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

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29 Interactions.

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**Abstract:**

The properties of (1,3)- $\beta$ -glucans (i.e. callose) remain largely unknown despite their importance in plant development and defence. Here we use mixtures of (1,3)- $\beta$ -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  $\beta$ -glucans which are essential for  
62 development and adaptation to a changing environment<sup>1, 2</sup>. The physical and mechanical  
63 properties of this diverse group of polysaccharides are applied in industry to obtain many  
64 valuable products. For example,  $\beta$ -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 products<sup>3</sup>. They are also applied in the development of recent technologies  
67 such as tissue engineering, prosthetics and nanostructured materials<sup>4, 5</sup>. In the cell wall  
68 matrix, the composition and structure of  $\beta$ -glucans underpin plant growth and development<sup>6</sup>.  
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)- $\beta$ -linked glucosyl residues) is the most abundant  $\beta$ -glucan  
74 polysaccharide, found in the cell wall of bacteria, algae, fungi and plants and even in  
75 urochordate animals<sup>7</sup>. Due to numerous intra- and intermolecular hydrogen bonds and van  
76 der Waals interactions<sup>8</sup> 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.<sup>6, 9, 10</sup>. The interactions of cellulose with diverse glycan polymers (including  
81 xyloglucans and xylans) have been reported but rarely validated in biological systems<sup>11-13</sup>. 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 interactions<sup>14-16</sup>.

85 In contrast to cellulose, plant (1,3)- $\beta$ -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<sup>17, 18</sup>. In normal unstressed conditions, callose appears mainly  
88 deposited around intercellular junctions named plasmodesmata<sup>19</sup>. Callose accumulation  
89 determines intercellular communication, affecting developmental and environmental

90 signalling<sup>20</sup>. Callose also has important regulatory functions in cell plate formation during cell  
91 division and in the sieve pores that connect the phloem vascular system<sup>21</sup>. It accumulates in  
92 response to wounding and exposure to biotic and abiotic stresses playing a key role in  
93 defence<sup>22, 23</sup>.

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 penetration<sup>24-26</sup>  
98 and to act as a matrix for the deposition of other cell wall components<sup>27</sup>. Digestion of callose  
99 appears to reduce cell wall stiffness leading to an increase in the diameter of the pollen tube<sup>28</sup>,  
100 <sup>29</sup>. 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 infections<sup>30</sup>.  
102 Pectin and cellulose distribution also change after digestion of callose in pollen tubes<sup>28</sup> but it  
103 is not clear if these changes in cell wall composition occur or if they affect plant-fungus  
104 interactions<sup>31</sup>. Recent studies visualized a 3D network of callose-cellulose microfibrils forming  
105 in the site of fungal penetration<sup>32</sup>, 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 polysaccharides<sup>33</sup> and were used here to prepare mixture  
112 solutions of microcrystalline cellulose and a (1,3)- $\beta$ -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 rule<sup>34</sup>, 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*)<sup>35</sup>. 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 elsewhere<sup>36</sup>. 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 Methods<sup>37</sup> 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 aerogels<sup>38</sup>. 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<sup>39, 40</sup> (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 <sup>39, 41</sup>. 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<sup>41, 42</sup>). 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  $86 \pm 1.6$  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  $42 \pm 1.6$  kPa at 100% callose. The  
168 theoretical Voigt upper and Reuss lower bounds are plotted for the elastic modulus<sup>43, 44</sup> (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 ( $\delta$ ) 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)<sup>45</sup> 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  $\gamma$  is shear rate,  $\eta_0$  is zero shear rate viscosity,  $\eta_\infty$  is the viscosity at infinite shear rate,  $n$   
231 is the flow index and  $\alpha$  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  $64.2 \pm 1.8$  kJ and  $70.8 \pm 9.6$  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 solution<sup>46</sup>. 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 water<sup>47</sup>.

259 The  $^1\text{H}$  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 methods<sup>46</sup>. 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 diffusion<sup>48</sup> 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  $44.1 \pm 0.8$  to  $41.3 \pm 0.9$  kJ mol<sup>-1</sup> 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 ordering<sup>49</sup>. 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<sup>-1</sup>) that informs about the hydrogen bonding pattern<sup>49,50</sup>. Using  
297 information from the spectra, the lateral order index (LOI, ratio between CH<sub>2</sub> bending and C–  
298 O–C stretching absorption bands) and the energy of the hydrogen bond ( $E_H$ ) was calculated

299 (see methods and reference<sup>50</sup>) (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*<sup>35, 51</sup>.  
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 cellulose<sup>9</sup>), 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)- $\beta$ -glucans (i.e. callose) plays an important role in cell biology impacting organism  
340 development and environmental responses<sup>1, 52, 53</sup>. 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<sup>27, 29,</sup>  
344 <sup>32</sup>. 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 <sup>1</sup>H 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 cellulose<sup>46</sup> 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 interactions<sup>48</sup>. 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<sup>47, 54, 55</sup>. 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)<sup>56</sup> 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 crystallinity<sup>57</sup> (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<sup>18 27</sup>. 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/ductility<sup>58</sup>. 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)- $\beta$ -D-glucan Pachyman, and aniline blue fluorochrome  
432 were obtained from Biosupplies Australia ([www.biosupplies.com.au](http://www.biosupplies.com.au)). The molecular weight  
433 of Avicel PH-101 is  $28\,400\text{ g mol}^{-1}$ <sup>59</sup> whereas of Pachyman it is as  $168\,000\text{ g mol}^{-1}$ <sup>60</sup>. The  
434 molecular weight distribution of Avicel PH-101 as well as polydispersity and crystallinity index  
435 were reported elsewhere<sup>61,62</sup>. Molecular weight and other properties (such as polydispersity,  
436 flexibility and conformation of polymers) were also reported elsewhere for Pachyman<sup>63</sup>.

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  $\beta$ -estradiol inducible pG1090 promoter<sup>51</sup>. In this transgenic line, callose  
441 amount is significantly higher 6-24 h after exposure to  $\beta$ -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  $\mu\text{M}$   $\beta$ -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  $\mu\text{M}$   $\beta$ -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)- $\beta$ -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  $\mu$ 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  $\mu$ 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  $\mu\text{m}$  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 method<sup>64</sup>

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  $\mu\text{m}$ ) 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  $\mu\text{ms}^{-1}$ ,  
522 ramp size of 1  $\mu\text{m}$  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  $\Phi$  is the indentation depth of the colloidal probe into the hydrogel,  $\alpha$  is the Poisson  
531 ratio of the hydrogel (here estimated  $\approx 0.35$ ), 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<sup>41, 42, 65</sup>

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

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 diameter<sup>66</sup>.  
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 model<sup>67</sup>, 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,  $\nu$  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 (4<sup>0</sup> - 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  $\delta$ ) 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 phase<sup>68</sup> 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<sup>-1</sup> 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  $\eta_\alpha$  is  
582 the zero shear rate viscosity,  $A$  is a constant,  $R$  is the universal gas constant (8.314 J mol<sup>-1</sup> K<sup>-1</sup>)  
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 <sup>1</sup>H NMR Spectroscopy

587 Diffusion coefficients of both the cation [C2mim]<sup>+</sup> and anion [OAc]<sup>-</sup> were determined as  
588 published before<sup>46</sup> by a pulsed-field gradient <sup>1</sup>H NMR technique using a widebore Avance II  
589 NMR Spectrometer (Bruker Biospin) operating at a <sup>1</sup>H resonant frequency of 400 MHz. A  
590 Diff50 diffusion probe (Bruker Biospin) capable of producing a maximum field gradient of 20  
591 T m<sup>-1</sup> 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<sup>-9</sup> m<sup>2</sup> s<sup>-1</sup>. 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.*<sup>69</sup> The recommendations set out by  
596 Annat *et al.*<sup>70</sup> 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 6<sup>71</sup>:

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

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,  $\gamma$  is the proton gyromagnetic ratio,  $\delta$  is the pulse

604 duration of a combined pair of bipolar pulses,  $\tau$  is the period between bipolar gradients,  $\Delta$  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  $\delta$  (2–5 ms) and  $\Delta$  (60 ms) remained constant.  $\tau$  was kept constant at 2 ms. The 90° pulse  
608 width was 6.6  $\mu$ s,  $g$  had maximum values between 200 and 600 G cm<sup>-1</sup>, 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 H<sub>2</sub>O) 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<sup>-1</sup> resolution, within frequency range of 400 - 4000 cm<sup>-1</sup>, 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<sup>-1</sup>.

627 The energy of the hydrogen bond ( $E_H$ ) was calculated using the equation <sup>749</sup>:

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

629 where  $\nu_0$  is the standard frequency corresponding to free –OH groups (3600 cm<sup>-1</sup>),  $\nu$  is the  
630 frequency of the bonded –OH groups, and  $K = 1.68 \times 10^{-2}$  kcal<sup>-1</sup>.

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

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  $\text{K}_2\text{HPO}_4$  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  $\text{K}_3\text{PO}_4$  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  $\text{K}_2\text{HPO}_4$  or  $\text{K}_3\text{PO}_4$  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**

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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)- $\beta$ -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  $\mu\text{m}$

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 ( $\delta$ , 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 ( $\eta$ ) 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 **<sup>1</sup>H 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  $1336\text{ cm}^{-1}$ . **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  $\mu\text{m}$ ; (**b,d,e,f)**=10  
1024  $\mu\text{m}$ ; (**b,d,e,f** close-ups)=5  $\mu\text{m}$ .  
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> *
<b>Callose content (10<sup>-3</sup> mg per mg AIR)</b>	6 ± 0.72	11 ± 0.91
<b>Hydrogen bond energy (kJ)</b>	4.05±0.1	3.38±0.2
<b>Lateral order index (LOI)</b>	6.12±0.19	3.85±0.77

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