities in never-dried and rehydrated cell walls**

To investigate whether dehydration and rehydration perturb polysaccharide mobility, we quantified the motional amplitudes and rates by measuring the $^{13}$C-$^1$H dipolar order parameters ($S_{CH}$) and $^{13}$C $T_1$ relaxation times. Supplemental **Fig. S3** shows that the C-H dipolar dephasing curves (Munowitz et al., 1981; Huster et al., 2001) are identical between the never-dried CW#1 and rehydrated CW#3 within experimental uncertainty. Fitting the dipolar decay curves yielded the bond order parameters, $S_{CH}$ (**Fig. 6a**). For both cell walls, the $S_{CH}$ values are the largest for cellulose (~0.9) and the lowest for pectins (~0.5), indicating that pectins have the largest
motional amplitudes. No significant difference is observed between the two samples, indicating 
that rehydration resumes the same polysaccharide mobilities as the never-dried wall. Similarly, 
$^{13}$C T$_1$ relaxation times (Fig. 6b-c, Fig. S4) are preserved between the never-dried and 
rehydrated walls, indicating that the nanosecond-timescale motions are similar between the two 
samples.

The $^{13}$C-T$_1$ relaxation times can be used to determine the motional heterogeneity in each 
wall polymer, which cannot be determined in the DIPSHIFT experiment, which reports only the 
averaged bond order parameters. Cellulose generally exhibits single-exponential decays with 
long relaxation times of ~5 s, while matrix polysaccharides have double-exponential decays with 
equal contribution of a short component (~0.3 s) and a long T$_1$ component (~4.5 s) (Tables 2, 3). 
Interior cellulose C6 (iC6) also has a fast-relaxing component with low population (~15%), 
which is likely caused by the influence from the surrounding pectins. The double-componential 
behavior is best seen in the pectin data, which show a distinct transition at ~0.5 s between the 
initial fast decay and the later slow decay. To assess if the matrix polysaccharides contain a third 
relaxation component that bridges the mobile and rigid portions, we fit the data using a triple-
exponential function. However, the resulting fits show large uncertainties in either the T$_1$ values 
or the fractions, or do not have sufficient differences in the T$_1$ values of the mobile and 
intermediate components (Table S1). Thus, there is no compelling evidence for a third dynamic 
component in the matrix polysaccharides. These results suggest that the cellulose-contacting 
pectic units are rigidified by the microfibrils while the pectic sugars that fill the interfibrillar 
space are dynamic. The main pectin signals detected here are RGI and HG backbones, because 
galactan sidechain signals are difficult to resolve from the signals of the surface cellulose, xyloglucan, and the concentrations of arabinan is low in this sample.

*Water interactions in the never-dried and rehydrated walls*

To investigate water-polysaccharide interactions, we measured 2D $^{13}$C-$^1$H heteronuclear 
correlation (HETCOR) spectra of never-dried CW#1 (Figs. 7a, S5). In the absence of $^1$H spin 
diffusion, only polysaccharide self-correlation peaks are seen. With a $^1$H spin diffusion mixing 
time of 1 ms, water cross peaks are observed for most polysaccharides at a $^1$H chemical shift of 
4.7 ppm. Since some polysaccharides protons have similar chemical shifts as the water proton, we 
further conducted a dipolar-filtered MELODI-HETCOR experiment (Yao et al., 2001; Li et al., 
2010) to remove the one-bond $^{13}$C-$^1$H cross peaks by means of their strong $^{13}$C-$^1$H dipolar 
coupling. In the absence of $^1$H spin diffusion, the MELODI-HETCOR spectrum (Fig. 7b) 
suppressed most polysaccharide signals except for a weak methyl ester peak at 3.8 ppm, as 
expected. With 1 ms $^1$H spin diffusion, multiple cross peaks at the $^1$H chemical shift of 4.7 ppm 
are detected while other cross peaks are suppressed (Fig. 7c). Thus, the 4.7-ppm $^1$H cross section 
indeed corresponds to water-polysaccharide correlations. Comparison of the HETCOR and 
MELODI-HETCOR spectra shows that the water-edited spectrum preferentially enhances the 
pectin signals (Fig. 7d), indicating that pectins constitute the majority of the hydrated 
polysaccharides, in excellent agreement with the results of a recent hydration study by $^1$H 
polarization transfer NMR (White et al., 2014). Both surface cellulose and interior cellulose (e.g. 
the 89 ppm C4 peak) show water cross peaks. Due to the 1 ms $^1$H spin diffusion period, this 
polarization most likely originates from water on the surface rather than interior of the 
microfibril. Interestingly, the water-correlated interior cellulose C4 peak has half the linewidth as 
the cellulose C4 peak in the HETCOR (Fig. 7e). Since the HETCOR spectrum exhibits all
interior-cellulose signals due to the presence of polysaccharide protons that resonate between 4.5 and 5.0 ppm (Kono et al., 2003) whereas the MELODI-HETCOR spectrum only detects water-contacting cellulose, the line narrowing indicates that the water-proximal interior cellulose chains adopt a subset of conformations that are present among all the glucan chains in the microfibril.

The rehydrated CW#3 shows similar HETCOR and MELODI-HETCOR spectra (Fig. S6) as the never-dried CW#1, except that the water-contacting pectins have 10-25% lower intensities when normalized by the iC4 peak (Fig. S7), indicating that pectin-water association is slightly reduced in the rehydrated cell wall.

**Investigation of molecular crowding using HG-extracted cell wall**

To probe whether the cellulose-pectin spatial contacts in the never-dried wall result from non-specific molecular crowding or specific interactions, we investigated a partially HG-extracted never-dried cell wall (CW#5). Previous monosaccharide analysis indicated that this sample contains ~40% less HG due to extraction by CDTA and sodium carbonate (White et al., 2014). Fig. 8 shows the 1-s 2D PDSD spectrum of this HG-reduced CW. Cellulose-pectin cross peaks such as GA/R/xC1-iC4 at (100, 89) ppm remain in the 2D spectrum. Representative cellulose and pectin cross sections show that the cellulose-pectin cross-peak intensities are similar between the intact and extracted walls, indicating that the removed HG does not contact cellulose in the original wall. Thus, pectin-cellulose spatial contacts are not caused by molecular crowding but by specific interactions, which may involve pectin entrapment within and between cellulose microfibrils.

**Discussion**

Our recent SSNMR studies of intact Arabidopsis primary cell walls (Dick-Perez et al., 2011; Dick-Perez et al., 2012; Wang et al., 2012; Wang et al., 2013; White et al., 2014) led to the proposal of a single-network model of wall polysaccharides, which differs from the prevailing CW model in which pectins do not form stable interactions with cellulose but constitute a gel-like milieu that embeds a cellulose-xyloglucan network and interacts with cellulose only via polymer entanglements (Carpita and Gibeaut, 1993; Cosgrove, 2001, 2005). The current study was designed to further test the authenticity of cellulose-pectin spatial contacts and gain insight into the structural details of such contacts. Are cellulose-pectin contacts artifacts of transient dehydration during cell-wall preparation? Do these contacts result from molecular crowding or specific binding that have escaped previous studies? The present SSNMR data show that quantitative $^{13}$C spectral intensities, $^{13}$C linewidths, 2D correlation peaks, and polysaccharide mobilities are indistinguishable between the never-dried and rehydrated walls, thus demonstrating that lyophilization followed by rehydration restores the wall polysaccharides to their original structure and dynamics. Dehydration did not cause irreversible changes in the wall polymer interactions. We propose that the reversibility of CW structure and dynamics upon rehydration is due to two factors: the rigidity of cellulose microfibrils, which provide a robust scaffold to the cell wall, and the ease of the charged, hydroxyl-rich, and Ca$^{2+}$-chelated matrix polysaccharides to associate with water (White et al., 2014).

Four pieces of experimental evidence support the existence of specific cellulose – pectin binding in the never-dried cell wall. First, pectin-cellulose cross peaks largely remain even after
40% of the HG has been extracted, thus molecular crowding is not the chief reason for the cellulose-pectin spatial contact. This result also means that about half of HG does not participate in cellulose binding, thus the main cellulose-binding pectins are either RGI or the remaining portion of HG or both. Second, a recent hydration study showed that the removal of Ca$^{2+}$ ions, which crosslink HG, slows down water $^1$H magnetization transfer to both cellulose and pectins instead of only pectins (White et al., 2014), indicating that pectins are in intimate contact with cellulose. Third, in the never-dried wall, pectins show a long $^{13}$C T$_1$ component of $\sim$4.5 s, comparable to the T$_1$ of cellulose ($\sim$5 s) (Table 2, 3). This rigid fraction represents $\sim$50% of pectins, similar to the fraction of HG that cannot be extracted by CDTA and sodium carbonate. These results strongly suggest that the rigid pectins are what remain in the wall, bound to cellulose. Indeed, a previous SSNMR study of a similarly depectinated Arabidopsis cell wall (Dick-Perez et al., 2012) showed significant increases in the $^{13}$C T$_1$ relaxation time and $^1$H rotation-frame longitudinal relaxation time (T$_{1p}$), confirming that the unextracted pectins are more rigid. Fourth, the cellulose-pectin cross peaks are observed at both low and high temperatures, thus they are not caused by freezing-induced alterations of polymer packing. The preservation of the cross peaks at high temperature further verifies the conclusion that the more rigid fraction of pectins is responsible for close contact with cellulose.

Taken together, these spectroscopic data indicate that part of HG and the majority of RGI have significant and specific contacts with cellulose microfibrils. We hypothesize that the pectins may be entrapped inside or between cellulose microfibrils, which may occur during cell wall biosynthesis: pectins, secreted from the Golgi, may interact with the plasma-membrane-synthesized glucan chains when both are transported to the cell wall.

The persistence of hydration- and temperature-independent pectin-cellulose cross peaks in multiple Arabidopsis primary wall samples raises the question why in-vitro binding assays found only weak association between these two polysaccharides (Chanliaud and Gidley, 1999; Zykwinska et al., 2007; Zykwinska et al., 2008). Pectins bind up to $\sim$10 $\mu$g per mg of cellulose, compared to xyluoglucon, which binds cellulose to $\sim$120 $\mu$g per mg of cellulose. An examination of the protocols for the binding studies suggests several possible reasons for this apparent discrepancy. First, the binding assays used polysaccharides that were extracted by strong acid and alkali solutions of 0.5 - 6 M in concentration, at temperatures of 40-80$^\circ$C, and with up to 95% ethanol. The strong chemical extraction procedures perturb the conformation, esterification, and methylation of the polysaccharides, and may irreversibly remove important intermolecular interactions. For example, in one study, the extracted sugar-beet pectin used for cellulose binding is predominantly HG and contains little Rha. Since neutral arabinan and galactan are known to have higher affinity to cellulose than anionic galacturonic acids, the reduced amount of RGI in the extracted pectins could reduce the measured binding level. Indeed, comparison of commercial pectins with few neutral sidechains and mildly treated natural pectins in plant cell walls that preserve the neutral sidechains show higher cellulose binding for the latter (Zykwinska et al., 2005).

Second, some binding assays used bacterial cellulose, whose structure differs from that of plant cellulose (Chanliaud and Gidley, 1999; Zykwinska et al., 2005). Bacterial cellulose is rich in the $I_a$ allomorph while the secondary walls of higher plants mainly contain the $I_b$ allomorph (Atalla and VanderHart, 1984), whose conformations and hydrogen-bonding patterns differ from
Those of the Iα allomorph (Nishiyama et al., 2002; Nishiyama et al., 2003). In Arabidopsis thaliana leaves, the cellulose 13C signals indicate the presence of both Iα and Iβ allomorphs, with slightly higher Iβ contents (Newman et al., 1996). The detailed structures of primary-wall microfibrils are still unknown, but in silico results suggest that cellulose chains in small microfibrils differ from those in extended crystalline phases in terms of chain tilts and various dihedral angles (Oehme et al., 2015). In addition, commercial Avicel microcrystalline cellulose has been shown to bind pectins more weakly than plant cell wall cellulose (Zykwinska et al., 2005), possibly due to its decreased surface area. Thus, the use of cellulose with different origins from the plant cell wall calls for caution in interpreting the results of in-vitro binding assays.

Third, in-vitro binding assays generally report much lower binding than in-vitro extraction assays (Ryden and Selvendran, 1990; Oehme et al., 2015), which also report the strength of the intermolecular interactions. This discrepancy also exists for xyloglucan, where both the amount and strength of binding to cellulose are much less for in-vitro assays than is the case in vivo in plant cell walls (Hayashi et al., 1987). On the molecular level, this discrepancy between the bottom-up and top-down assays is conceivable because absorption studies limit possible binding to the cellulose surface, while polysaccharide entrapment within or between microfibrils can occur during cell wall biosynthesis. It is known that primary wall cellulose has a lower crystallinity than the crystallinity of cellulose in synthetic composites, further suggesting that in-vitro binding assays cannot reproduce the complexity of the polysaccharide interactions in the native wall. Taken together, these considerations suggest that the cellulose-pectin NMR cross peaks observed in never-dried walls at ambient temperature are more authentic indicators of the wall structure than in-vitro assays of cellulose-pectin composite formation.

While the current study demonstrates the existence of cellulose-pectin close contacts in the never-dried primary wall, the nature of this intermolecular interaction requires further investigations. One question is whether RGI or HG is more responsible for interacting with cellulose. At present it is difficult to fully resolve the HG and RGI 13C chemical shifts. Polysaccharide-specific isotopic labeling and/or mutant plants deficient in one of the two pectins would be desirable for answering this question. It is also unclear whether pectins can interact with multiple microfibrils at the same time, which is a prerequisite for the load-bearing function in both the tethered-network and the ‘hotspots’ model.

Material and Methods

Plant material

Never-dried Arabidopsis thaliana primary cell walls (CW#1) were prepared and 13C labeled at Penn State University as described recently (White et al., 2014). Briefly, Arabidopsis seedlings were harvested after dark growth for 14 days in liquid culture containing 13C-labeled glucose as the sole carbon source. The seedlings were frozen, ground in liquid nitrogen and washed with 1.5% SDS for 3 h to solubilize cell membranes, proteins and to inactivate endogenous wall-degrading enzymes (Zablackis et al., 1995; Gibeaut et al., 2005). The material was washed extensively in water, incubated with α-amylase (5000 units in 30 mL) from porcine pancreas (Sigma Adrich) in sodium MES buffer (pH 6.8) to remove starch, then digested with Pronase (Roche Life Sciences; 200 units, 5 mg in 20 mL) in sodium MES buffer (pH 7.5) to digest proteins. The Pronase was finally inactivated by incubating the sample with shaking in 1.5% SDS at room temperature for 1 h. 0.02% NaN3 was used in all solutions to inhibit microbial growth.
growth. Bulk water was removed by centrifugation (White et al., 2014). This sample preparation procedure avoided extensive washes with organic solvents and air drying, which were used in an earlier sample prepared at Iowa State University (CW#4) (Dick-Perez et al., 2011). Walls that have been frozen, thawed and washed with detergents retain their original mechanical characteristics as well as microscopic appearance as seen by atomic force microscopy (Zhang et al., 2014), so the molecular interactions are believed to be largely perturbed by such treatment. Approximately 60 mg of the never-dried CW#1 was packed into 4 mm magic-angle-spinning (MAS) rotors for SSNMR experiments.

As controls, another 60 mg of the same batch of never-dried wall was packed into an MAS rotor and lyophilized for one day. 47 mg water was removed and a $^1$H NMR spectrum confirmed the loss of bulk water. $^{13}$C spectra of this dehydrated CW#2 sample were measured. Subsequently, an equivalent amount of water was added back to this dehydrated sample and mixed homogeneously for a few minutes to produce CW#3. The dried wall absorbed water readily.

We also re-measured the organic-solvent washed, air-dried and rehydrated CW#4. This sample was used to obtain the previously reported cellulose-pectin cross peaks (Dick-Perez et al., 2011; Wang et al., 2012). We measured relaxation-compensated difference 2D spectra on this CW#4 to verify the previous results and to compare with the never-dried CW#1.

A partially depectinated sample, CW#5, was prepared by treating the never-dried CW#1 with 50 mM trans-1,2-cyclohexanediaminetetraacetic acid (CDTA) for 16 h at 25°C with three changes of solution to chelate Ca$^{2+}$ ions and solubilize HG, then with 50 mM sodium carbonate with 20 mM NaBH$_4$ (to minimize polysaccharide degradation) at 22°C for 16 h with three changes of solution to neutralize galacturonic acid and hydrolyze methyl esters to carboxylate ions. Quantitative $^{13}$C NMR spectra show that ~40% of HG was removed by this protocol while the majority of RGI (~95%) remained (White et al., 2014). This HG-depleted sample was measured to compare with the never-dried CW#1.

**Solid-state NMR experiments**

Most SSNMR experiments were conducted on a Bruker Avance II 600 MHz spectrometer (14.1 Tesla) using a 4 mm MAS probe. $^{13}$C-$^1$H HETCOR spectra were measured on a Bruker 900 MHz spectrometer (21.1 T) using a 3.2 mm MAS probe. PDSD experiments of CW#5 at -18°C and CW#1 at 20°C were measured on a Bruker Avance III 400 MHz spectrometer using a 4 mm MAS probe. Typical radiofrequency (rf) field strengths were 50-83 kHz for $^1$H and 40-50 kHz for $^{13}$C. $^{13}$C chemical shifts were externally referenced to the adamantane CH$_2$ signal at 38.48 ppm on the TMS scale.

1D $^{13}$C MAS spectra were measured at 20°C under 9 kHz MAS using either DP or CP to create the initial $^{13}$C magnetization. DP experiments were conducted with a long recycle delay of 25 s to obtain quantitative spectra or a short recycle delay of 2 s to selectively detect dynamic polysaccharides, while CP experiments were used to preferentially detect rigid polysaccharides’ signals.
2D $^{13}$C-$^{13}$C PDSD experiments were conducted on the never-dried CW#1, rehydrated CW#3, and HG-depleted CW#5 with mixing times of 1.0 – 1.5 s to detect long-range (~1 nm) intermolecular correlations under varying hydration histories and polysaccharide densities. These spectra were measured at -20°C where both pectins and cellulose were immobilized, which facilitate magnetization transfer (Wang et al., 2012). The 2D experiments were also conducted at 20°C on CW#1 with mixing times of 5–500 ms to verify whether the intermolecular contacts also exist at ambient temperature. The relaxation-compensated PDSD experiment was conducted on CW#4 to obtain clean difference spectra showing only intermolecular cross peaks (Wang et al., 2015). The experiment inserts a z-filter before the evolution period, so that the sum of the z-filter and the spin diffusion mixing time is a constant value of 1.005 s. Two parent spectra, with mixing times of 1.0 s and 200 ms, were measured, and a difference spectrum was obtained by subtracting the 200 ms spectrum after scaling by 0.83 from the 1.0 s spectrum.

The amplitudes and rates of polysaccharide motion were measured on the never-dried CW#1 and rehydrated CW#3. $^{13}$C-$^{1}$H dipolar chemical-shift (DIPSHIFT) correlation experiments (Munowitz et al., 1981) were conducted at 20°C under 7 kHz MAS to measure the C-H order parameters ($S_{CH}$). The FSLG sequence (Bielecki et al., 1989) was used for $^{1}$H homonuclear decoupling. This theoretical scaling factor of 0.577 was verified using the model peptide formyl-Met-Leu-Phe (MLF) (Rienstra et al., 2002). The ratio of the true coupling to the rigid-limit value of 22.7 kHz gives the $S_{CH}$ (Fig. S3). $^{13}$C T$_1$ relaxation times were measured using a z-filter sequence (Torchia, 1978) at 20°C under 9 kHz MAS. Most relaxation decays are well fit by a double exponential function (Tables 2, 3, Fig. S4).

2D $^{1}$H-$^{13}$C HETCOR experiments were carried out at 20°C under 14.8 kHz MAS on a 900 MHz spectrometer. For the Lee-Goldburg (LG) HETCOR experiment, $^{1}$H homonuclear decoupling was achieved using the FSLG sequence with a $^{1}$H transverse field strength of 80 kHz during $t_1$ evolution, followed by a mixing period for $^{1}$H spin diffusion. The $^{1}$H magnetization was transferred to $^{13}$C by 300 μs LG-CP (van Rossum et al., 2000). The $^{1}$H chemical shift was calibrated using MLF (Li et al., 2010). To detect unambiguous polysaccharide-water cross peaks, we also measured dipolar-edited medium- and long-distance (MELODI) HETCOR spectra (Yao and Hong, 2001; Yao et al., 2001). Eight rotor periods of C-H dipolar dephasing were used before the $^{1}$H $t_1$ evolution period to dephase the $^{1}$H signals of $^{13}$C-labeled polysaccharides and retain only the water $^{1}$H cross peaks to the polysaccharide $^{13}$C signals. The 180° pulse lengths were 10 μs for $^{13}$C and 6.25 μs for $^{1}$H. Hartman-Hahn (HH) CP with 300 μs contact time was used for the MELODI-HETCOR experiments.
1D $^{13}$C quantitative DP spectra (a) and $^1$H spectra (b) of *Arabidopsis* cell walls at 20°C under 9 kHz MAS. (a) $^{13}$C spectra of never-dried CW#1, dried CW#2 and rehydrated CW#3. (b) $^1$H spectra, showing the relative water intensities. Lyophilization removed all bulk water. The remaining $^1$H signals in the dried wall result from lipids. The dried CW#2 exhibits severely broadened $^{13}$C signals, but the linewidths are fully restored by rehydration in CW#3. The abbreviations for assignment are given.
Figure 2. 1D $^{13}$C CP (a) and DP (b) spectra of never-dried, dried, and rehydrated *Arabidopsis* primary walls at 20°C. The CP spectra selectively detect the signals of rigid polysaccharides, while the DP spectra with 2 s recycle delays preferentially detect the signals of dynamic polysaccharides. Rehydration fully restored polysaccharide linewidths to those seen for the never-dried CW#1.
Figure 3. 1.5 s 2D PDSD spectra of *Arabidopsis* cell walls. (a) Never-dried CW#1. (b) Rehydrated CW#3. The spectra were measured at -20°C under 9 kHz MAS. (c) Representative cellulose cross sections at various mixing times, normalized to the diagonal peaks (asterisk). (d) Representative pectin cross sections. Intermolecular cellulose-pectin cross peaks are annotated. All cross peaks detected in the rehydrated CW#3 are also observed in the never-dried CW#1 with similar intensities, confirming that the close pectin-cellulose contact is intrinsic to the primary cell wall.
Figure 4. Difference 2D $^{13}$C PDSD spectra of CW#4 with $T_1$-relaxation compensation to detect intermolecular correlations. (a) Difference 2D spectrum between 1.0 s and 200 ms, measured with a constant z-period of 1.005 s, 8 kHz MAS and at -20°C. Negative intensities along the diagonal are shown in green. Red shaded areas indicate intramolecular peaks that are present in the parent spectra, which are removed in the difference spectrum. (b) Selected 1D cross sections of cellulose, showing the suppression of intramolecular cross peaks to better resolve the intermolecular cross peaks. Asterisks denote the diagonal peaks. (c) Representative 1D cross sections of pectins.
Figure 5. 2D PDSD spectra of never-dried CW#1 at ambient temperature, showing retention of pectin-cellulose cross peaks. (a) 0.5 s PDSD spectrum at 20°C. A few cellulose-pectin cross peaks are assigned. (b) Representative cellulose cross sections from 2D spectra with mixing times of 30 ms and 0.5 s. The 1.5 s spectrum measured at -20°C is superimposed for comparison. Pectin-cellulose cross peaks are present at high temperature, indicating that the intermolecular contacts detected at -20°C are not due to freezing. The high-temperature cross peaks are lower than at low temperature due to conformational dynamics of the pectins. Asterisks indicate diagonal peaks.
Figure 6. Comparison of polysaccharide mobility between the never-dried CW#1 (left) and the rehydrated CW#3 (right). (a) C-H order parameters $S_{CH}$, obtained from a quantitative DIPSHIFT spectrum measured at 20°C. The $S_{CH}$'s are very similar between the two samples, indicating similar motional amplitudes. (b) $^{13}$C $T_1$ relaxation times. Matrix polysaccharides exhibit a short (cyan) and long (magenta) $T_1$ component, which likely correspond to cellulose-unbound and bound fractions. (c) Fractions of the short and long $T_1$ components. The never-dried and rehydrated walls have similar $T_1$ distributions.
Figure 7. 2D $^{13}$C-$^1$H HETCOR spectra of never-dried CW#1 to probe water-polysaccharide interactions. The spectra were measured at 20°C under 14.8 kHz MAS. (a) 2D HETCOR spectrum without and with $^1$H spin diffusion. (b) MELODI-HETCOR spectrum without and with spin diffusion. (c) Representative $^1$H cross sections from the HETCOR (black) and MELODI-HETCOR (blue) spectra with 0 and 1 ms $^1$H spin diffusion. (d) $^1$H projection of the 1 ms HETCOR spectrum (black) compared with the water $^1$H cross section of the 1 ms MELODI-HETCOR spectrum (blue). The water cross section shows higher pectin intensities. (e) Expanded cellulose region of (d). The water-contacting cellulose has narrower linewidth than the average cellulose.
Figure 8. Observation of cellulose-pectin cross peaks in HG-reduced CW#5. (a) 2D PDSD spectrum with 1 s $^{13}$C spin diffusion. Some of the cellulose-pectin cross peaks are assigned. (b) Comparison of the main cellulose cross sections between CW#5 (blue) and CW#1 (black). (c) Comparison of the main pectin cross sections between CW#5 and CW#1. The HG-reduced wall shows similar cellulose-pectin cross-peak intensities as the intact wall. The two spectra were measured at about -20°C under 9 kHz MAS.
Table 1. Cell wall samples and preparation conditions.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Preparation conditions</th>
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<tr>
<td>CW#1</td>
<td>Never-dried cell wall produced at Penn State</td>
</tr>
<tr>
<td>CW#2</td>
<td>Lyophilized cell wall from the same batch as CW#1</td>
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<tr>
<td>CW#3</td>
<td>CW#2 rehydrated</td>
</tr>
<tr>
<td>CW#4</td>
<td>Cell wall produced at Iowa State, washed with organic solvents, air-dried and rehydrated</td>
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<tr>
<td>CW#5</td>
<td>CW#1 that was extracted with CDTA and Na₂CO₃</td>
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</table>

Table 2. $^{13}$C $T_1$ relaxation times of never-dried CW#1. The single and double exponential functions used to fit the data are $I(t) = e^{-t/T_{1a}}$ and $I(t) = ae^{-t/T_{1a}} + be^{-t/T_{1b}}$ where $b = 1-a$.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>$\delta_C$ (ppm)</th>
<th>a (mobile)</th>
<th>b (rigid)</th>
<th>$T_{1a}$ (s)</th>
<th>$T_{1b}$ (s)</th>
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<tbody>
<tr>
<td>i/s/G/GalC1</td>
<td>105.0</td>
<td>1</td>
<td></td>
<td>5.1±0.1</td>
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<tr>
<td>HG/RC1</td>
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<td>0.39±0.01</td>
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<tr>
<td>x/GAC1</td>
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<td>0.50±0.02</td>
<td>0.44±0.03</td>
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<tr>
<td>iC4</td>
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<td>1</td>
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<tr>
<td>s/GC4</td>
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<td>xC4</td>
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<tr>
<td>(H)GAC2/RC5</td>
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<td>0.82±0.01</td>
<td>0.20±0.03</td>
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Table 3. $^{13}$C $T_1$ relaxation times of Arabidopsis CW#3.

<table>
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<tr>
<th>Assignment</th>
<th>$\delta_C$ (ppm)</th>
<th>a (mobile)</th>
<th>b (rigid)</th>
<th>$T_{1a}$ (s)</th>
<th>$T_{1b}$ (s)</th>
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<tr>
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<td>1</td>
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<tr>
<td>s/GC4</td>
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<tr>
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<td>0.09±0.02</td>
<td>4.2±0.1</td>
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