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► To cite this version:

Radwa Abou-Saleh, Mercedes Hernandez-Gomez, Sam Amsbury, Candelas Paniagua, Matthieu Bourdon, et al.. Interactions between callose and cellulose revealed through the analysis of biopolymer mixtures. *Nature Communications*, 2018, 9 (1), 10.1038/s41467-018-06820-y . hal-02419183

HAL Id: hal-02419183

<https://hal.science/hal-02419183>

Submitted on 20 Mar 2023

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1 Interactions between callose and cellulose revealed
2 through the analysis of biopolymer mixtures

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27 **Keywords:** Cellulose, Avicel, (1,3)- β -glucans, Callose, Pachyman, Ionic Liquid, Cell Walls,
28 Hydrogels, Mixtures, Biopolymers, Rheology, AFM-nanoindentation, ¹H-NMR, FTIR, Polymer
29 Interactions.

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Abstract:

The properties of (1,3)- β -glucans (i.e. callose) remain largely unknown despite their importance in plant development and defence. Here we use mixtures of (1,3)- β -glucan and cellulose, in ionic liquid solution and hydrogels, as proxies to understand the physico-mechanical properties of callose. We show that after callose addition the stiffness of cellulose hydrogels is reduced at a greater extent than predicted from the ideal mixing rule (i.e. the weighted average of the individual components' properties). In contrast, yield behaviour after the elastic limit is more ductile in cellulose-callose hydrogels compared with sudden failure in 100% cellulose hydrogels. The viscoelastic behaviour and the diffusion of the ions in mixed ionic liquid solutions strongly indicate interactions between the polymers. Fourier-transformed infrared analysis suggest that these interactions impact cellulose organization in hydrogels and cell walls. We conclude that polymer interactions alter the properties of callose-cellulose mixtures beyond what it is expected by ideal mixing.

60 **Introduction**

61 The cell wall of plant, bacteria, and fungi are enriched with β -glucans which are essential for
62 development and adaptation to a changing environment^{1, 2}. The physical and mechanical
63 properties of this diverse group of polysaccharides are applied in industry to obtain many
64 valuable products. For example, β -glucans cell wall biopolymers are used as raw materials for
65 paper, films and textiles, as gelling agents and natural immune stimulants in food and in
66 pharmaceutical products³. They are also applied in the development of recent technologies
67 such as tissue engineering, prosthetics and nanostructured materials^{4, 5}. In the cell wall
68 matrix, the composition and structure of β -glucans underpin plant growth and development⁶.
69 Understanding how these glycans are assembled, how they interact at the molecular level,
70 and the specific physical and mechanical properties they confer, is a major challenge due to
71 the complexities of their natural systems. This knowledge could open a whole new range of
72 applications in biotechnology and in the design of new biodegradable materials.

73 Cellulose (a homopolymer of (1,4)- β -linked glucosyl residues) is the most abundant β -glucan
74 polysaccharide, found in the cell wall of bacteria, algae, fungi and plants and even in
75 urochordate animals⁷. Due to numerous intra- and intermolecular hydrogen bonds and van
76 der Waals interactions⁸ cellulose chains self-assemble into microfibrils which provide strength
77 and determines the direction of cell growth through increasing the stiffness of transverse cell
78 walls. Interactions between cell wall polymers modify the ordering, spacing or slippage
79 between microfibrils affecting cell wall mechanics and thereby cell behaviour such as growth
80 rate, shape etc.^{6, 9, 10}. The interactions of cellulose with diverse glycan polymers (including
81 xyloglucans and xylans) have been reported but rarely validated in biological systems¹¹⁻¹³. The
82 association of these macromolecules into a composite structure is dependent upon
83 intermolecular bonding, hence stability depends on several factors including the polymer
84 side-chains and the number and spatial configuration of these interactions¹⁴⁻¹⁶.

85 In contrast to cellulose, plant (1,3)- β -glucans (termed callose) are a minor component in
86 developing cell walls but have important regulatory functions in cell-to-cell signalling,
87 organogenesis and defence^{17, 18}. In normal unstressed conditions, callose appears mainly
88 deposited around intercellular junctions named plasmodesmata¹⁹. Callose accumulation
89 determines intercellular communication, affecting developmental and environmental

90 signalling²⁰. Callose also has important regulatory functions in cell plate formation during cell
91 division and in the sieve pores that connect the phloem vascular system²¹. It accumulates in
92 response to wounding and exposure to biotic and abiotic stresses playing a key role in
93 defence^{22, 23}.

94 Despite its importance, the physical properties of callose and its interactions with other
95 biopolymers within the cell wall matrix remain unknown. Most of the work in this area focuses
96 on callose deposited during pathogenic attacks or during pollen tube growth. Callose is
97 thought to strengthen the cell wall forming a physical barrier against fungal penetration²⁴⁻²⁶
98 and to act as a matrix for the deposition of other cell wall components²⁷. Digestion of callose
99 appears to reduce cell wall stiffness leading to an increase in the diameter of the pollen tube²⁸,
100 ²⁹. Conversely, a mutant in a callose biosynthetic enzyme (named *powdery mildew resistant*
101 *4, pmr4*), shows reduced callose levels but appears resistant to some fungal infections³⁰.
102 Pectin and cellulose distribution also change after digestion of callose in pollen tubes²⁸ but it
103 is not clear if these changes in cell wall composition occur or if they affect plant-fungus
104 interactions³¹. Recent studies visualized a 3D network of callose-cellulose microfibrils forming
105 in the site of fungal penetration³², but what factors affect the formation of these networks
106 and their significance in the success of fungal infection are unknown.

107 Given the importance of callose to regulate developmental and adaptive processes it is
108 imperative to clarify whether it can interact with cellulose at the molecular level and what are
109 the mechano-physical properties that derive from this interaction. Ionic liquids, including 1-
110 ethyl-3-methylimidazolium acetate ([C2mim][OAc]), have emerged as a powerful solvent for
111 cellulose and other water-insoluble polysaccharides³³ and were used here to prepare mixture
112 solutions of microcrystalline cellulose and a (1,3)- β -glucan polymer named Pachyman, as a
113 commercial form of callose. A combination of techniques including: Atomic Force Microscopy
114 (AFM) and nanoindentation analysis; Scanning Electron Microscopy (SEM), liquid state
115 Nuclear Magnetic Resonance (NMR); rheology and Fourier Transformed InfraRed (FTIR)
116 spectroscopy were used to analyse the properties of these polymer mixes in solution and in
117 the form of hydrogels. The results unveil molecular interaction between cellulose and callose
118 in both the liquid and gel state, forming systems with dynamics and mechanical properties
119 that are not adequately described by a simple ideal mixing rule³⁴, i.e. the mixtures properties
120 are not just the weighted average of the individual component properties. We consider the

121 relevance of these interactions in cell walls by comparing wild type and transgenic plant
122 material expressing a hyperactive version of the biosynthetic enzyme callose synthase 3 in an
123 inducible system (*icals3m*)³⁵. Based on the results, we propose that interactions occur
124 between callose and cellulose that influence cell wall structure (and thereby properties). The
125 implications of these results for the regulation of cell-to-cell communication and other
126 biological processes are discussed as well as their potential applications for the design of new
127 cellulosic biomaterials.

128 **Results**

129 **Mechanical properties of cellulose-callose hydrogels**

130 To gain insight into how callose modulates the physico-mechanical properties of plant cell
131 walls, we used a simplified model of composite hydrogels, as reported elsewhere³⁶. The
132 samples were made with different proportions of commercial microcrystalline cellulose and
133 the callose analogue Pachyman dissolved in the ionic liquid [C2mim][OAc] and coagulated in
134 water (see details in the methods section). It should be noted that cellulose and callose are
135 insoluble in water, and thus when water is added to their solutions in ionic liquid, phase
136 separation occurs leading to a formation of a 3D network. We shall use the term hydrogel in
137 the following keeping in mind that the samples were precipitated polymer networks with
138 water in the pores. Mixtures contained a 10% total weight (wt) of the polysaccharides with
139 different proportions of the individual polymers. These samples are referred to as wt percent
140 of cellulose-callose in the mixtures. Pure cellulose is labelled 0%, 20:80 is 20% callose, 50:50
141 is 50% callose, 80:20 is 80% callose, and pure callose is labelled as 100%. The concentration
142 of callose in hydrogels was fluorescently quantified as described in the Methods³⁷ in order to
143 confirm that callose was not washed out during exchange of ionic liquid to water. The results
144 showed that callose was not removed during solvent exchange and its concentration in
145 hydrogel is the same as in the mixtures with cellulose (see Supplementary Fig. 1).

146 The morphology of supercritically dried hydrogels was visualized using SEM (Fig.1) to identify
147 potential changes in their structure as a function of mixture composition. 0% callose shows
148 the classical structure of macroporous cellulose aerogels³⁸. Significant differences in
149 morphology were detected between 0%, 50%, 80% callose and the 100% callose. The increase
150 in callose concentration leads to a finer morphology with fewer and fewer macropores.

151 The mechanical properties of the hydrogels were measured using AFM nanoindentation with
152 a colloidal probe. Force maps were collected and force-indentation curves were fitted to the
153 Hertz model^{39, 40} (Fig.2a and Supplementary Fig.2) to calculate the Young's modulus for the
154 five different hydrogels. The plastic behaviour of the hydrogels (deformation in response to
155 applied forces) can be compared in a relative fashion by calculating the index of plasticity
156 from the hysteresis, derived from the recovery of the indented surface after the force is
157 relaxed, as described in the Methods ^{39, 41}. Mean values of Young's modulus and plasticity
158 index are presented in Figures 2b and c, and were derived from statistical analysis of hundreds
159 of force curves (Supplementary Fig. 3).

160 The plasticity index for all samples was between 0.2 and 0.5 (where 0 represents a purely
161 elastic response, and 1 a purely viscous, or plastic response with no recovery^{41, 42}). Both the
162 highest modulus value (stiffness) and plasticity index were measured for cellulose hydrogels
163 in comparison to 100% callose. Plasticity values remained around 0.45 from 0% to 20%
164 callose, and slowly reduced thereafter. The elastic modulus dropped dramatically from $200 \pm$
165 6.6 to 86 ± 1.6 kPa (errors are standard error of the mean) when comparing cellulose (0 %
166 callose) to 20% callose hydrogel, respectively. The modulus continues to decrease with
167 increasing callose concentration reaching a value of 42 ± 1.6 kPa at 100% callose. The
168 theoretical Voigt upper and Reuss lower bounds are plotted for the elastic modulus^{43, 44} (Fig.
169 2d). Although these models are typically used for composites reinforced with fibres, and in
170 our samples there are no fibres, they mathematically describe the mechanical response of
171 two idealized combinations of materials (not exclusive to fibres): one to maximise the
172 stiffness by having the stiffer component spanning the sample in the direction of strain
173 (Voigt), and one to minimise the stiffness by having the stiffer component in layers orthogonal
174 to the strain direction (Reuss). It is therefore useful to compare our results with the limits
175 established by these models. In the hydrogels, the experimental value of the Young's modulus
176 corresponding to 20% callose does not fall in between these boundaries. This indicates that
177 the large reduction in elastic modulus upon addition of 20% callose cannot be explained by a
178 physical arrangement of the callose and cellulose components, leading to the conclusion it
179 must be an effect at the molecular level.

180 Mechanical properties of the cellulose-callose hydrogels were further explored using a 2mm
181 flat-ended probe in a Texture Analyzer (as described in the methods) suitable for soft

182 materials. This method allows testing beyond the elastic limit through to failure at high strain
183 (Figure 3). Analysis of the linear elastic region revealed an identical relationship to AFM-
184 nanoindentation, with higher modulus at 0% callose, dropping rapidly at 10% - 20% callose.
185 The absolute values are about 50% of the AFM values, which is reasonable considering the
186 magnitude difference in the scale of measurement, different contact geometry, contact
187 mechanics model and boundary conditions. It is also possible that the 50% modulus reduction
188 is a function of the indentation depth, with the 50 -150 nm deformation in AFM reporting on
189 near-surface properties, and the 2mm flat-ended probe giving a bulk measurement with an
190 elastic response up to around 1 mm deformation (25% strain). The largest difference between
191 samples occurred beyond the elastic limit. Although the addition of a small amount of callose
192 reduced the yield stress by 2-3 times, it also abolished the catastrophic failure of the pure
193 cellulose. At the yield point (when plastic non-linear deformations begin), the pure cellulose
194 gel suddenly cleaves to a depth of > 1 mm. At 10% callose this sudden cracking is entirely
195 abolished, and the hydrogel yields smoothly and plastically all the way through the depth,
196 maintaining its integrity. This behaviour is similar at 20%, but the hydrogel is much weakened
197 at 50% and greater callose concentrations, with a 20x reduction in yield stress. The uptick in
198 force at the end of each trace is caused by the proximity of the underlying surface on which
199 the gel sits.

200 In summary, mechanical testing using both AFM-nanoindentation and a Texture Analyzer in
201 compression mode indicates that the addition of a small amount of callose strongly reduces
202 the elastic modulus of the resultant hydrogels beyond what is predicted by the rule of
203 mixtures (see Fig. 2d). Plasticity (and therefore the ability to absorb energy to cope with
204 deformations) is maintained in cellulose-callose hydrogels until it drops at higher callose
205 concentrations (Fig. 2b). The yield behaviour is also very different, with the pure cellulose gel
206 allowing the stress to build up before the material catastrophically fails and breaks apart,
207 whereas an addition of 10% callose allows a smooth yield and plastic deformation without a
208 sudden failure event. The results suggest that a small amount of callose disrupts the cellulose
209 network changing the hydrogel mechanical properties, which points to potential microscopic
210 interactions between these two polymers. The yield behaviour might suggest that inter-
211 molecular bonding is weakened such that the polymer chains can slide more easily and relieve

212 the build-up of stress. Hence addition of small amount of callose allows the material to be
213 more resilient to both high stress and strain which otherwise might lead to material failure.

214 **Viscoelastic behaviour of cellulose-callose mixtures**

215 Mechanical testing of the hydrogels shows a significant drop in plasticity at concentrations
216 >50% callose. The next step was to examine the viscoelastic properties of cellulose-callose
217 mixtures in the liquid state. The phase angle (δ) and storage (G') and loss modulus (G'') of
218 neat cellulose and callose solutions and of their mixtures are represented in Figure 4; the
219 complex modulus (G^*) is shown in Supplementary Figure 4.

220 The data suggest gel-like properties for the neat callose solution as the phase angle remained
221 almost independent of the frequency. This is also reflected by the overlap in the storage and
222 loss modulus at low frequency for 100% callose (Figure 4b).

223 Steady state viscosity of the mixtures was measured as a function of shear rate at different
224 temperatures. Flow curves demonstrate mostly Newtonian behaviour and a very weak shear
225 thinning, which is more pronounced for 100% callose solutions, likely as a consequence of
226 high molecular weight chains' alignment in the flow (Supplementary Fig. 5). The Cross model
227 (equation 1)⁴⁵ was used to fit the data and calculate the viscosity of the samples at zero shear
228 rate:

$$229 \quad \eta = \frac{\eta_0 + \eta_\infty \alpha \gamma^n}{1 + \alpha \gamma^n} \quad (1)$$

230 where γ is shear rate, η_0 is zero shear rate viscosity, η_∞ is the viscosity at infinite shear rate, n
231 is the flow index and α is the consistency index.

232 The results show, as expected, that viscosity increased with decreasing temperature for each
233 system studied and, consistently, with the increase of callose concentration (Fig. 5).
234 Interestingly, at all temperature tested, values at 80% callose show lower viscosity than
235 predicted by a simple ideal mixture rule, as it overlaps with the ideal theoretical values for
236 50% callose (calculated as the average of the 0% and 100% $\ln \eta_0$ values) (Fig. 5b). On the
237 contrary, the experimental value of the zero shear rate viscosity of the mixture with 50 % of
238 callose is higher than ideal mixing rule prediction. It is clear that there are structural
239 reorganisations in the mixtures with more than 50% of callose.

240 The activation energy of viscous flow was calculated using the Arrhenius approach but no
241 significant influence of mixture composition was detected across the entire concentration
242 range, with values between 64.2 ± 1.8 kJ and 70.8 ± 9.6 kJ.

243 To summarize, the rheological analysis suggests gel-like properties for 10 % wt callose-ionic
244 liquid solution. The viscosity of the 80% callose samples coincides with the experimental and
245 theoretical values for 50% callose suggesting that the mixtures do not obey the ideal mixing
246 rule.

247 **Liquid state $^1\text{H-NMR}$ suggests cellulose-callose interactions**

248 Molecular interactions between the two polymers could explain the non-ideal mixing
249 behaviour in the mechanical and viscoelastic properties of the mixes. To test this hypothesis,
250 NMR was used to determine the self-diffusion coefficients of the ions as a function of
251 composition of mixed solutions. The ions act as probe molecules in these systems. Their
252 diffusion coefficients depend on their environment, which can be affected by the interactions
253 between the polymers. For cellulose, it has been shown that the ions interact with the
254 hydroxyl groups (not engaged in forming intra or inter- molecular hydrogen bonds between
255 the polymers) and that this interaction reduces the diffusion coefficient of the ions in
256 solution⁴⁶. If callose and cellulose interact, a change is expected in the number of OH groups
257 available for the ions to interact with and thereby their diffusion coefficient to deviate from
258 the ideal mixing rule, such as, for example, in the case of [C2mim][Oac] and water⁴⁷.

259 The ^1H NMR spectra for the different mixtures were determined within a temperature range
260 between 20 and 60 °C inclusive. The peaks corresponding to the cation and the anion were
261 used to calculate the diffusion coefficients as described in methods⁴⁶. Figure 6a shows the
262 cation and anion diffusion coefficients as a function of mixture composition at 30 °C.
263 Predicted values for log-linear correlations (ideal mixing) were drawn based on the values
264 obtained for 100% cellulose and 100% callose. Notice that both the diffusion coefficients of
265 cations and anions behave similarly as a function of mixture composition and significantly
266 deviate from the mixing rule prediction at around 20% and 80% callose. The deviations are
267 highlighted when comparing the percentage difference between the theoretical values
268 (calculated based on the ideal mixing rule) and the experimental values and these changes
269 were also found at 40 °C (Supplementary Figure 6). Figure 6b shows the factor $D_{\text{anion}}/D_{\text{cation}} < 1$

270 suggesting that in these mixtures anions diffuse slower than cations despite being smaller.
271 This is known as anomalous diffusion⁴⁸ of the ions in ionic liquid and occurs at all tested
272 temperatures. Consistent with deviations from ideal mixing, the anomalous diffusion become
273 more prominent at 20% and 80% callose concentrations.

274 The activation energy of the ions diffusing in polymer solutions and their mixtures was
275 calculated using the Arrhenius equation. The results (Supplementary Figure 7) show a slight
276 reduction from 44.1 ± 0.8 to 41.3 ± 0.9 kJ mol⁻¹ when comparing 100% cellulose to 100%
277 callose, respectively. This slight decrease in activation energy reflects the increase in the
278 diffusion coefficients when comparing 100% cellulose and 100% callose.

279 To summarize, the results show that at certain callose concentrations in the mixture (20% and
280 80%) there is a deviation of both anion and cation diffusion coefficients from the expected
281 ideal mixing rule, which is the evidence of the changes in the number/accessibility of OH
282 groups on carbohydrate chain that interact with the ionic liquid. Since the mixtures are of the
283 same total polymer concentration (10% total weight), these changes can only be explained by
284 molecular interactions between the polymers, modifying, for example, the number of
285 inter/intra-molecular hydrogen bonds. The significance of this result, together with the
286 mechanical and the viscoelastic properties of mixtures, are discussed below.

287 **Changes in cellulose-callose organization exposed by FTIR**

288 To further understand the effect of callose interactions with cellulose in hydrogels, we used
289 Fourier Transform InfraRed spectroscopy (FTIR) to analyse the spectra of the callose:cellulose
290 mixtures (Figure 7a). The spectra depicted main characteristic vibrational bands
291 corresponding to cellulose II which allow characterization of polymer mixture ordering⁴⁹. The
292 peak positions for the raw powders of microcrystalline cellulose (Avicel) and callose
293 (Pachyman) as well as their 50% mixture were also obtained (Supplementary Figure 8,
294 Supplementary Table 1).

295 Significant differences were observed in the position of the OH- stretching vibration broad
296 band ($3100-3600$ cm⁻¹) that informs about the hydrogen bonding pattern^{49,50}. Using
297 information from the spectra, the lateral order index (LOI, ratio between CH₂ bending and C–
298 O–C stretching absorption bands) and the energy of the hydrogen bond (E_H) was calculated

299 (see methods and reference⁵⁰) (Figure 7b-c). It was found that the cellulose sample has the
300 highest LOI (also known as crystallinity index) and E_H values which correlates with forming a
301 higher organized network in relation to callose. Both values decreased with the increase of
302 callose concentration in hydrogel but deviations from an ideal behaviour (linear correlation
303 with concentration, Figure 7b) were significant at 20% callose. This suggests that addition of
304 callose modifies cellulose structuring during coagulation in a manner that affects the
305 hydrogen bonding and the ordering of the polymer chains in the hydrogel. This result supports
306 the interpretation of the results obtained by NMR.

307 **Callose synthesis in *A. thaliana* modifies the FTIR profile**

308 Callose co-exists with cellulose in specific plant cell wall domains and cell types. To determine
309 if callose concentration *in planta* modify cellulose organization (as found in hydrogels) we
310 used a transgenic tool to induce ectopic callose synthesis in seedlings of *Arabidopsis thaliana*.
311 A vector containing an estradiol-dependent promoter (*pG1090:XVE*) and a mutated
312 hyperactive version of the callose synthetic enzyme CALS3 (*cals3m*) was introduced into
313 *Arabidopsis thaliana* to produce a stable transgenic line named *pG1090::icals3m*^{35, 51}.
314 Phenotypic comparison after 24 h treatment of *pG1090::icals3m* with either the chemical
315 inducer estradiol or with DMSO (control experiment) revealed a substantial increase in callose
316 concentration in different plant tissues (detected using confocal fluorescent microscopy after
317 aniline blue staining, Figure 8 and Supplementary Figure 9). Callose was quantified in cell walls
318 isolated from 24h-estradiol treated *pG1090::icals3m* seedlings. The amount of callose was
319 significantly higher than wild type after *icals3m* induction (Table 1). Callose mainly
320 accumulated on the vasculature and associated cells as well as in the elongation and
321 differentiation zones of the root (Supplementary Figure 9). Confocal microscopy of cells at
322 the root cap regions, co-stained with aniline blue and Direct Red 23 (a fluorescent dye that
323 enables visualization of cellulose⁹), showed a pattern that suggest integration and/or close
324 spatial proximity of callose deposits and cellulose fibrils where interactions can potentially
325 occur (Figure 8). Callose and cellulose concentrations and organization in cell walls are
326 described to affect cell growth. Supplementary Figure 10 shows a strong restriction in root
327 length as soon as 48h after estradiol treatment/induction of callose.

328 FTIR was also carried out to reveal the structural organization of cell walls after callose
329 induction (Figure 9). The spectra revealed peaks that coincide in position to those found in
330 the microcrystalline cellulose (Avicel) and Pachyman (Supplementary Fig. 8). The energy of
331 the hydrogen bonds and the LOI were quantified and significant reductions were observed
332 after callose induction in the *pG1090::icals3m* when compared to wild type control (Table 1).
333 The results further support the conclusion that an increase in callose decreases hydrogen
334 bonding coincident with more disordered cell walls. This effect might be linked to the
335 restriction observed on root growth as discussed below.

336

337

338 Discussion:

339 (1,3)- β -glucans (i.e. callose) plays an important role in cell biology impacting organism
340 development and environmental responses^{1, 52, 53}. The accumulation of this polymer is
341 associated with changes in cell wall architecture and mechanical properties but it is not clear
342 if the effect is direct or indirect. There is evidence pointing to callose function as a sealant, as
343 a load-bearing structure or as a matrix for the deposition of other cell wall components^{27, 29,}
344 ³². Our study, using a hydrogel model, shows that the addition of callose to cellulose leads to
345 a dramatic reduction in elastic modulus and changes the yield behaviour. Callose acts as a
346 sort of cellulose plasticizer abolishing the dramatic failure which would otherwise occur when
347 stress increases to the yield point and the material cleaves deeply. Rheology analysis of
348 mixtures in ionic liquid supports a gel-like behaviour (viscoelastic response) for callose
349 concentrated solutions. We also demonstrate that the mechano-physical properties (elastic
350 modulus, plasticity and viscosity) of the hydrogels and mixtures in ionic liquid diverged from
351 linear ideal mixing rules. The diffusion of the ions, measured using liquid state ¹H NMR,
352 strongly suggests that callose and cellulose engage in molecular interactions showing a non-
353 ideal decreasing pattern at concentrations of 20% and between 70-90% callose. Deviations
354 from ideal mixing in the hydrogen bond pattern and lateral order index were also found in
355 20% callose hydrogel mixtures using FTIR. Although more complex in interpretation, changes
356 in cell walls ordering and hydrogen bond pattern also occur after increasing callose
357 biosynthesis in plant cell walls using as a tool a transgenic estradiol-inducible callose synthase
358 system (*pG1090::icals3m*). Together, the results indicate that the accumulation of callose
359 causes disruption in the organization of cellulose and cell wall networks which can influence
360 cell wall mechanical properties and is likely linked to cell growth.

361 Molecular interactions between callose and cellulose are supported by the non-ideal patterns
362 in ionic liquid across all temperature measurements recorded in the mixtures using NMR. The
363 characteristic anomalous diffusion ($D_{\text{anion}}/D_{\text{cation}} < 1$) reported for cellulose⁴⁶ becomes more
364 prominent at the concentrations where the diffusion of the ions follows a non-ideal pattern.
365 The anion interacts predominantly with the carbohydrate and thus is expected to be more
366 sensitive to the polymer-polymer interactions⁴⁸. A decrease of $D_{\text{anion}}/D_{\text{cation}}$ indicates that the
367 diffusion of the anion is preferentially reduced providing a strong evidence for interactions.
368 The non-ideal behaviours of either the diffusion coefficient of the ions and of the mechanical

369 properties of the mixtures are concentration dependent, which likely reflect the complexity
370 of these systems where cellulose-cellulose, cellulose-callose and callose-callose interactions
371 can contribute differently to the molecular environment and properties of the mixtures. The
372 fact that interactions appear to occur at certain stoichiometric ratios has been previously
373 reported for other blends^{47, 54, 55}. In callose-cellulose mixtures, interactions at 20% and 80%
374 wt appear to deviate more strongly for the diffusion coefficient of the ions in ionic liquid
375 mixtures, i.e. more OH groups are available for the ions to interact with. The outcome of these
376 interactions is also observed in the FTIR results where the hydrogen bond energy and lateral
377 order index (which relate to the OH groups engaged in H-bonds) is significantly decreased in
378 20% callose hydrogels in relation to what was expected by the mere changes in polymer
379 concentrations.

380 The biological importance of the findings using hydrogels as a model is still a point of debate.
381 In our study, we showed that increasing callose concentration in developing cell walls of the
382 model plant *Arabidopsis thaliana* significantly affects the hydrogen bond energy and lateral
383 order index (considered as crystallinity index by other authors)⁵⁶ calculated using FTIR spectra
384 of cell walls (Figure 9, table 1). Moreover, confocal imaging after induction of callose using
385 the *pG1090::icals3m* line shows close proximity between newly synthesized callose and
386 cellulose microfibrils and 48 h induction times produce phenotypes that resemble those
387 reported in plants defective in cellulose organization and cell wall crystallinity⁵⁷ (Figure 8 and
388 Supplementary Fig. 10). These results support the potential applicability of our hydrogel
389 model in the understanding callose-cellulose interactions *in vivo*.

390 Traditionally, callose production has been associated with cell wall reinforcement to restrict
391 the penetration of pathogens or in response to elicitors and other stress conditions^{18 27}. Our
392 results using hydrogel models suggest that increasing callose concentration significantly
393 reduces the Young's modulus of neat cellulose by about three fold, increasing flexibility, whilst
394 not affecting the viscous nature (plasticity values), hence its ability to absorb deformation
395 energy, until quite high concentrations. It also changes the material properties beyond the
396 yield point, acting as a plasticizer and preventing the catastrophic failure of the material under
397 a high strain condition. Adding callose appears to increase cellulose resilience to high strain
398 through a plastic deformation after the yield point rather than the total failure and fracture
399 that occurs in a more crystalline material. Based on this data, we speculate that callose

400 enhances cell walls resilience in high strain conditions by increasing flexibility preventing the
401 build-up of high stress, and by allowing a failure mode that involves a gradual deformation
402 rather than a catastrophic tearing apart. Toughness, in mechanical engineering terms, is the
403 ability to deform without breaking, and at the same time absorbing and dissipating the
404 energy, which requires a balance of deformability and energy absorption via viscous damping
405 or plastic deformation/ductility⁵⁸. Whilst the addition of callose does not increase toughness
406 in strict terms (the area under the stress-strain curve to the yield point is not increased) it
407 appears to increase ductility under high strain condition. Cell wall mechanics is tightly
408 dependent on the cell wall environment and, based on our research in hydrogel models,
409 callose has the capacity to increase the resilience of cellulosic materials to large deformations.
410 How important are these features of callose in the regulation of cell wall microdomains (such
411 as plasmodesmata) is difficult to predict without more knowledge on the structural
412 architecture and mechanical properties of these cell walls domains. The complexities of this
413 challenge increase when considering cell wall metabolic cross-talks and dynamic response to
414 signals.

415 Our results challenge models where callose is viewed as a carrier of cell wall stiffness and
416 support the establishment of interactions between callose, cellulose (and potentially other
417 polymers) as determinant of cell walls responses to developmental and environmental
418 signals. It also highlights the importance of considering cell wall composition and dynamics
419 and how these affect callose biological function. More studies are required to dissect callose
420 properties and potential role as cellulose modifier in cell walls. From the material science
421 perspective, the interactions between callose and cellulose, exposed by this research, open
422 the path for the development of new applications for this natural biopolymer to create, for
423 example, composites that can be used as biodegradable substitutes for other less
424 environmentally friendly polymers.

425

426 **Methods:**

427 **Materials**

428 Microcrystalline cellulose Avicel PH-101, the ionic liquid 1-ethyl-3-methyl-imidazolium
429 acetate ([C2mim][OAc]) (97% purity), FTIR grade KBr, ethanol (absolute, ≥99.8%), acetone

430 ($\geq 99.9\%$), methanol ($\geq 99.9\%$) and chloroform ($\geq 99\%$) were all obtained from Sigma Aldrich.
431 Callose commercial analogue, the (1-3)- β -D-glucan Pachyman, and aniline blue fluorochrome
432 were obtained from Biosupplies Australia (www.biosupplies.com.au). The molecular weight
433 of Avicel PH-101 is $28\,400\text{ g mol}^{-1}$ ⁵⁹ whereas of Pachyman it is as $168\,000\text{ g mol}^{-1}$ ⁶⁰. The
434 molecular weight distribution of Avicel PH-101 as well as polydispersity and crystallinity index
435 were reported elsewhere^{61,62}. Molecular weight and other properties (such as polydispersity,
436 flexibility and conformation of polymers) were also reported elsewhere for Pachyman⁶³.

437 All plant work used *Arabidopsis thaliana* in the Columbia (Col-0) background. Transgenic
438 pG1090::*icals3m* was generated by Agrobacterium-mediated transformation of Arabidopsis
439 plants with a vector containing a hyperactive callose synthase mutant version (*icals3m*) under
440 the control of a β -estradiol inducible pG1090 promoter⁵¹. In this transgenic line, callose
441 amount is significantly higher 6-24 h after exposure to β -estradiol in comparison to wild type
442 subjected to the same conditions. Wild type and transgenic seeds were surfaced sterilised in
443 bleach solution (20% thin bleach, 0.01% tween-20) for 10 minutes and stratified in the dark
444 at 4°C for 5 days. Seeds were germinated on $\frac{1}{2}$ Murashige and Skoog (MS) medium plates
445 containing 0.22% Murashige and Skoog basal medium (Sigma Aldrich), 1% sucrose (Sigma
446 Aldrich), 0.8% plant agar (Sigma Aldrich) and grown under long day conditions (16 hour
447 photoperiod) at a constant temperature of 20°C. 10 days old pG1090::*icals3m* seedlings were
448 transferred for induction to $\frac{1}{2}$ MS plates supplemented with 10 μM β -estradiol (Sigma Aldrich,
449 diluted from a stock solution in DMSO). Controls experiments (with basal/non-induced
450 amount of callose) were performed by exposing pG1090::*icals3m* to the corresponding
451 solvent (DMSO) concentration or by transferring wild type seedlings to 10 μM β -estradiol.
452 Plants were analysed or harvested at different times after transfer as indicated in Results. For
453 FTIR analysis, plants were collected 24 h post-transfer and flash-frozen in liquid nitrogen to
454 stop metabolic activity before undergoing freeze drying.

455 **Preparation of ionic liquid mixtures**

456 Callose was milled at 50 Hz for 2 min in a TissueLyser LT (Qiagen, Hilden, Germany) to break
457 agglomerations formed due to ambient humidity. Cellulose and callose were then dried in a
458 vacuum oven at 50 °C for 2 days prior to preparing the mixes. Five different samples were
459 prepared with concentrations of 0, 20, 50, 80, and 100% callose, all at a final concentration of

460 10 wt% of total carbohydrate weight in [C2mim][OAc]. First, dry polymers were mixed in the
461 proportions mentioned above and then the mixed powders were dissolved in ionic liquid. All
462 solutions were prepared in an MBraun Labmaster 130 atmospheric chamber under nitrogen,
463 providing a dry environment, with the chamber being maintained at a dew point level
464 between -70 and -40 °C. The [C2mim][OAc] and callose/cellulose were mixed and kept on
465 magnetic stirrer at 50 °C until producing clear solutions. All samples were kept sealed when
466 not in use to prevent any moisture contamination.

467 **Hydrogel preparation and callose quantification**

468 The mixtures were hydrated to form hydrogels by adding deionised water, which was
469 renewed every few hours in the course of two days until full removal of the ionic liquid.

470 The percentage of callose in the hydrogels was determined using aniline blue. The
471 fluorochrome produces a signal directly proportional to (1,3)- β -glucan concentration that can
472 be measured using a fluorescence microscope and/or fluorometer. For confocal imaging,
473 hydrogel samples were cut into 3x3x1 mm sections and incubated for 30 minutes at room
474 temperature in 1 ml of staining solution containing 25 mg/ml of aniline blue fluorochrome in
475 distilled water. Stained samples were washed with 3 changes of distilled water (5 minutes per
476 change) and mounted on microscope slides. Samples were imaged on a Zeiss LSM880 Upright
477 confocal microscope (Zeiss, Cambridge, UK) using a 40x objective with 405 nm excitation and
478 450 nm emission. Imaging settings (pinhole, gain, etc.) were kept constant in all images.

479 For fluorometric quantification, hydrogels were freeze dried, then ground using a TissueLyser
480 LT (Qiagen, Hilden, Germany). 0.2 mg of the powder was incubated in 200 μ l of 1 M NaOH at
481 80 °C for 30 minutes then centrifuged at 13000 rpm for 15 minutes to remove any undissolved
482 material. The supernatant was added to 1.25 ml of aniline blue staining solution (0.25 M
483 glycine, pH 9 with 2.25% aniline blue) and incubated for 20 minutes at 50 °C followed by 30
484 minutes at room temperature. 200 μ l of sample was aliquoted to a dark multiwell plate and
485 fluorescence was assayed at 405 nm excitation, 512 nm emission using a POLARstar OPTIMA
486 microplate reader (BMG labtech, Ortenberg, Germany).

487 A calibration curve was created using different starting weights (0.05, 0.1, 0.15 and 0.25 mg)
488 of callose (powder obtained from the 100% callose hydrogel). Changes in fluorescence values

489 in relation to callose weight (mg) were represented to generate a linear regression curve. This
490 was used to calculate callose content in each of the hydrogels as described above.

491 **Characterization of hydrogels using SEM**

492 Hydrogels were fixed for 2 hours in 2.5% glutaraldehyde in 0.1 M phosphate buffer, then
493 washed twice in 0.1 M phosphate buffer for 30 min each. They were then incubated with 1 %
494 osmium tetroxide in 0.1 M phosphate buffer overnight. Gels were dehydrated using an
495 ascending acetone series (20-40-60-80-100%) for 30 minutes each change, followed by critical
496 point drying with a Polaron E3000 apparatus using carbon dioxide.

497 The specimens were then mounted on 13 mm diameter pin stubs using double sided adhesive
498 carbon tape tabs. These were then coated with platinum to a thickness of 5 nm using a
499 Cressington 208HR high resolution sputter coating unit. Images were then obtained using a
500 FEI Quanta 200F FEGSEM (field emission gun environmental scanning electron microscope).

501 **AFM-nanoindentation analysis**

502 AFM coupled with a colloidal probe was used to probe the mechanical properties of the
503 hydrogels. A few drops of cellulose-callose mixtures in ionic liquid were placed in an O-ring
504 fixed on a glass slide, and then the whole slide was immersed in a petri dish with methanol
505 for 5 hours with continuous exchange of methanol every 30 min. When the alcogel disc was
506 formed, it was then placed in an eppendorf tube filled with deionised water to exchange the
507 methanol for water. The samples were stored in water for a minimum of one day, and usually
508 two days, to ensure complete exchange. This was important as even residual swelling at rates
509 of nanometres per second would take the sample surface beyond the limited z-range of the
510 AFM scanners with the long acquisition times of the force spectroscopy maps. In preparation
511 for the AFM, the hydrogels were briefly placed onto a filter paper to remove excess water on
512 the underside only and then glued on a glass slide using two-part epoxy.

513 An Asylum Research MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA) and a PNP-TR
514 silicon nitride cantilever with polystyrene colloidal probe (SQube, Surface Science Support,
515 Germany) with diameter of 1.98 μm was used for measuring the elastic modulus of the
516 hydrogels. The cantilever was calibrated for sensitivity and spring constant before starting any
517 measurement. The spring constant was calibrated in water using the thermal tuning method⁶⁴

518 giving 0.09 N/m. The gel disc for each sample was prepared twice and for each sample force
519 volumes were collected on an average of 5 different areas of the gel. An average of 5 Force
520 maps (20 x 20 μm) were acquired for each sample on different areas, with each force volume
521 map providing 400 individual force measurements. The loading velocity was fixed to 1 μms^{-1} ,
522 ramp size of 1 μm and a maximum applied load of 20 nN. For all samples, the same probe was
523 used and all the parameters were kept constant. Between experiments the cantilever was
524 rinsed with Isopropanol, then ozone cleaning was performed to ensure the colloidal probe is
525 not contaminated.

526 The Hertz model was used for calculating a Young's modulus E of the hydrogels. This models
527 a sphere indenting a flat plane, the exact geometry of our system. The force F applied is given
528 by:

$$529 \quad F = \frac{4E\sqrt{R}}{3(1-\alpha)} \Phi^{3/2} \quad (2)$$

530 where Φ is the indentation depth of the colloidal probe into the hydrogel, α is the Poisson
531 ratio of the hydrogel (here estimated ≈ 0.35), and R is the radius of the probe sphere. The
532 proprietary AFM (Asylum Research) software was used to fit the Hertz model to the first 20
533 % indentation only (Supplementary Figure 2, black trace on the force curve around the
534 contact area).

535 The viscoelastic properties have been calculated from the hysteresis between loading and
536 unloading for all samples, which is related to the dissipation of energy during sample
537 deformation. The plastic behaviour of the hydrogel can be quantified by calculating the
538 plasticity factor (P) from the ratio between the areas under the unloading (A_U) and loading
539 curves (A_L) as given by equation 3 which reflects the relative plastic/elastic behaviour of the
540 material under force^{41, 42, 65}

$$541 \quad P = 1 - \left(\frac{A_U}{A_L} \right) \quad (3)$$

542 For purely elastic samples, where the matter recovers immediately and elastically after
543 indentation, the loading and unloading curves overlap and the two areas are equal ($A_U=A_L$),
544 there is no hysteresis and $P = 0$. In contrast, $P = 1$ indicates fully plastic properties where the
545 area under the unloading curve is zero, and the deformation of the hydrogel remains constant
546 when the load is removed, meaning it has been plastically deformed over a timescale of
547 seconds (it might recover at longer time scales).

548

549 **Macro-indentation measurement**

550 Cellulose-callose mixtures in ionic liquid were produced and rehydrated similarly as described
551 above for AFM disc-hydrogels but on a larger scale. 2 ml of cellulose-callose mixtures in ionic
552 liquid were prepared in 5 ml vials and then immersed in methanol for 5 hours with continuous
553 exchange of methanol every 30 min. The samples were then incubated in deionized water for
554 24 h. Hydrogel discs of 10 mm diameter were cut out with a hole borer, the thickness of the
555 gels was approximately 4 mm. 100% callose hydrogels could not be made by the method. The
556 hydrogel discs were glued onto a petri dishes using two-part epoxy and measurements (force-
557 distance) were performed with a TA-TX2 Texture Analyser (stable Micro Systems, Surrey, UK)
558 in compression mode equipped with a flat-bottomed probe of 2.0 mm diameter⁶⁶.
559 Indentation was strain controlled to an indentation depth of around 80% of the full sample
560 depth. The raw force-distance values were exported and analysed. Young's modulus was
561 calculated according to the Sneddon model⁶⁷, which is suitable for deep indentation into soft
562 materials that conform to the indenter probe geometry. The Elastic modulus (E) was obtained
563 using the following equation (4)

$$564 \quad E = \frac{\sqrt{\pi}}{2} \cdot \frac{S \cdot (1-\nu^2)}{\sqrt{A}} \quad (4)$$

565 where A is the contact area, ν is the Poisson ratio (0.3) and S is the stiffness calculated by $\frac{\delta P}{\delta h}$,
566 where P is the indenter load and h is the displacement of the indenter.

567

568 **Rheological measurements**

569 Rheological measurements of the neat solutions and of cellulose/callose mixtures in
570 [C2mim][OAc] were performed on Kinexus rheometer (Malvern Instrument Ltd.,
571 Worcestershire, England, UK), equipped with cone-plate geometry (4⁰ - 40 mm) with 2 mm
572 gap and a temperature controlled system. Viscoelastic spectra of the samples (elastic
573 component, G' , viscous component, G'' , and phase angle δ) were detected with the oscillation
574 frequency sweep method, within a frequency range 0.01-100 Hz, at 25 °C. For a pure elastic
575 material (solid-like behaviour) the stress and strain are in phase with each other resulting in

576 $\delta = 0$, but for a pure viscous material (liquid-like behaviour) the stress and strain are out of
577 phase⁶⁸ with $\delta = 90^\circ$.

578 Steady state viscosity of each sample was measured using the same rheometer at increasing
579 shear rate in the range of 0.01-100 s⁻¹ across the temperature range 30-60 °C, in 10 °C
580 increments.

581 The activation energy was calculated using the Arrhenius equation (equation 5), where η_α is
582 the zero shear rate viscosity, A is a constant, R is the universal gas constant (8.314 J mol⁻¹ K⁻¹)
583 and T is temperature in K.

$$584 \quad \eta_\alpha = A e^{\left(\frac{E_a}{RT}\right)} \quad (5)$$

585

586 Pulsed-Field Gradient ¹H NMR Spectroscopy

587 Diffusion coefficients of both the cation [C2mim]⁺ and anion [OAc]⁻ were determined as
588 published before⁴⁶ by a pulsed-field gradient ¹H NMR technique using a widebore Avance II
589 NMR Spectrometer (Bruker Biospin) operating at a ¹H resonant frequency of 400 MHz. A
590 Diff50 diffusion probe (Bruker Biospin) capable of producing a maximum field gradient of 20
591 T m⁻¹ was used in the experiments. The calibration of the gradient field strength was
592 performed by measuring the self-diffusion coefficient of water at 20.0 ± 0.1 °C, which has the
593 value (2.03 ± 0.01) × 10⁻⁹ m² s⁻¹. A subsequent check of the sample environment temperature
594 was performed by measuring the temperature dependence of the diffusion coefficient of
595 water with reference to results published by Holz *et al.*⁶⁹ The recommendations set out by
596 Annat *et al.*⁷⁰ were followed such as keeping sample depths to less than 1 cm to minimize
597 convection currents on heating in the NMR spectrometer. The uncertainty in these diffusion
598 coefficient values is estimated to be approximately 3%. In this study a stimulated echo pulse
599 sequence with bipolar gradients was used. The attenuation of the signal intensity in this
600 pulsed field gradient NMR experiment follows equation 6⁷¹:

$$601 \quad \ln\left(\frac{S_i}{S_{i0}}\right) = -D_i \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} - \frac{\tau}{2}\right) \quad (6)$$

602 where S_i is the measured signal intensity of species i and D_i is the diffusion coefficient of that
603 species, S_{i0} defines the initial signal intensity, γ is the proton gyromagnetic ratio, δ is the pulse

604 duration of a combined pair of bipolar pulses, τ is the period between bipolar gradients, Δ is
605 the period separating the beginning of each pulse-pair (*i.e.* diffusion time), and g is the
606 gradient strength. In each experiment the strength of the gradient pulse was incremented,
607 while δ (2–5 ms) and Δ (60 ms) remained constant. τ was kept constant at 2 ms. The 90° pulse
608 width was 6.6 μ s, g had maximum values between 200 and 600 G cm⁻¹, the number of scans
609 was 16, and the repetition time was 6 s. The T_1 relaxation times for the various resonances
610 ranged from 600 to 1200 ms. Samples were studied in steps of 10 °C over the temperature
611 range 20–60 °C inclusive.

612 FTIR Spectroscopy

613 Hydrogel samples were snap-frozen in liquid nitrogen and then dehydrated in a freeze drier
614 at -80 °C for 24 hours. The dried hydrogels were ground using tissue lyser LT (Qiagen, Hilden,
615 Germany) for 20 min each. For the plant samples, alcohol insoluble residues (AIR) were
616 obtained from frozen tissues via extraction in an ascending ethanol series (70, 80, 90, 100 %
617 v/v in H₂O) for 1 hour per change, followed by extraction with 100 % acetone and
618 methanol:chloroform (3:2) (1 hour per change). The pellet was recovered by centrifugation
619 between each step. The resulting pellet was dried and used directly for FTIR. 1 mg of each
620 sample was mixed with 300 mg of fresh FTIR grade KBr, ground together, and then
621 compressed to a disc under 10 tons applied weight for 2 min.

622 FTIR spectra were obtained for all samples using Bruker IFS-66 spectrometer equipped with a
623 liquid nitrogen cooled MCT detector, with all optics under vacuum. Scans were taken at a 2
624 cm⁻¹ resolution, within frequency range of 400 - 4000 cm⁻¹, in absorption mode, and 1000
625 spectra were co-added to yield spectra of high signal-to-noise ratio. Obtained spectra were
626 normalized to the absorbance of the O–H in-plane deformation band at 1336 cm⁻¹.

627 The energy of the hydrogen bond (E_H) was calculated using the equation ⁷⁴⁹:

$$628 \quad E_H = \left(\frac{1}{K}\right) \left[\frac{(\nu_0 - \nu)}{\nu_0}\right] \quad (7)$$

629 where ν_0 is the standard frequency corresponding to free –OH groups (3600 cm⁻¹), ν is the
630 frequency of the bonded –OH groups, and $K = 1.68 \times 10^{-2}$ kcal⁻¹.

631 Lateral Order Index (LOI) was calculated as a ratio of intensity of the peaks, $\alpha_{1429/893}$ ⁷².

632 **Analysis and confocal imaging of the plant material**

633 Plants were grown as indicated in the methods section. For phenotyping, seedlings were
634 stained in a two-step procedure by aniline blue followed with Direct Red 23 (Sigma Aldrich,
635 USA). Roots were dissected from the plant hypocotyls and incubated for 2 h at room
636 temperature in a $25 \mu\text{g mL}^{-1}$ ($42.66 \mu\text{M}$) solution of aniline blue fluorochrome (Biosupplies,
637 AU) in a 50mM K_2HPO_4 buffer under vacuum (60 MPa). A 1% wt filtered ($0.45\mu\text{m}$ filter) stock
638 solution of Direct Red 23 (Sigma Aldrich, USA) was then added to the aniline blue solution in
639 order to reach a 0.1 % wt final concentration, and allow to stain one more hour in the same
640 vacuum conditions (60 MPa). Slight changes were made to perform this double staining in
641 leaves: leaves were dehydrated in 96 % vol EtOH until cleared, quickly rehydrated in water
642 prior to a 4 h incubation at room temperature in a $25 \mu\text{g/ml}$ ($42.66 \mu\text{M}$) solution of aniline
643 blue fluorochrome in a 50mM K_3PO_4 buffer under vacuum (60 MPa). Direct Red 23 was then
644 added as described above to the aniline blue solution and allowed to stain 2 h more. Roots
645 and leaves were then mounted in a 1:1 vol solution of AF1 antifadent (Citifluor, USA) and
646 50mM K_2HPO_4 or K_3PO_4 buffer, and subsequently imaged by confocal laser scanning
647 microscopy (Zeiss LSM700). Aniline blue staining was imaged with a 405 nm solid state laser,
648 whose emission was recovered with a short pass 490 nm filter, and Direct Red 23 staining was
649 imaged with a 555 nm solid state laser, whose emission was recovered with a long pass 560
650 nm filter. Pictures were processed using the deconvolution software Huygens and ImageJ
651 1.51s (for z-stacks projections and contrast). Adobe Photoshop CS5 was used to do the
652 montages.

653 Callose amounts were quantified in AIR extracts obtained 24 hours after estradiol treatment
654 using aniline blue fluorescence as described for the hydrogels above.

655

656 **Materials and correspondence**

657 Correspondence to Yoselin Benitez-Alfonso or Michael Ries.

658

659 **Data availability**

660 All raw and processed data, associated with both main and supplementary figures and tables, are
661 accessible in '[<http://doi.org/10.5518/225>]'. All other data supporting the findings of this study are
662 available within the manuscript or are available from the corresponding authors upon request.

663

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915 **END NOTES**

916 **Acknowledgements**

917 This work was supported by EPSRC Grant EF/M027740/1 (which funded M.C.H-G. work) and
918 the Leverhulme Trust Grant RPG-2016-136 (which funded R.H.A-S., C.P. and S.A. work). We
919 thank Prof. Peter Hine, Prof. Richard Bushby and Dr. Richard Blackburn for help with the
920 analysis of the data and comments on the manuscript. M.E.R. is grateful to the Royal Society
921 for funding his Industrial Fellowship. Y.H. have been supported by Finnish Centre of Excellence
922 in Molecular Biology of Primary Producers (Academy of Finland CoE program 2014-2019)
923 decision #271832. Y.H. laboratory was funded by the Gatsby Foundation [GAT3395/PR3]; the
924 National Science Foundation Biotechnology and Biological Sciences Research Council grant
925 [BB/N013158/1]; University of Helsinki [award 799992091], the European Research Council
926 Advanced Investigator Grant SYMDEV [No. 323052].

927

928 **Author contributions**

929 All authors contributed to manuscript preparation. R.H.A-S. and M.C.H-G. did most of the
930 experiments and contributed to data analysis and interpretation of results. S.A.
931 experimentally determined (1,3)- β -glucan concentration, did all plant work and isolated cell
932 walls extracts for FTIR. C.P. did the macro-indentation measurements. M.F. did the SEM
933 experiments and T.B. contributed to the analysis and interpretation of the data. M.B., S.M.
934 and Y.H. obtained and analysed the *pG1090::icals3m* lines and contributed to the writing of
935 this manuscript. Y.B-A, M.E.R. and S.D.C. conceived and designed research, interpreted the
936 data and supervised the experiments.

937

938 **Competing interests**

939 The authors declare no competing financial interests.

940

941 **Summary of Figures and Figures legends**

942

943 **Figure 1: Morphology of hydrogels with different callose concentrations.** Cross-sections of
944 dried hydrogels with different amounts of cellulose and callose were prepared as described in
945 the methods and visualized using scanning electron microscopy (SEM). The pictures (a-d) are
946 representative of each mixture composition and are labelled as percentage of callose. Scale
947 bar = 3 μm

948

949 **Figure 2: Nano-mechanical properties of the hydrogels.** a) shows typical loading and
950 unloading force curves obtained for the different hydrogels (0% callose in black, 20% in red,
951 50% in blue, 80% in green and 100% in orange) using AFM-nanoindentation. b) shows
952 plasticity values as a function of callose concentration; the solid line is presented to guide
953 the eye. c) shows the calculated Young's modulus (data points) as a function of the callose
954 concentration in the hydrogel (solid line is to guide the eye). d) Mean Young's modulus (blue
955 circles, individual data points displayed in c) are represented in relation to values obtained as
956 result of applying the Voigt and Reuss hypothetical model (dashed lines in black and red
957 respectively). Boxes in b and c represent the first (25%) and third (75%) quartiles, the central
958 line is the mean, and outliers at the 1% and 99% level are indicated by the whiskers, the data
959 points are averages for 3 individual samples, with 5 areas imaged for each sample.

960

961 **Figure 3: Mechanical properties of cellulose-callose hydrogels.** a) shows force-displacement
962 curves obtained for the hydrogels at different callose concentrations (0% in black, 10% in red,
963 20% in blue, 50% in green and 80% in magenta) using a Texture Analyzer and a 2mm flat-ended
964 probe in compression mode. The yield point for 100% cellulose (0% callose), which coincides
965 with a yield stress of 1.4 MPa, is arrowed. b) shows Young's modulus values as a function of
966 callose concentration represented in a box plot. The solid line is drawn to guide the eye. The
967 Young's modulus was calculated according to the Sneddon model as described in methods.
968 Boxes represent the first (25%) and third (75%) quartiles, the central line is the mean, and
969 outliers at the 1% and 99% level are indicated by the whiskers. Individual data points for four-
970 five independent replicas are shown.

971 **Figure 4: Viscoelastic properties of the cellulose-callose mixtures in ionic liquid solution at**
972 **25 °C.** a) represents changes in the phase angle (δ , open circles) as a function of frequency (f)
973 for the five callose concentrations. b) Changes in the viscous (G'' , open squares) and elastic
974 (G' , filled squares) components as a function of frequency at different percentage (%) of
975 callose in the mixtures. Graph elements in black correspond to 0% callose, in dark-red to 20%,
976 orange to 50%, blue to 80% and green to 100%. The arrows indicate the approximate
977 frequency where G'' and G' overlap.

978 **Figure 5: Zero shear rate viscosity values as a function of callose concentration and**
979 **temperature in the mixtures of ionic liquid solution.** a) shows the Ln of the viscosity (η) at
980 the different callose percentage and within the temperature range 30-60 °C. A linear fitting
981 (mixing rule) is presented in discontinuous lines in brown for 30 °C, blue for 40 °C, orange for
982 50 °C and green for 60 °C. The graph in b) shows changes in the viscosity values as a function

983 of temperature at the different callose concentrations (%). The discontinuous pink line in **b)**
984 shows the theoretical values predicted for the 50% composition as calculated using ideal
985 mixing rule. Boxes represent the first (25%) and third (75%) quartiles, the central line is the
986 mean, and outliers at the 1% and 99% level are indicated by the whiskers. Individual data
987 points for four-five independent replicas are shown. Error bars represent the standard
988 deviation.

989 **Figure 6: Illustration of the non-ideal behaviour of the diffusion of the ions determined by**
990 **¹H NMR.** **a)** represents the Ln diffusion coefficient (D) of the anion (red circles) and the cation
991 (black circles) in [C2mim][OAc] at 30 °C as a function of mixture composition (% of callose).
992 The blue dashed lines represent the theoretical values calculated based on the ideal mixing
993 rule. Average percentage errors (calculated based on the uncertainty in the diffusion values
994 for the cation) are below 2%. **b)** shows the ratio of diffusion coefficients for the anion and the
995 cation ($D_{\text{anion}}/D_{\text{cation}}$) as a function of callose concentrations at 20 °C (black square), 30 °C (red
996 circle), 40 °C (green triangle), 50 °C (blue inverted triangle) and 60 °C (light blue diamond).
997 The values are individual data points. Percentage differences in technical replicas at 30 °C and
998 40 °C are shown in Supplementary Figure 6.
999

1000 **Figure 7: Fourier- transformed infrared spectra show changes in the ordering of the**
1001 **polymers in the hydrogels with increasing callose concentration.** **a)** The image shows the
1002 superimposed FTIR spectra obtained for hydrogel samples with 0 % (black trace), 20% (red),
1003 50% (blue), 80% (magenta) and 100% (green) concentrations of callose. For representation
1004 the spectra were normalized to the absorbance of the O–H in-plane deformation band at
1005 1336 cm^{-1} . **b)** shows the lateral order index (LOI) and **c)** shows hydrogen bond energy as a
1006 function of the different callose concentration. These parameters measure the degree of
1007 structural organization. In both cases, values at 20% callose deviate from ideal mixing (blue
1008 dashed line). Boxes represent the first (25%) and third (75%) quartiles, the central line is the
1009 mean, and outliers at the 1% and 99% level are indicated by the whiskers. Individual data
1010 points for three independent replicas are shown. Error bars represent the standard deviation
1011 for three independent experimental replicas.
1012

1013 **Figure 8: Induction of *pG1090::icals3m* increases callose which decorates cellulose**
1014 **microfibrils.** DMSO-treated and estradiol-treated *icals3m* roots were co-stained with aniline
1015 blue (blue signal) and direct red 23 (red signal) which fluorescently stain callose and
1016 cellulose respectively. **a, b)** show confocal images of a region in the elongation zone of the
1017 root 24 h after treatment with DMSO (control). **b)** and close-up show merged signal at higher
1018 magnification of a cell as indicated. **c-f)** show comparative regions in *icals3m* roots 24h after
1019 estradiol induction (activation of *icals3m*). Notice the increase accumulation of callose (blue
1020 signal) in **c)** comparing to **a)**. Consult also Supplementary Figure 9. **d, e)** and **f)** show
1021 enlargements of a cell from **c)**, with their associated close-ups in the separate channels (**d)**,
1022 direct red 23; **e)**, aniline blue and **f)**, merged image). Arrowhead highlights an area where
1023 callose integrates very closely to cellulose microfibrils. Scale bars (**a,b)**= 25 μm ; (**b,d,e,f)**=10
1024 μm ; (**b,d,e,f** close-ups)=5 μm .
1025

1026 **Figure 9: Fourier- transformed infrared analysis of cell walls 24 h after estradiol treatment.**
1027 The image shows an example of the FTIR profile for one replica per each genotype: wild type

1028 (black trace) and transgenic line *pG1090::icals3m* (*icals3m*, in red). Estradiol-treated *icals3m*
1029 accumulates callose in comparison to control (Figure 8 and Supplemental Figure 9). Notice
1030 that the absorbance of the OH- stretching vibration broad band ($3100\text{-}3600\text{ cm}^{-1}$) is reduced
1031 in *icals3m*.
1032

1033

1034

1035 **Tables**

1036 **Table 1.** Callose concentration, hydrogen bond energy and lateral order index quantified in
 1037 alcohol insoluble residues (AIR) from *wild type* and transgenic *pG1090::icals3m* plants

	<i>Wild type</i> *	<i>pG1090::icals3m</i> *
Callose content (10⁻³ mg per mg AIR)	6 ± 0.72	11 ± 0.91
Hydrogen bond energy (kJ)	4.05±0.1	3.38±0.2
Lateral order index (LOI)	6.12±0.19	3.85±0.77

1042 * mean values ± standard deviation calculated for four biological replicas.