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¹ Interactions between callose and cellulose revealed

² through the analysis of biopolymer mixtures

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 Interactions.

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36 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 physicomechanical 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.

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60 Introduction

The cell wall of plant, bacteria, and fungi are enriched with β -glucans which are essential for 61 development and adaptation to a changing environment^{1, 2}. The physical and mechanical 62 properties of this diverse group of polysaccharides are applied in industry to obtain many 63 valuable products. For example, β-glucans cell wall biopolymers are used as raw materials for 64 paper, films and textiles, as gelling agents and natural immune stimulants in food and in 65 66 pharmaceutical products ³. They are also applied in the development of recent technologies such as tissue engineering, prosthetics and nanostructured materials^{4, 5}. In the cell wall 67 matrix, the composition and structure of β -glucans underpin plant growth and development⁶. 68 Understanding how these glycans are assembled, how they interact at the molecular level, 69 and the specific physical and mechanical properties they confer, is a major challenge due to 70 71 the complexities of their natural systems. This knowledge could open a whole new range of applications in biotechnology and in the design of new biodegradable materials. 72

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

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

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

Despite its importance, the physical properties of callose and its interactions with other 94 95 biopolymers within the cell wall matrix remain unknown. Most of the work in this area focuses on callose deposited during pathogenic attacks or during pollen tube growth. Callose is 96 thought to strengthen the cell wall forming a physical barrier against fungal penetration²⁴⁻²⁶ 97 and to act as a matrix for the deposition of other cell wall components²⁷. Digestion of callose 98 appears to reduce cell wall stiffness leading to an increase in the diameter of the pollen tube^{28,} 99 ²⁹. Conversely, a mutant in a callose biosynthetic enzyme (named *powdery mildew resistant* 100 101 4, pmr4), shows reduced callose levels but appears resistant to some fungal infections³⁰. Pectin and cellulose distribution also change after digestion of callose in pollen tubes²⁸ but it 102 is not clear if these changes in cell wall composition occur or if they affect plant-fungus 103 104 interactions³¹. Recent studies visualized a 3D network of callose-cellulose microfibrils forming in the site of fungal penetration³², but what factors affect the formation of these networks 105 and their significance in the success of fungal infection are unknown. 106

107 Given the importance of callose to regulate developmental and adaptive processes it is imperative to clarify whether it can interact with cellulose at the molecular level and what are 108 109 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 cellulose and other water-insoluble polysaccharides³³ and were used here to prepare mixture 111 112 solutions of microcrystalline cellulose and a (1,3)- β -glucan polymer named Pachyman, as a commercial form of callose. A combination of techniques including: Atomic Force Microscopy 113 (AFM) and nanoindentation analysis; Scanning Electron Microscopy (SEM), liquid state 114 Nuclear Magnetic Resonance (NMR); rheology and Fourier Transformed InfraRed (FTIR) 115 116 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 in both the liquid and gel state, forming systems with dynamics and mechanical properties 118 that are not adequately described by a simple ideal mixing rule³⁴, i.e. the mixtures properties 119 120 are not just the weighted average of the individual component properties. We consider the relevance of these interactions in cell walls by comparing wild type and transgenic plant material expressing a hyperactive version of the biosynthetic enzyme callose synthase 3 in an inducible system (*icals3m*)³⁵. Based on the results, we propose that interactions occur between callose and cellulose that influence cell wall structure (and thereby properties). The implications of these results for the regulation of cell-to-cell communication and other biological processes are discussed as well as their potential applications for the design of new 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 walls, we used a simplified model of composite hydrogels, as reported elsewhere³⁶. The 131 samples were made with different proportions of commercial microcrystalline cellulose and 132 the callose analogue Pachyman dissolved in the ionic liquid [C2mim][OAc] and coagulated in 133 water (see details in the methods section). It should be noted that cellulose and callose are 134 135 insoluble in water, and thus when water is added to their solutions in ionic liquid, phase separation occurs leading to a formation of a 3D network. We shall use the term hydrogel in 136 137 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 of cellulose-callose in the mixtures. Pure cellulose is labelled 0%, 20:80 is 20% callose, 50:50 140 is 50% callose, 80:20 is 80% callose, and pure callose is labelled as 100%. The concentration 141 of callose in hydrogels was fluorescently quantified as described in the Methods ³⁷ in order to 142 confirm that callose was not washed out during exchange of ionic liquid to water. The results 143 144 showed that callose was not removed during solvent exchange and its concentration in hydrogel is the same as in the mixtures with cellulose (see Supplementary Fig. 1). 145

The morphology of supercritically dried hydrogels was visualized using SEM (Fig.1) to identify potential changes in their structure as a function of mixture composition. 0% callose shows the classical structure of macroporous cellulose aerogels³⁸. Significant differences in morphology were detected between 0%, 50%, 80% callose and the 100% callose. The increase 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 a colloidal probe. Force maps were collected and force-indentation curves were fitted to the 152 Hertz model^{39, 40} (Fig.2a and Supplementary Fig.2) to calculate the Young's modulus for the 153 154 five different hydrogels. The plastic behaviour of the hydrogels (deformation in response to applied forces) can be compared in a relative fashion by calculating the index of plasticity 155 156 from the hysteresis, derived from the recovery of the indented surface after the force is relaxed, as described in the Methods ^{39, 41}. Mean values of Young's modulus and plasticity 157 index are presented in Figures 2b and c, and were derived from statistical analysis of hundreds 158 159 of force curves (Supplementary Fig. 3).

The plasticity index for all samples was between 0.2 and 0.5 (where 0 represents a purely 160 elastic response, and 1 a purely viscous, or plastic response with no recovery^{41, 42}). Both the 161 highest modulus value (stiffness) and plasticity index were measured for cellulose hydrogels 162 163 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 6.6 to 86 \pm 1.6 kPa (errors are standard error of the mean) when comparing cellulose (0 % 165 166 callose) to 20% callose hydrogel, respectively. The modulus continues to decrease with increasing callose concentration reaching a value of 42± 1.6 kPa at 100% callose. The 167 168 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 to the strain direction (Reuss). It is therefore useful to compare our results with the limits 174 established by these models. In the hydrogels, the experimental value of the Young's modulus 175 corresponding to 20% callose does not fall in between these boundaries. This indicates that 176 177 the large reduction in elastic modulus upon addition of 20% callose cannot be explained by a physical arrangement of the callose and cellulose components, leading to the conclusion it 178 179 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

materials. This method allows testing beyond the elastic limit through to failure at high strain 182 (Figure 3). Analysis of the linear elastic region revealed an identical relationship to AFM-183 nanoindentation, with higher modulus at 0% callose, dropping rapidly at 10% - 20% callose. 184 185 The absolute values are about 50% of the AFM values, which is reasonable considering the magnitude difference in the scale of measurement, different contact geometry, contact 186 187 mechanics model and boundary conditions. It is also possible that the 50% modulus reduction is a function of the indentation depth, with the 50 -150 nm deformation in AFM reporting on 188 near-surface properties, and the 2mm flat-ended probe giving a bulk measurement with an 189 190 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 concentrations (Fig. 2b). The yield behaviour is also very different, with the pure cellulose gel 205 allowing the stress to build up before the material catastrophically fails and breaks apart, 206 207 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

the build-up of stress. Hence addition of small amount of callose allows the material to bemore 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.

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

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

229
$$\eta = \frac{\eta_0 + \eta_\infty \, \alpha \gamma^n}{1 + \alpha \gamma^n} \tag{1}$$

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.

The results show, as expected, that viscosity increased with decreasing temperature for each 232 system studied and, consistently, with the increase of callose concentration (Fig. 5). 233 Interestingly, at all temperature tested, values at 80% callose show lower viscosity than 234 235 predicted by a simple ideal mixture rule, as it overlaps with the ideal theoretical values for 50% callose (calculated as the average of the 0% and 100% ln η_0 values) (Fig. 5b). On the 236 contrary, the experimental value of the zero shear rate viscosity of the mixture with 50 % of 237 238 callose is higher than ideal mixing rule prediction. It is clear that there are structural reorganisations in the mixtures with more than 50% of callose. 239

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

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

247 Liquid state ¹H-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 composition of mixed solutions. The ions act as probe molecules in these systems. Their 251 252 diffusion coefficients depend on their environment, which can be affected by the interactions between the polymers. For cellulose, it has been shown that the ions interact with the 253 hydroxyl groups (not engaged in forming intra or inter- molecular hydrogen bonds between 254 the polymers) and that this interaction reduces the diffusion coefficient of the ions in 255 solution⁴⁶. If callose and cellulose interact, a change is expected in the number of OH groups 256 257 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.

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

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

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

To summarize, the results show that at certain callose concentrations in the mixture (20% and 279 280 80%) there is a deviation of both anion and cation diffusion coefficients from the expected ideal mixing rule, which is the evidence of the changes in the number/accessibility of OH 281 282 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 molecular interactions between the polymers, modifying, for example, the number of 284 inter/intra-molecular hydrogen bonds. The significance of this result, together with the 285 mechanical and the viscoelastic properties of mixtures, are discussed below. 286

287 Changes in cellulose-callose organization exposed by FTIR

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

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

(see methods and reference⁵⁰) (Figure 7b-c). It was found that the cellulose sample has the 299 highest LOI (also known as crystallinity index) and E_H values which correlates with forming a 300 301 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 with concentration, Figure 7b) were significant at 20% callose. This suggests that addition of 303 304 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 the interpretation of the results obtained by NMR. 306

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

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

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

336

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338 **Discussion:**

(1,3)-β-glucans (i.e. callose) plays an important role in cell biology impacting organism 339 development and environmental responses^{1, 52, 53}. The accumulation of this polymer is 340 associated with changes in cell wall architecture and mechanical properties but it is not clear 341 if the effect is direct or indirect. There is evidence pointing to callose function as a sealant, as 342 a load-bearing structure or as a matrix for the deposition of other cell wall components^{27, 29,} 343 344 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 sort of cellulose plasticizer abolishing the dramatic failure which would otherwise occur when 346 stress increases to the yield point and the material cleaves deeply. Rheology analysis of 347 mixtures in ionic liquid supports a gel-like behaviour (viscoelastic response) for callose 348 concentrated solutions. We also demonstrate that the mechano-physical properties (elastic 349 modulus, plasticity and viscosity) of the hydrogels and mixtures in ionic liquid diverged from 350 351 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 nonideal decreasing pattern at concentrations of 20% and between 70-90% callose. Deviations 353 from ideal mixing in the hydrogen bond pattern and lateral order index were also found in 354 20% callose hydrogel mixtures using FTIR. Although more complex in interpretation, changes 355 in cell walls ordering and hydrogen bond pattern also occur after increasing callose 356 357 biosynthesis in plant cell walls using as a tool a transgenic estradiol-inducible callose synthase system (*pG1090::icals3m*). Together, the results indicate that the accumulation of callose 358 359 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 in ionic liquid across all temperature measurements recorded in the mixtures using NMR. The 362 characteristic anomalous diffusion ($D_{anion}/D_{cation} < 1$) reported for cellulose⁴⁶ becomes more 363 364 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 sensitive to the polymer-polymer interactions⁴⁸. A decrease of D_{anion}/D_{cation} indicates that the 366 diffusion of the anion is preferentially reduced providing a strong evidence for interactions. 367 The non-ideal behaviours of either the diffusion coefficient of the ions and of the mechanical 368

properties of the mixtures are concentration dependent, which likely reflect the complexity 369 of these systems where cellulose-cellulose, cellulose-callose and callose-callose interactions 370 371 can contribute differently to the molecular environment and properties of the mixtures. The fact that interactions appear to occur at certain stoichiometric ratios has been previously 372 reported for other blends^{47, 54, 55}. In callose-cellulose mixtures, interactions at 20% and 80% 373 374 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 model plant Arabidopsis thaliana significantly affects the hydrogen bond energy and lateral 382 order index (considered as crystallinity index by other authors)⁵⁶ calculated using FTIR spectra 383 384 of cell walls (Figure 9, table 1). Moreover, confocal imaging after induction of callose using the pG1090::icals3m line shows close proximity between newly synthesized callose and 385 386 cellulose microfibrils and 48 h induction times produce phenotypes that resemble those reported in plants defective in cellulose organization and cell wall crystallinity⁵⁷ (Figure 8 and 387 Supplementary Fig. 10). These results support the potential applicability of our hydrogel 388 389 model in the understanding callose-cellulose interactions in vivo.

Traditionally, callose production has been associated with cell wall reinforcement to restrict 390 the penetration of pathogens or in response to elicitors and other stress conditions^{18 27}. Our 391 results using hydrogel models suggest that increasing callose concentration significantly 392 reduces the Young's modulus of neat cellulose by about three fold, increasing flexibility, whilst 393 394 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 a high strain condition. Adding callose appears to increases cellulose resilience to high strain 397 398 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 build-up of high stress, and by allowing a failure mode that involves a gradual deformation 401 402 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 or plastic deformation/ductility⁵⁸. Whilst the addition of callose does not increase toughness 405 406 in strict terms (the area under the stress-strain curve to the yield point is not increased) it appears to increase ductility under high strain condition. Cell wall mechanics is tightly 407 408 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 as plasmodesmata) is difficult to predict without more knowledge on the structural 411 412 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 and how these affect callose biological function. More studies are required to dissect callose 419 420 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 example, composites that can be used as biodegradable substitutes for other less 423 environmentally friendly polymers. 424

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 (≥99.9%), methanol (≥99.9%) and chloroform (≥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 g mol^{-1 59} whereas of Pachyman it is as 168 000 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 pG1090::icals3m was generated by Agrobacterium-mediated transformation of Arabidopsis 438 plants with a vector containing a hyperactive callose synthase mutant version (*cals3m*) under 439 the control of a β -estradiol inducible pG1090 promoter⁵¹. In this transgenic line, callose 440 441 amount is significantly higher 6-24 h after exposure to β-estradiol in comparison to wild type subjected to the same conditions. Wild type and transgenic seeds were surfaced sterilised in 442 bleach solution (20% thin bleach, 0.01% tween-20) for 10 minutes and stratified in the dark 443 at 4°C for 5 days. Seeds were germinated on ½ Murashige and Skoog (MS) medium plates 444 445 containing 0.22% Murashige and Skoog basal medium (Sigma Aldrich), 1% sucrose (Sigma Aldrich), 0.8% plant agar (Sigma Aldrich) and grown under long day conditions (16 hour 446 447 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 μ M 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 μ M β -estradiol. 452 Plants were analysed or harvested at different times after transfer as indicated in Results. For FTIR analysis, plants were collected 24 h post-transfer and flash-frozen in liquid nitrogen to 453 stop metabolic activity before undergoing freeze drying. 454

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, hydrogel samples were cut into 3x3x1 mm sections and incubated for 30 minutes at room 473 temperature in 1 ml of staining solution containing 25 mg/ml of aniline blue fluorochrome in 474 distilled water. Stained samples were washed with 3 changes of distilled water (5 minutes per 475 change) and mounted on microscope slides. Samples were imaged on a Zeiss LSM880 Upright 476 477 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 LT (Qiagen, Hilden, Germany). 0.2 mg of the powder was incubated in 200 µl of 1 M NaOH at 480 481 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 minutes at room temperature. 200 µl of sample was aliquoted to a dark multiwell plate and 484 fluorescence was assayed at 405 nm excitation, 512 nm emission using a POLARstar OPTIMA 485 microplate reader (BMG labtech, Ortenberg, Germany). 486

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

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

491 Characterization of hydrogels using SEM

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

The specimens were then mounted on 13 mm diameter pin stubs using double sided adhesive carbon tape tabs. These were then coated with platinum to a thickness of 5 nm using a Cressington 208HR high resolution sputter coating unit. Images were then obtained using a FEI Quanta 200F FEGESEM (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 hydrogels. A few drops of cellulose-callose mixtures in ionic liquid were placed in an O-ring 503 fixed on a glass slide, and then the whole slide was immersed in a petri dish with methanol 504 for 5 hours with continuous exchange of methanol every 30 min. When the alcogel disc was 505 506 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 AFM scanners with the long acquisition times of the force spectroscopy maps. In preparation 510 for the AFM, the hydrogels were briefly placed onto a filter paper to remove excess water on 511 512 the underside only and then glued on a glass slide using two-part epoxy.

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

giving 0.09 N/m. The gel disc for each sample was prepared twice and for each sample force 518 volumes were collected on an average of 5 different areas of the gel. An average of 5 Force 519 520 maps (20 x 20 μ 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 μ ms⁻¹, ramp size of 1 µm and a maximum applied load of 20 nN. For all samples, the same probe was 522 used and all the parameters were kept constant. Between experiments the cantilever was 523 524 rinsed with Isopropanol, then ozone cleaning was performed to ensure the colloidal probe is not contaminated. 525

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

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

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

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

541
$$P = 1 - \left(\frac{A_{\rm u}}{A_{\rm L}}\right) \tag{3}$$

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

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549 Macro-indentation measurement

Cellulose-callose mixtures in ionic liquid were produced and rehydrated similarly as described 550 above for AFM disc-hydrogels but on a larger scale. 2 ml of cellulose-callose mixtures in ionic 551 liquid were prepared in 5 ml vials and then immersed in methanol for 5 hours with continuous 552 553 exchange of methanol every 30 min. The samples were then incubated in deionized water for 24 h. Hydrogel discs of 10 mm diameter were cut out with a hole borer, the thickness of the 554 gels was approximately 4 mm. 100% callose hydrogels could not be made by the method. The 555 556 hydrogel discs were glued onto a petri dishes using two-part epoxy and measurements (forcedistance) were performed with a TA-TX2 Texture Analyser (stable Micro Systems, Surrey, UK) 557 in compression mode equipped with a flat-bottomed probe of 2.0 mm diameter⁶⁶. 558 559 Indentation was strain controlled to an indentation depth of around 80% of the full sample depth. The raw force-distance values were exported and analysed. Young's modulus was 560 calculated according to the Sneddon model ⁶⁷, which is suitable for deep indentation into soft 561 materials that conform to the indenter probe geometry. The Elastic modulus (E) was obtained 562 563 using the following equation (4)

564

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

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

567

568 Rheological measurements

569Rheological measurements of the neat solutions and of cellulose/callose mixtures in570[C2mim][OAc] were performed on Kinexus rheometer (Malvern Instrument Ltd.,571Worcestershire, England, UK), equipped with cone-plate geometry (4^0 - 40 mm) with 2 mm572gap and a temperature controlled system. Viscoelastic spectra of the samples (elastic573component, G', viscous component, G'', and phase angle δ) were detected with the oscillation574frequency sweep method, within a frequency range 0.01-100 Hz, at 25 °C. For a pure elastic575material (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.

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

584
$$\eta_a = Ae^{\left(-\frac{E_a}{RT}\right)}$$
 (5)

585

586 Pulsed-Field Gradient ¹H NMR Spectroscopy

Diffusion coefficients of both the cation [C2mim]⁺ and anion [OAc]⁻ were determined as 587 published before ⁴⁶ by a pulsed-field gradient ¹H NMR technique using a widebore Avance II 588 NMR Spectrometer (Bruker Biospin) operating at a ¹H resonant frequency of 400 MHz. A 589 Diff50 diffusion probe (Bruker Biospin) capable of producing a maximum field gradient of 20 590 T m⁻¹ was used in the experiments. The calibration of the gradient field strength was 591 performed by measuring the self-diffusion coefficient of water at 20.0 ± 0.1 °C, which has the 592 value $(2.03 \pm 0.01) \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$. A subsequent check of the sample environment temperature 593 was performed by measuring the temperature dependence of the diffusion coefficient of 594 water with reference to results published by Holz *et al.*⁶⁹ The recommendations set out by 595 Annat *et al.* ⁷⁰ were followed such as keeping sample depths to less than 1 cm to minimize 596 convection currents on heating in the NMR spectrometer. The uncertainty in these diffusion 597 coefficient values is estimated to be approximately 3%. In this study a stimulated echo pulse 598 599 sequence with bipolar gradients was used. The attenuation of the signal intensity in this pulsed field gradient NMR experiment follows equation 6⁷¹: 600

601
$$\ln\left(\frac{s_i}{s_{i0}}\right) = -D_i \gamma^2 g^2 \delta^2 \left(\Delta - \frac{\delta}{3} - \frac{\tau}{2}\right)$$
 (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

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

612 FTIR Spectroscopy

Hydrogel samples were snap-frozen in liquid nitrogen and then dehydrated in a freeze drier 613 614 at -80 °C for 24 hours. The dried hydrogels were ground using tissue lyser LT (Qiagen, Hilden, Germany) for 20 min each. For the plant samples, alcohol insoluble residues (AIR) were 615 obtained from frozen tissues via extraction in an ascending ethanol series (70, 80, 90, 100 % 616 617 v/v in H₂0) 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 between each step. The resulting pellet was dried and used directly for FTIR. 1 mg of each 619 sample was mixed with 300 mg of fresh FTIR grade KBr, ground together, and then 620 compressed to a disc under 10 tons applied weight for 2 min. 621

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

527 The energy of the hydrogen bond (E_H) was calculated using the equation 7^{49} :

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

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

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 stained in a two-step procedure by aniline blue followed with Direct Red 23 (Sigma Aldrich, 634 USA). Roots were dissected from the plant hypocotyls and incubated for 2 h at room 635 temperature in a 25 µg mL⁻¹ (42.66 µM) solution of aniline blue fluorochrome (Biosupplies, 636 AU) in a 50mM K₂HPO₄ buffer under vacuum (60 MPa). A 1% wt filtered (0.45µm filter) stock 637 solution of Direct Red 23 (Sigma Aldrich, USA) was then added to the aniline blue solution in 638 639 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 leaves: leaves were dehydrated in 96 % vol EtOH until cleared, quickly rehydrated in water 641 prior to a 4 h incubation at room temperature in a 25 μ g/ml (42.66 μ M) solution of aniline 642 643 blue fluorochrome in a 50mM K₃PO₄ buffer under vacuum (60 MPa). Direct Red 23 was then added as described above to the aniline blue solution and allowed to stain 2 h more. Roots 644 645 and leaves were then mounted in a 1:1 vol solution of AF1 antifadent (Citifluor, USA) and 50mM K₂HPO₄ or K₃PO₄ buffer, and subsequently imaged by confocal laser scanning 646 647 microscopy (Zeiss LSM700). Aniline blue staining was imaged with a 405 nm solid state laser, whose emission was recovered with a short pass 490 nm filter, and Direct Red 23 staining was 648 649 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 treatment654 using aniline blue fluorescence as described for the hydrogels above.

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656 Materials and correspondence

657 Correspondence to Yoselin Benitez-Alfonso or Michael Ries.

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Data availability 659 660 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 References 664 Bacic A., Fincher G. B., Stone B. A. Chemistry, Biochemistry, and Biology of 1-3 Beta Glucans 665 1. 666 and Related Polysaccharides, first edn. Academic press, Elsevier: USA, (2009). 667 2. Brown G. D., Gordon S. Fungal beta-glucans and mammalian immunity. *Immunity*, 19(3): 668 311-315,(2003). 669 670 671 3. Ferreira A. R. V., Alves V. D., Coelhoso I. M. Polysaccharide-Based Membranes in Food 672 Packaging Applications. *Membranes*, **6**(2): (2016). 673 674 4. Carreno N. L. V., Barbosa A. M., Normberg B. S., Salas M. M. S., Fernandes S. C. M., Labidi J. 675 Advances in nanostructured cellulose-based biomaterials, 1 edn. Springer international 676 publishing, (2017). 677 678 5. Zhang R. R., Edgar K. J. Properties, Chemistry, and Applications of the Bioactive 679 Polysaccharide Curdlan. *Biomacromolecules*, **15**(4): 1079-1096,(2014). 680 681 6. Cosgrove D. J. Growth of the plant cell wall. Nat Rev Mol Cell Biol, 6(11): 850-861,(2005). 682 7. Nakashima K., Yamada L., Satou Y., Azuma J., Satoh N. The evolutionary origin of animal 683 684 cellulose synthase. Dev Genes Evol, 214(2): 81-88,(2004). 685 Thomas L. H., Forsyth V. T., Šturcová A., Kennedy C. J., May R. P., Altaner C. M., et al. 686 8. 687 Structure of Cellulose Microfibrils in Primary Cell Walls from Collenchyma. Plant Physiology, **161**(1): 465-476,(2013). 688 689 690 9. Anderson C. T., Carroll A., Akhmetova L., Somerville C. Real-Time Imaging of Cellulose 691 Reorientation during Cell Wall Expansion in Arabidopsis Roots. Plant Physiology, 152(2): 787-692 796,(2010). 693 694 Marga F., Grandbois M., Cosgrove D. J., Baskin T. I. Cell wall extension results in the 10. 695 coordinate separation of parallel microfibrils: evidence from scanning electron microscopy and atomic force microscopy. *The Plant Journal*, **43**(2): 181-190,(2005). 696 697 698 11. Kiemle S. N., Zhang X., Esker A. R., Toriz G., Gatenholm P., Cosgrove D. J. Role of (1,3)(1,4)- β -699 Glucan in Cell Walls: Interaction with Cellulose. Biomacromolecules, 15(5): 1727-700 1736,(2014).

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915 END NOTES

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928 Author contributions

All authors contributed to manuscript preparation. R.H.A-S. and M.C.H-G. did most of the 929 experiments and contributed to data analysis and interpretation of results. S.A. 930 931 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. and Y.H. obtained and analysed the *pG1090::icals3m* lines and contributed to the writing of 934 this manuscript. Y.B-A, M.E.R. and S.D.C. conceived and designed research, interpreted the 935 936 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

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

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949 Figure 2: Nano-mechanical properties of the hydrogels. a) shows typical loading and unloading force curves obtained for the different hydrogels (0% callose in black, 20% in red, 950 951 50% in blue, 80% in green an 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 circles, individual data points displayed in c) are represented in relation to values obtained as 955 956 result of applying the Voigt and Reuss hypothetical model (dashed lines in black and red respectively). Boxes in **b** and **c** represent the first (25%) and third (75%) quartiles, the central 957 line is the mean, and outliers at the 1% and 99% level are indicated by the whiskers, the data 958 959 points are averages for 3 individual samples, with 5 areas imaged for each sample.

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

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

Figure 5: Zero shear rate viscosity values as a function of callose concentration and temperature in the mixtures of ionic liquid solution. a) shows the Ln of the viscosity (η) at the different callose percentage and within the temperature range 30-60 °C. A linear fitting (mixing rule) is presented in discontinuous lines in brown for 30 °C, blue for 40 °C, orange for 50 °C and green for 60 °C. The graph in **b**) shows changes in the viscosity values as a function of temperature at the different callose concentrations (%). The discontinuous pink line in **b**) shows the theoretical values predicted for the 50% composition as calculated using ideal mixing rule. Boxes represent the first (25%) and third (75%) quartiles, the central line is the mean, and outliers at the 1% and 99% level are indicated by the whiskers. Individual data points for four-five independent replicas are shown. Error bars represent the standard deviation.

989 Figure 6: Illustration of the non-ideal behaviour of the diffusion of the ions determined by

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

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1000 Figure 7: Fourier- transformed infrared spectra show changes in the ordering of the

polymers in the hydrogels with increasing callose concentration. a) The image shows the
 superimposed FTIR spectra obtained for hydrogel samples with 0 % (black trace), 20% (red),
 50% (blue), 80% (magenta) and 100% (green) concentrations of callose. For representation
 the spectra were normalized to the absorbance of the O–H in-plain deformation band at

1336 cm⁻¹. b) shows the lateral order index (LOI) and c) shows hydrogen bond energy as a
function of the different callose concentration. These parameters measure the degree of
structural organization. In both cases, values at 20% callose deviate from ideal mixing (blue
dashed line). Boxes represent the first (25%) and third (75%) quartiles, the central line is the
mean, and outliers at the 1% and 99% level are indicated by the whiskers. Individual data
points for three independent replicas are shown. Error bars represent the standard deviation
for three independent experimental replicas.

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1013 Figure 8: Induction of *pG1090::icals3m* increases callose which decorates cellulose

microfibrils. DMSO-treated and estradiol-treated *icals3m* roots were co-stained with aniline
 blue (blue signal) and direct red 23 (red signal) which fluorescently stain callose and
 cellulose respectively. a, b show confocal images of a region in the elongation zone of the
 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
- signal) in **c** comparing to **a**. Consult also Supplementary Figure 9. **d**, **e** and **f** show
- 1021 enlargements of a cell from \mathbf{c} , with their associated close-ups in the separate channels (\mathbf{d} , direct red 22): \mathbf{c} applies blue and \mathbf{f} merged image). Arrowhead highlights an area where
- direct red 23); e, aniline blue and f, merged image). Arrowhead highlights an area where
 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*
- accumulates callose in comparison to control (Figure 8 and Supplemental Figure 9). Notice
- 1030 that the absorbance of the OH- stretching vibration broad band (3100-3600 cm⁻¹) is reduced 1031 in *icals3m*.
- 1032
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- 1034

Tables

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

	Wild type*	pG1090::icals3m*
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

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