Water—Polysaccharide Interactions in the Primary Cell Wall of *Arabidopsis thaliana* from Polarization Transfer Solid-State NMR

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**ABSTRACT:** Polysaccharide-rich plant cell walls are hydrated under functional conditions, but the molecular interactions between water and polysaccharides in the wall have not been investigated. In this work, we employ polarization transfer solid-state NMR techniques to study the hydration of primary-wall polysaccharides of the model plant, *Arabidopsis thaliana*. By transferring water $^{1}$H polarization to polysaccharides through distance- and mobility-dependent $^{1}$H—$^{1}$H dipolar couplings and detecting it through polysaccharide $^{13}$C signals, we obtain information about water proximity to cellulose, hemicellulose, and pectins as well as water mobility. Both intact and partially extracted cell wall samples are studied. Our results show that water—pectin polarization transfer is much faster than water—cellulose polarization transfer in all samples, but the extent of extraction has a profound impact on the water—polysaccharide spin diffusion. Removal of calcium ions and the consequent extraction of homogalacturonan (HG) significantly slowed down spin diffusion, while further extraction of matrix polysaccharides restored the spin diffusion rate. These trends are observed in cell walls with similar water content, thus they reflect inherent differences in the mobility and spatial distribution of water. Combined with quantitative analysis of the polysaccharide contents, our results indicate that calcium ions and HG gelation increase the amount of bound water, which facilitates spin diffusion, while calcium removal disrupts the gel and gives rise to highly dynamic water, which slows down spin diffusion. The recovery of spin diffusion rates after more extensive extraction is attributed to increased water-exposed surface areas of the polysaccharides. Water—pectin spin diffusion precedes water—cellulose spin diffusion, lending support to the single-network model of plant primary walls in which a substantial fraction of the cellulose surface is surrounded by pectins.

**INTRODUCTION**

The primary cell wall of growing plants contains a mixture of polysaccharides and glycoproteins that provide mechanical strength to the cell, protect cells against biotic and environmental stresses, and allow cell—cell adhesion.1,2 At the core of this polysaccharide mixture are nanometer-sized cellulose microfibrils, which interact with matrix polysaccharides. In dicotyledonous plants such as *Arabidopsis thaliana*, these matrix polysaccharides mainly consist of the neutral hemicellulose, xyloglucan (XyG), and negatively charged pectins, which are galacturonic acid (GalA)-rich polymers. Two major types of pectins are found in dicot primary walls: the linear homogalacturonan (HG) and rhamnogalacturonan I (RGI), which contains arabinan (Ara) and galactan (Gal) side chains of varying lengths. The molecular packing and three-dimensional architecture of primary wall polysaccharides have been extensively characterized by chemical extraction and biochemical assays. Recent evidence from 2D and 3D solid-state NMR spectroscopy3,4,9,10 and biomechanical studies of endoglucanase-treated cell walls7 suggests that, in contrast to the conventional tethered network model,8 cellulose microfibrils are in close contact with both pectins and hemicellulose on the sub-nanometer scale, but the microfibrils are neither mechanically tethered by XyG nor extensively coated with XyG. This polysaccharide network exhibits heterogeneous mobility: the cellulose chains are immobilized by intra- and intermolecular hydrogen bonding and hydrophobic interactions, whereas pectins exhibit large-amplitude motion. This motional heterogeneity is manifested by the selective detection of pectin signals in certain solid-state NMR spectra3,4,9,10 and by different nuclear-spin relaxation times.11—13 The mobility of hemicellulose is intermediate between those of cellulose and pectins.9

A different approach for investigating the structure and dynamics of the plant cell wall is to probe the water accessibilities of polysaccharides. Water is a prerequisite for wall-degrading enzymes and is essential for polymer creep of the primary wall during growth. Cell wall hydration depends on the ionic content and intermolecular packing of the polysaccharides. It is well-known that anionic pectins strongly attract water and are the main swelling agent of the cell wall.14 Indirect evidence of preferential hydration of pectins in the wall is seen in the reduced pectin intensities in $^{13}$C cross...
solid-state NMR techniques that transfer the water $^1$H polarization to polysaccharides. But direct measurement of pectin–water interaction has not been reported. Cellulose–water interactions in native cell walls are even less studied. Molecular mechanics simulations of cellulose–water interactions found that water changes the hydroxymethyl (C6) conformation of cellulose I −. These simulations also showed that water can cause twisting of cellulose I −. Experimental conditions that water changes the hydroxymethyl (C6) conformation of cellulose I − have been reported. Cellulose − water and hemicellulose − water interactions in Arabidopsis thaliana primary walls have not been measured. However, hydration-induced pectin − water interaction has not been measured. The polarization transfer rates are strongly affected by the concentration and extent of HG esterification and by the relative amounts of matrix polysaccharides to cellulose. These results reveal a profound effect of HG on water mobility and the impact of extraction on the water-exposed surface areas of polysaccharides in the wall.

### Table 1. Relative Intensities of Polysaccharide $^{13}$C Signals of Arabidopsis Primary Walls from Quantitative 1D $^{13}$C Spectra

<table>
<thead>
<tr>
<th>$^{13}$C chemical shift (ppm)</th>
<th>assignment</th>
<th>sample 1</th>
<th>assignment changes</th>
<th>sample 2</th>
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<tr>
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<td>A C1</td>
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<td>0.79</td>
<td>0.53</td>
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<tr>
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<td>1.00</td>
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<tr>
<td>65</td>
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<td></td>
<td>0.99</td>
<td>0.89</td>
<td>0.96</td>
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<tr>
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<td>i/s/Gal/G C6, x/A C5</td>
<td>1.00</td>
<td></td>
<td>1.02</td>
<td>0.83</td>
<td>0.79</td>
</tr>
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</table>

The intensities are normalized to the height of the 89 ppm cellulose C4 peak in each sample, and then normalized to the value of sample 1.

### Experimental Section

**Plant Material.** Uniformly $^{13}$C-labeled Arabidopsis thaliana primary walls were prepared from seedlings grown in liquid culture, using a procedure adapted from Gibeaut and co-workers. The walls were never dried throughout the preparation. Plant tissue was frozen at −80 °C, ground in liquid nitrogen, and incubated with shaking in 1.5% sodium dodecyl sulfate (SDS) at room temperature (RT) for 3 h to inactivate endogenous wall-degrading enzymes. The tissue was washed 10 times with double-distilled water (ddH2O), incubated with shaking at 37 °C for 12 h in 50 mM MES buffer (pH 6.8) that contains porcine pancreas α-amylase (5000 units, 30 mL −1) to remove starch and 0.02% NaN3 to inhibit microbial growth. The walls were washed again three times with ddH2O, incubated with shaking at 40 °C for 12 h in 50 mM MES buffer (pH 7.5) that contains Pronase (200 units, 5 mg mL −1) to further digest proteins and 0.02% NaN3 to inhibit microbial growth. The sample was washed again three times with ddH2O, incubated with shaking in 1.5% SDS at RT for 1 h to inactivate Pronase, and washed 10 times with 0.02% NaN3, as a final step to prevent microbial contamination during storage and NMR experiments.

The intact cell walls prepared above were subjected to sequential extraction to create a total of four NMR samples. Sample 1 is the unextracted wall, while the other three samples were sequentially treated with trans-1,2-cyclohexanediiminetetraacetic acid (CDTA), sodium carbonate (Na2CO3), xyloglucanase (XEG), and Cel12A to selectively remove matrix polysaccharides. Sample 2 was extracted with 50 mM CDTA (pH 6.5) overnight (with three exchanges), followed by a second overnight extraction with three exchanges of 50 mM Na2CO3 containing 20 mM NaBH4. CDTA chelates calcium ions (Ca2+), which removes the ionic cross-links between the carboxyl groups of HG, thus solubilizing HG. Na2CO3 neutralizes GaA and hydrolyzes methyl esters to carboxylate ions, further solubilizing HG. Na2CO3 also hydrolyzes the esters in RGI. However, most GaA in RGI is not methyl-esterified but is acetylated at O3, and acetyl groups are more resistant to hydrolysis because of the bulkiness of the sugar, the electronic effects of the secondary alcohol, and the presence of neutral side chains in RGI, which make RGI more hemicellulose-like than HG. The sample was washed again three times with ddH2O, incubated with shaking in 1.5% SDS at RT for 1 h to inactivate Pronase, and washed 10 times with 0.02% NaN3, as a final step to prevent microbial contamination during storage and NMR experiments.

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Sample 3 was sequentially treated with CDTA, Na2CO3, and XEG (200 μg mL −1), a recombinant XyG-specific endoglucanase. The treatment with XEG was carried out in 100 mM ammonium acetate (pH 5.0) with 0.02% NaN3 at 37 °C for 48 h to remove the majority of non-load-bearing XyG. Sample 4 was sequentially treated with CDTA, Na2CO3, XEG, and an evolved Cel12A (200 μg mL −1). Cel12A is a recombinant endoglucanase that digests some of the load-bearing XyG. The sample was washed again three times with ddH2O, incubated with shaking in 1.5% SDS at RT for 1 h to inactivate Pronase, and washed 10 times with 0.02% NaN3, as a final step to prevent microbial contamination during storage and NMR experiments.
Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, at 37 °C for 48 h. Avicel PH-101 was purchased from Sigma-Aldrich and used as received.

The cell wall samples were centrifuged at 1000g for 5 min and 5000g for 90 min to reach a hydration level of ~40 wt%, as verified by gravimetric analysis of the unextracted wall. Monosaccharide compositions of the four samples were analyzed as described previously, but with the addition of a methanolysis step before hydrolysis with trifluoroacetic acid, which otherwise reduces galacturonic acid content by up to 70%.

About 65 mg of each cell wall sample was packed into 4 mm magic-angle-spinning (MAS) rotors for solid-state NMR experiments. Intensity quantification (Table 1) and sugar composition analysis (Table 2) were used to estimate the relative concentrations of pectins, 

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fuc</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc\textsuperscript{b}</th>
<th>Xyl/Man\textsuperscript{b}</th>
<th>GaLA</th>
<th>GluA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5 ± 0.03</td>
<td>10.0 ± 0.09</td>
<td>10.4 ± 0.18</td>
<td>9.5 ± 0.14</td>
<td>8.0 ± 0.13 (20.2 ± 0.75)</td>
<td>15.8 ± 0.37 (0.9 ± 0.02)</td>
<td>23.8 ± 0.38</td>
<td>0.3 ± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>1.7 ± 0.06</td>
<td>9.9 ± 0.16</td>
<td>12.9 ± 0.21</td>
<td>10.8 ± 0.13</td>
<td>10.7 ± 0.18 (22.9 ± 0.29)</td>
<td>17.9 ± 0.09 (1.6 ± 0.14)</td>
<td>11.6 ± 0.10</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>1.5 ± 0.05</td>
<td>10.3 ± 0.05</td>
<td>13.6 ± 0.19</td>
<td>10.5 ± 0.11</td>
<td>8.6 ± 0.10 (26.2 ± 0.33)</td>
<td>18.1 ± 0.42 (2.1 ± 0.27)</td>
<td>11.7 ± 0.28</td>
<td>0.2 ± 0.07</td>
</tr>
<tr>
<td>4</td>
<td>1.4 ± 0.03</td>
<td>9.9 ± 0.15</td>
<td>13.1 ± 0.12</td>
<td>10.1 ± 0.03</td>
<td>5.5 ± 0.07 (29.1 ± 0.67)</td>
<td>17.5 ± 0.6 (1.9 ± 0.20)</td>
<td>11.4 ± 0.27</td>
<td>0.1 ± 0.07</td>
</tr>
</tbody>
</table>

**Table 2. (Top) Monosaccharide Compositions (Mol % of Total Sugars, ±SEM, n = 3 Technical Replicates) of the Four Cell Wall Samples Used for NMR Analysis and (Bottom) Cell Wall Compositions (%) Estimated from the Sugar Analysis**

The number in each entry shows the sugars, primarily from matrix polysaccharides, released by methanolysis and hydrolysis of the samples with 2 M trifluoroacetic acid (TFA). The bottom number, in parentheses, shows sugars in the residues (mainly cellulose). Our analysis did not separate xylose from mannose. * Using a method described by Wang et al. in 2013.**

**RESULTS**

**Polysaccharide Compositions of Sequentially Extracted Cell Walls.** Quantitative \(^{13}\)C spectra were measured using recycle delays of 25–30 s to determine the polysaccharide contents and their changes due to sequential extraction. The intact wall shows well-resolved \(^{13}\)C signals for matrix polysaccharides and crystalline cellulose (Figure 2a). Participation...
GalA, galacturonic acid; Gal, galactan.

Glucose in xyloglucan; x, xylose; A, arabinan; R, rhamnose; GA and RGI are suppressed by a significant increase of the 100 ppm peak, suggesting that RGI was retained to ~90% in sample 2 whereas HG concentration decreased to ~64% (Tables S1 and S2). Interestingly, although the total pectin concentration is lower in sample 2, the integrated COO⁻ signals in the quantitative spectra are similar between the two samples (Figure 2a,b), indicating that the loss of HG is offset by the conversion of methyl esters to carboxylates. The 176 ppm COO⁻ peak is also much narrower in sample 2, suggesting that the non-cross-linked HG chains have either larger mobility or more uniform conformation.

Additional digestion by XEG reduced the intensities of the 99 ppm peak, the 79 ppm peak, and the 68 ppm peak (Figure 2c), which correspond to GalA/Xyl C1, GalA C4 and Rha/Ara C2, and Rha/Ara C5 and GalA/HG C2, respectively. These changes indicate that XyG as well as RGI are extracted by XEG, which may occur to XyG-bound pectins during the washing step. Assuming that the cellulose concentration is constant in these samples, the total pectin concentration in sample 3 is about half the pectin concentration of the intact wall, whereas the XyG concentration in sample 3 is ~70% that of the intact wall (Tables S2 and S3). Finally, the Cel12A-extracted sample 4 shows only 30% and 40% of the pectin and hemicellulose quantities as the intact sample. For comparison, the ¹³C CP-MAS spectra of the four samples are shown in Figure S2.

Monosaccharide analysis (Table 2) agrees well with the NMR spectra about the changes of the XyG concentrations with extraction (Tables S2 and S3), but reports lower absolute amounts of cellulose and XyG and higher amounts of pectins compared to the quantitative NMR spectra (Tables 2 and S4). One possible origin of this discrepancy is that sugar analysis reports the amount of RGI side chains (Ara and Gal) well, but Gal and Ara signals are not well resolved in the NMR spectra. Thus, the NMR-deduced total mass concentration of RGI is less accurate than the NMR-deduced backbone concentration of pectins. We estimated the total pectin molar amounts using a side chain/backbone mole ratio of 2.4–2.7, which was obtained from sugar analysis. Despite these uncertainties, the NMR and monosaccharide analyses agree on the trend of pectin reduction by sequential extraction.

**Polysaccharide Hydration in the Intact Cell Wall.** We first investigated the water content and water mobility of the four cell wall samples using ¹H NMR spectra (Figure 3). The spectra show similar integrated intensities of water within 20%, indicating that the total water content of the four cell walls is similar. However, the water ¹H line widths and T₂’s differ significantly. The intact wall has the largest line width of 76 Hz and the shortest ¹H T₂ (0.12 s) (Table S5), whereas the extracted cell walls exhibit narrower water line widths of 9–27 Hz and longer T₂’s of 0.9–1.4 s, which are closer to bulk water properties. Sample 2 has the narrowest water ¹H line width and the highest integrated intensity among the four samples,

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**Figure 1.** (a) Pulse sequence of the water–polysaccharide spin diffusion experiment. The 2D version is shown here. Removal of the t₁ and t₂ periods converts the experiment to 1D. (b) ¹H spectra of sample 1 without (black) and with (red) a T₂ filter (1.6 ms). Inset shows a 32-fold y-axis enlarged spectrum of the lipid ¹H chemical shift region. (c) ¹³C CP-MAS spectra of sample 1 without T₂ filter (black), with a 1.6 ms ¹H T₂ filter but no spin diffusion (SD) (red), and with a 1.6 ms T₂ filter and 2 ms spin diffusion (blue). The ¹H and ¹³C spectra were measured at 263 K under 9 kHz MAS. Assignment abbreviations: i, interior crystalline cellulose; s, surface cellulose; G, glucose in xyl glucan; x, xylose; A, arabinan; R, rhamnose; GA and GalA, galacturonic acid; Gal, galactan.
indicating that it contains the most dynamic water. These line
widths and $T_1$ differences indicate that water is more tightly
bound to the intact wall than to the extracted walls, causing
lower mobility and higher chemical exchange rates with the
polysaccharides.42

Representative water−polysaccharide $^1$H spin diffusion data
are shown in Figure 4 for the intact wall sample. Water protons
were selected as the magnetization source using a $^1$H $T_2$
delay of 1.2−2.6 ms, which was optimized to preserve water
magnetization maximally while suppressing most of the
polysaccharide $^{13}$C signals. The $T_2$ filter was unsynchronized
with MAS so that recoupled anisotropic interactions better
suppress the polysaccharide $^1$H magnetization. Under these
conditions, the polysaccharide $^{13}$C signals were suppressed to
<3% while 60−80% of the water intensity was retained. Some
lipid $^1$H signals also remained but accounted for less than 2% of
the total $^1$H magnetization; thus, they have negligible effects on
water−polysaccharide spin diffusion. Due to the need to
transfer the polysaccharide $^1$H magnetization to $^{13}$C by dipolar
CP, the spin diffusion spectra preferentially enhance the signals
of the rigid cellulose while reducing the signals of the dynamic
pectins. However, at the experimental temperature of 263 K,
the CP spectral deviation from the quantitative DP spectra is
small, as shown in Figure S2. Thus, the majority of the matrix
polysaccharides are captured in the spin diffusion data.

Turning on the spin diffusion mixing time for as little as 2 ms
already caused significant polysaccharide $^{13}$C signals (Figure
1c). The matrix polysaccharide intensities are enhanced relative
to cellulose intensities. A series of 1D $^{13}$C spectra with varying
mixing times showed site-specific intensity buildup of the
polysaccharides (Figure 4). Plotted as the square root of the
mixing time to reflect the relayed nature of spin diffusion,43,44
most $^{13}$C signals exhibit a sigmoidal buildup: an initial slow
transfer is followed by a fast linear regime that terminates in a
plateau. All $^{13}$C sites reached equilibrium by $\sim$50 ms, and no
intensity drop was observed, consistent with the fact that the
water $^{1}$H $T_1$ is much longer than 50 ms. The matrix
polysaccharide peaks have the shortest equilibration times of
10−20 ms. In comparison, the crystalline cellulose peaks at 89
and 65 ppm display the slowest buildup, reaching equilibrium
after $\sim$35 ms. Since crystalline cellulose is not exposed to the
microfibril surface, water polarization transfer is most likely
relayed via surface cellulose through the $^1$H−$^1$H dipolar
coupling network of the microfibril. The buildup curves for
the mixed matrix polysaccharide and cellulose peaks such as the
84 and 62 ppm peaks equilibrated at intermediate times of $\sim$30
ms, consistent with the mixed nature of these peaks. Pectin and XyG showed the largest intensity differences from cellulose in the initial regime of 2−6 ms: the former recovered 70−80% of the full magnetization while cellulose attained only 30−40% of the maximum intensity. XyG and pectin signals are not well resolved in these 1D $^{13}$C spectra, thus only the average water contact of both matrix polysaccharides is reported from these 1D spin diffusion spectra. Taken together, these buildup curves indicate that water is much closer, and/or binds with much longer residence times, to pectins and hemicelluloses than to cellulose.

**Hydration of Extracted Cell Walls.** Partial extraction of pectins and hemicelluloses caused strikingly different water polarization transfer behaviors. For the CDTA/Na$_2$CO$_3$-treated sample 2, spin diffusion to both matrix polysaccharides and cellulose slowed down dramatically (Figure 5a). For example, the 69 ppm peak of GalA C2 and Rha C5 reached equilibrium at $\sim$150 ms instead of 15 ms in the intact wall, and the 89 ppm peak of crystalline cellulose equilibrated at 200 ms instead of 35 ms. Thus, the removal of calcium-cross-linked HG retarded water spin diffusion to the remaining polysaccharides. However, when half of the XyG is removed by XEG in sample 3, the spin diffusion rate partially recovered. The equilibration times shortened to $\sim$50 ms for the 69 ppm peak and $\sim$100 ms for cellulose, which are intermediate between the equilibration times of samples 1 and 2. Finally, further digestion by Cel12A in sample 4 fully restored the buildup rates, and cellulose intensities plateaued at about the same time as in the intact sample (Figure 5b). These buildup rate differences are not caused by centrifugal forces under 9 kHz spinning, since

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**Figure 3.** $^1$H spectra of the four cell wall samples at 296 K. Numbers associated with each peak indicate the integrated intensities of the water peak as normalized to the value of the intact wall of sample 1. Sample 1 exhibits the largest water line width and the shortest $^1$H $T_1$, indicating that water is well bound to the wall polysaccharides. The three extracted walls have narrower water line widths and longer $T_1$ values, indicating that water is less tightly bound to the polysaccharides.

**Figure 4.** Water−polysaccharide spin diffusion buildup curves of sample 1. Mixing times ranged from 0.1 to 49 ms. (a) Assigned $^{13}$C spectrum. (b) Buildup curves of the various peaks.
slowing down the MAS frequency to 4.5 kHz reproduced the spin diffusion curves (Figure S3).

The fact that more extensive extractions in samples 3 and 4 caused more similar water–cellulose spin diffusion rates as sample 1 is interesting. This may be coincidental, but may also suggest that HG and RGI cause a similar hydration environment for cellulose compared to extracted walls with lower levels of both pectins. We then asked the question whether in the limit of no matrix polysaccharides, water–cellulose spin diffusion would be similar to that of the most extracted wall of sample 4. We chose commercial Avicel Ph-101, which is microcrystalline cellulose obtained from acid hydrolysis of wood pulp.45,46 The 13C CP-MAS spectrum (Figure 2e) of unlabeled and 40% hydrated Avicel shows well-resolved 13C signals predominantly at crystalline cellulose chemical shifts, confirming that there are minimal amorphous cellulose and other polysaccharides.47 Water spin diffusion to Avicel equilibrated at ~100 ms (Figure S3b), similar to the buildup time of sample 3 but longer than the buildup time of sample 4.

Since water spin diffusion to polysaccharides depends on not only water proximity but also water mobility, we measured the 1H line widths of polysaccharide-proximal water follow the trend of the bulk water 1H line widths: the intact wall has a broad 1H line width of 118 Hz, whereas sample 2 has a much narrower line width of 31 Hz. Thus, the polysaccharide-proximal water is more dynamic in the extracted wall of sample 2 than in the intact wall. Interestingly, both samples show 0.1 ppm smaller 1H chemical shifts for the polysaccharide-bound water in the 2D spectra than the bulk water in the 1D spectra. This 1H chemical shift change may be caused by exchange between water and sugar moieties or weaker water–polysaccharide hydrogen-bonding compared to hydrogen-bonding in bulk water.

**Water-Transferred 2D 13C–13C Correlation Spectra.**

Water–polysaccharide spin diffusion also provides a method to simplify the 2D correlation spectra. Figure 7 compares the full 2D spectrum with the water-transferred 2D spectrum measured with mixing times of 4 ms for sample 1 and 25 ms for sample 2. To allow comparison, we plotted the 2D spectra with equal multiplication factors from a minimum contour level of 1.3% of the maximum intensity, which is found at (72.2, 72.2) ppm. The full 2D spectrum of sample 1 showed prominent cellulose peaks such as C1–C2 (105, 72 ppm) and C4–C2/5 (89, 75 ppm), whereas matrix polysaccharide peaks such as Xyl C1–C2...
and C4–C5 (70.0, 62.3 ppm) are much weaker. In the water-transferred 2D spectrum, the matrix polysaccharide intensities increased relative to the cellulose peaks. The (79.4, 68.8 ppm) peak of Rha C2–C3/5 and GalA C2–C4 showed the largest intensity increase, and the XyG and pectin cross peaks at (99.5, 72.2 ppm) and (100.3, 68.7 ppm) are also enhanced. Interestingly, the water-transferred spectrum of sample 2 showed preferential enhancement of the pectin signals relative to XyG. For example, the RGI and HG cross peak at (100.3, 68.7 ppm) is ∼50% higher than the Xyl cross peak at (99.5, 72.2 ppm). This contrasts with the trend that this pectin peak is ∼20% weaker than the Xyl peak in the full 2D spectrum. Indeed, when the water-transferred 2D spectra were measured as a function of mixing time, the pectin peaks show slightly faster water transfer than the hemicellulose peaks (Figure S4). This difference was not observable from the 1D spin diffusion spectra (Figure 4) because of insufficient resolution. Thus, water interacts with pectins slightly more strongly than with hemicellulose.

■ DISCUSSION

These water–polysaccharide ¹H polarization transfer results can be summarized as follows. First, water polarization transfer to pectins is always faster than to cellulose in every sample, despite the fact that pectins are more mobile than cellulose. The latter is shown by the smaller C–H order parameters of pectins at ambient temperature (Figure S5) compared to cellulose. This pectin mobility partially remains at 263 K, which attenuates the ¹H–¹H dipolar couplings between water and pectins. Despite this pectin mobility, water–pectin polarization transfer is more rapid than water–cellulose transfer, indicating that water is either closer to pectins to give stronger ¹H–¹H dipolar couplings, and/or has a longer residence time on pectins than on cellulose. We attribute this time-averaged water–pectin proximity to the charged carboxylates and the polar methyl esters and acetyl groups in HG and RGI. In comparison, the lower hydration of cellulose can be attributed to the water-excluding assembly of glucan chains in the microfibril, and matrix polysaccharides may further reduce the water-exposed surface area of cellulose covering the cellulose microfibrils.

The second observation, which is more unexpected, is that the water–polysaccharide spin diffusion rates are significantly impacted by extraction: sample 2 exhibited the slowest spin diffusion while samples 1 and 4 showed the fastest spin diffusion. Since the total water content is similar for the four samples, and since sample 2 is the least extracted among the three extracted walls, this spin diffusion trend neither correlates with the extent of extraction nor correlates with the total water content.

We first considered the model where the slow spin diffusion of sample 2 may result from a smaller amount of polysaccharide-bound water after preferential extraction of HG. Although the total water content is similar among the four
samples, in principle the amount of polysaccharide-associated water could differ, with some water being separated into bulk. Among all polysaccharides, HG has the largest number of carboxyl and methyl ester groups, thus its preferential loss in sample 2 (Tables S1 and S2) might decrease the amount of water between cellulose microfibrils. However, close inspection of the quantitative $\textsuperscript{13}C$ spectra (Figure 2) shows that the carboxylate concentration is similar between samples 1 and 2, since the 176 ppm peak has similar integrated intensities. Thus, the model of wall dehydration due to reduced anion content does not agree with the NMR data.

Instead, the model that explains both the slow polarization transfer of sample 2 and the quantitative NMR spectra is that loss of Ca$^{2+}$ by extraction significantly increased the water mobility in the wall so that spin diffusion is less efficient in sample 2. In the intact cell wall, Ca$^{2+}$ ions tightly coordinate carboxylates and mediate HG gelation. Both the carboxylates and the HG network partly immobilize and entrap water molecules, whose magnetization can thus be efficiently transferred to the polysaccharides due to stronger $^{1}H−^{1}H$ dipolar couplings (Figure 8a). The bound nature of water in the intact wall is manifested by the larger water $^{1}H$ line width of sample 1 in both the 1D $^{1}H$ spectrum (Figure 3) and the 2D spectrum that detects polysaccharide-associated water (Figure 7).

After Ca$^{2+}$ ions were removed by CDTA, charge neutrality was maintained by sodium ions (Na$^{+}$) in solution, since the carboxylate concentrations remained constant between samples 1 and 2. These sodium ions are more weakly coordinated to the carboxylates and better solvated than Ca$^{2+}$.58 The different bond strength between calcium carboxylate and sodium carboxylate is due to the higher absolute electronegativity of Ca$^{2+}$ (31.6 eV) than Na$^{+}$ (26.2 eV).49 Indeed, electrical conductance of sodium-pectin samples is higher than that of calcium-pectin samples at the same hydration level.48 In addition to ion solvation effects, monovalent ions such as Na$^{+}$ and K$^{+}$ do not cause HG gelation,50 which further reduces the amount of bound water. The lower bound-water content of sample 2 is manifested by the narrower water $^{1}H$ line widths (Figures 3 and 6). Therefore, although the water content of sample 2 is slightly higher than sample 1, the larger water mobility slows down spin diffusion (Figure 8b).

It is worthwhile noting that the effect of Ca$^{2+}$ extraction on water mobility should not be confused with the effect of pectin de-esterification, and in fact the two effects are opposite of each other. Hydrolysis of methyl esters to carboxylates would promote the formation of calcium-chelated HG if Ca$^{2+}$ ions were present, thus it should increase HG gelation, not decrease it, which would in turn increase the bound water content and speed up spin diffusion. The fact that the opposite was observed must thus be attributed uniquely to Ca$^{2+}$ extraction and the consequent loss of HG and the loss of gelation.

The recovery of spin diffusion rates in samples 3 and 4 indicates a second factor regulating water—polysaccharide spin diffusion, which is the water-exposed surface area of the biopolymers. The increased digestions generate shorter XyG fragments and solubilize pectins, thus increasing the surface area of these matrix polysaccharides (Figure 8c). The digestion should also increase the water-exposed area of cellulose. It is difficult to estimate the relative increase of water exposure between matrix polysaccharides and cellulose, since water—cellulosic spin diffusion is likely relayed through matrix polysaccharides. This is suggested by the fact that spin diffusion rates changed uniformly and in the same direction for matrix polysaccharides and cellulose, and cellulose buildup lags behind matrix buildup in each sample.

The current results lend support to the single-network model of native plant walls concluded from 2D and 3D solid-state NMR spectra.3−5 The fact that the water—cellulose spin diffusion rate changes in the same direction as water—pectin spin diffusion indicates a significant fraction of the microfibril surface to be loosely surrounded by pectins. Similarly, the fact that pectins are solubilized by XEG and Cel12A digestion supports the notion that pectins and XyG are intimately entangled, as seen by the intermolecular cross peaks in previous 2D and 3D spectra.3−5

Microcrystalline cellulose, which has a mixture of hydrophobic and hydrophilic surfaces with different hydration properties and bound-water structures,15 showed comparable spin diffusion rates as cellulose in sample 3, despite the fact that sample 3 still contains matrix polysaccharides. This result suggests that the hydrophilicity of the cellulose surfaces in Avicel may be similar to the hydrophilicity of the cellulose microfibrils in the extracted cell wall.

![Figure 8. Model of water mobility and polysaccharide hydration in the Arabidopsis primary cell wall. (a) In the intact wall, Ca$^{2+}$-cross-linked HG chains create tightly bound water molecules that transfer polarization to polysaccharides efficiently. (b) Extraction of Ca$^{2+}$ and solubilization of HG reduce the amount of bound water, thus slowing down spin diffusion. (c) When XyG is extracted together with other pectins, the remaining polysaccharides have larger water-exposed surface area, thus speeding up spin diffusion.](dx.doi.org/10.1021/ja504108h)
The water mobility and accessibility information obtained here has no simple correlation with polysaccharide mobility. High polysaccharide mobility does not necessarily lead to fast or slow polarization transfer from water. For example, water spin diffusion to mobile pectins is faster than spin diffusion to the rigid cellulose in each sample. On the other hand, spin diffusion to the rigid cellulose in sample 3 is faster than spin diffusion to mobile pectins in sample 2. Magnetic-resonance studies of water dynamics and water interactions in a wide range of biological and engineering materials such as cartilage and cement have unraveled rich information on the physicochemical properties of these materials. Most of these studies measure water relaxation times to infer water interactions with the matrix. The current study has the advantage of directly correlating water and matrix protons in a site-specific manner. This correlation and spin diffusion NMR approach can be readily extended into relaxation NMR studies of other hydrated chemical and biological systems.

CONCLUSIONS

These water–polysaccharide 1H polarization transfer data provide molecular insights into the mobility of water and the amount of water-exposed surface areas of polysaccharides in non-grass primary cell walls. We found that calcium cross-links and HG gelation cause the formation of strongly bound water that transfers its polarization efficiency to surrounding polysaccharides. Calcium extraction and replacement by sodium ions reduce the number of tightly bound water molecules, thus slowing down spin diffusion to polysaccharides. Further extraction of matrix polysaccharides speeds up spin diffusion by increasing the water-exposed surface areas of polysaccharides. Thus, polysaccharide hydration is improved at later stages of extraction, after both pectins and hemicellulose polysaccharides. Thus, polysaccharide hydration is improved at later stages of extraction, after both pectins and hemicellulose polysaccharides. This is where pectin and hemicellulose polysaccharides have extensive molecular contact with cellulose. Comparative studies of the hydration of other cell walls such as grass cell walls and bacterial composites will be interesting in the future.

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Supporting Information

Water – Polysaccharide Interactions in the Primary Cell Wall of Arabidopsis thaliana from Polarization Transfer Solid-State NMR

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Table S1. Relative intensities of polysaccharide peaks from 2D \(^{13}\text{C}\) correlation spectra at 253 K.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Interior cellulose ((89, 75)) ppm (^1)</th>
<th>Xyl in XyG ((100, 72)) ppm (^2)</th>
<th>Pectin backbone ((100, 69)) ppm (^3)</th>
<th>Rha ((68, 17)) ppm (^4)</th>
<th>RG backbone ((68, 17)) ppm (^5)</th>
<th>RG backbone 17 ppm (^6)</th>
<th>Average RG backbone (^7)</th>
<th>HGA (^8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>0.19</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.94</td>
<td>0.95</td>
<td>0.18</td>
<td>0.99</td>
<td>0.79</td>
<td>0.9±0.1</td>
<td>0.64</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.75</td>
<td>0.74</td>
<td>0.10</td>
<td>0.56</td>
<td>0.51</td>
<td>0.54±0.03</td>
<td>0.59</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>0.43</td>
<td>0.52</td>
<td>0.08</td>
<td>0.44</td>
<td>0.32</td>
<td>0.38±0.06</td>
<td>0.39</td>
</tr>
</tbody>
</table>

1. The interior cellulose C4-C3 cross peak at \((89, 75)\) ppm is used to normalize matrix polysaccharide intensities.
2. The \((100, 72)\) ppm Xyl C1-C2 peak is used to quantify XyG amounts relative to cellulose.
\[ I_{XyG} = \frac{I_{(100,72)}}{I_{(89,75)}} \]
3. The \((100, 69)\) ppm cross peak of GalA C1-C2 in RG I and HGA and Rha C1-C5 is used to represent the total pectin backbone concentration.
\[ I_{Pectin} = \frac{I_{(100,69)}}{I_{(89,75)}} \]
4. The \((68, 17)\) ppm cross peak of Rha C5-C6 represents the Rha amount.
\[ I_{Rha} = \frac{I_{(68,17)}}{I_{(89,75)}} \]
5. The RG backbone concentration change from sample 1 is calculated by normalizing the Rha values with respect to that of sample 1.
6. The RG backbone concentration change was also estimated from the Rha C6 peak (17 ppm) intensity from the 1D quantitative \(^{13}\text{C}\) spectra.
7. Consensus values of the RG concentration changes are calculated as the average of the 1D and 2D values.
8. The HGA concentration change is calculated as
\[ I_{HGA} = \frac{\left( \frac{I_{100,69} - 2 \cdot I_{68,17}}{I_{100,69} - 2 \cdot I_{68,17}} \right)_{sample1}}{\left( \frac{I_{100,69} - 2 \cdot I_{68,17}}{I_{100,69} - 2 \cdot I_{68,17}} \right)_{sample1}}. \]

The factor of 2 accounts for the fact that the \((100, 69)\) ppm cross peak contains one HGA peak and two RG peaks.
Table S2. Quantification of pectins based on 2D NMR spectral intensities of Rha and GalA.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Pectin backbone (100, 69) ppm</th>
<th>Rha (68, 17) ppm</th>
<th>RG sidechain $^1$</th>
<th>RG amount $^2$</th>
<th>HGA amount $^3$</th>
<th>Pectin amount $^4$</th>
<th>Pectin/Cellulose NMR</th>
<th>Sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>0.19</td>
<td>0.55</td>
<td>0.93</td>
<td>0.92</td>
<td>1.9</td>
<td>0.83 (100%)</td>
<td>2.7 (100%)</td>
</tr>
<tr>
<td>2</td>
<td>0.95</td>
<td>0.18</td>
<td>0.52</td>
<td>0.88</td>
<td>0.59</td>
<td>1.5</td>
<td>0.65 (78%)</td>
<td>1.7 (63%)</td>
</tr>
<tr>
<td>3</td>
<td>0.74</td>
<td>0.10</td>
<td>0.27</td>
<td>0.47</td>
<td>0.54</td>
<td>1.0</td>
<td>0.44 (53%)</td>
<td>2.1 (78%)</td>
</tr>
<tr>
<td>4</td>
<td>0.52</td>
<td>0.08</td>
<td>0.19</td>
<td>0.24</td>
<td>0.36</td>
<td>0.60</td>
<td>0.26 (31%)</td>
<td>1.7 (63%)</td>
</tr>
</tbody>
</table>

1. The RG sidechain quantity is calculated as the Rha amount times 2.4-2.7, which is the ratio obtained from sugar analysis.

2. The RG amount is calculated as $I_{RG} = I_{RG, sidechain} + 2I_{Rha}$.

3. The HGA amount is calculated as $I_{HGA} = I_{pectin} - 2I_{Rha}$.

4. The NMR-derived pectin amount is calculated as $I_{Pectin} = I_{RG} + I_{HGA}$.

Table S3. XyG quantification from 2D SSNMR data and from sugar composition analysis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Interior cellulose (89, 75) ppm</th>
<th>All cellulose $^1$ (100, 72) ppm</th>
<th>Xyl in XyG $^2$</th>
<th>XyG $^3$</th>
<th>XyG : Cellulose ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NMR $^4$</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>2.3</td>
<td>1.1</td>
<td>2.5</td>
<td>1.1 (100%)</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>2.3</td>
<td>0.94</td>
<td>2.2</td>
<td>0.94 (85%)</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>2.3</td>
<td>0.75</td>
<td>1.7</td>
<td>0.75 (68%)</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>2.3</td>
<td>0.43</td>
<td>0.86</td>
<td>0.43 (39%)</td>
</tr>
</tbody>
</table>

1. The total amount of cellulose is estimated with a ratio of 1.0 : 1.3 between interior and surface cellulose. This ratio is obtained from a simulated cellulose model $^1$ and from 1D quantitative $^{13}$C spectra.

2. The Xyl amount is estimated from the Xyl C1-C2 (100, 72) ppm peak intensity and normalized to the cellulose concentration.

3. The total amount of XyG is calculated as the sum of Glc and Xyl amounts, assuming a Glc : Xyl ratio of 4 : 3.

4. The NMR-derived XyG: cellulose molar ratio is calculated as the ratio of the XyG and all-cellulose concentrations. The relative value to sample 1 is given in brackets. The NMR quantification result overall agrees well with the sugar composition result.
Table S4. Amounts of the three classes of polysaccharides from all $^{13}$C SSNMR data.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Cellulose$^1$</th>
<th>XyG$^2$</th>
<th>Pectins$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34%</td>
<td>37%</td>
<td>29%</td>
</tr>
<tr>
<td>2</td>
<td>39%</td>
<td>36%</td>
<td>25%</td>
</tr>
<tr>
<td>3</td>
<td>46%</td>
<td>34%</td>
<td>20%</td>
</tr>
<tr>
<td>4</td>
<td>57%</td>
<td>24%</td>
<td>19%</td>
</tr>
</tbody>
</table>

1. The cellulose amount includes both surface and crystalline cellulose.
2. The XyG amount includes both the Glc backbone and Xyl sidechains. The XyG: cellulose ratio is calculated in Table S3.
3. The pectin amount includes both HGA and RG-I, reported in Table S2.

Table S5. Water intensities and relaxation times in the four CW samples at 296 K $^1$.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Intensity$^2$</th>
<th>$^1$H T$_1$ (s)$^3$</th>
<th>$^1$H T$_2$ (ms)</th>
<th>$\Delta$ (Hz)$^4$</th>
<th>$\Delta^*$ (Hz)$^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.12</td>
<td>5.7±0.4</td>
<td>56</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>1.2</td>
<td>194±17</td>
<td>1.6</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>0.88</td>
<td>33.3±0.5</td>
<td>9.6</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.38</td>
<td>25±1</td>
<td>13</td>
<td>27</td>
</tr>
</tbody>
</table>

1. Water intensities and relaxation times were measured on a Bruker DSX-400 MHz (9.4 T) spectrometer.
2. Water intensity is the integrated area of the water peak.
3. The $^1$H T$_1$ was measured using a standard inversion recovery sequence.
4. The homogeneous linewidth is calculated from the measured $^1$H T$_2$ of the water peak as $1/\pi T_2$.
5. The apparent linewidth is obtained from the full width at half maximum of the water peak.
Figure S1. Pectin $^{13}$C chemical shift changes between sample 1 and sample 2 due to extraction by chelating agents CDTA and sodium carbonate.
Figure S2. $^{13}$C MAS spectra of sequentially digested Arabidopsis cell walls (a-e) and microcrystalline Avicel PH-101 (f). All spectra were measured at 296 K using CP except for (b), which was measured with DP in a quantitative manner. (a) CP spectrum of the intact cell wall (sample 1). (b) Quantitative $^{13}$C DP spectrum of the intact cell wall. (c) CP spectrum of CDTA and sodium carbonate treated cell wall (sample 2). (d) CP spectrum of additional XEG-treated cell wall (sample 3). (e) CP spectrum of additional Cel12A treated sample (sample 4). (f) Avicel $^{13}$C CP spectrum.
Figure S3. The water-to-polysaccharide spin diffusion is independent of the MAS frequency in the range used here (< 10 kHz). Buildup curves obtained from 9 kHz MAS (filled symbols) are indistinguishable from buildup curves obtained at the slower MAS frequency of 4.5 kHz. Thus, the centrifugal force due to MAS has negligible effects on water-to-polysaccharide spin diffusion.
Figure S4. 2D water-edited $^{13}$C correlation spectra resolve water spin diffusion to pectins and hemicellulose. The 1D buildup curves of the 100-ppm mixed peak of hemicellulose and pectins (filled green circles) and the 89-ppm peak of interior cellulose C4 (filled red circles) are compared with various 2D cross peaks. (a) Buildup curves of the (100, 69) ppm pectin cross peak and the (99, 72) ppm xylose cross peak straddle the buildup curve of the 100-ppm peak in the 1D spectra. The pectin buildup is faster than the hemicellulose buildup. (b) Buildup curves of several 2D cross peaks. Water spin diffusion to pectins is faster than to hemicellulose. (c) Buildup curve of the (89, 72) ppm cross peak of interior cellulose superimposes well with the buildup curve of the 1D 89-ppm peak.
Figure S5. $^{13}$C-$^1$H dipolar couplings of polysaccharides in three cell wall samples measured by the doubled DIPSHIFT experiment at 296 K under 7 kHz MAS. The C-H dipolar couplings (and order parameters) of matrix polysaccharides (a) decreased due to sequential extraction, indicating increased mobility, while the couplings of cellulose (b) are unaffected by digestion. The reported couplings are true values after taking into account the homonuclear decoupling scaling factor and the doubling factor.

References