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1 # Structural imaging of native cryo-preserved secondary cell walls
2 # reveals presence of macrofibrils composed of cellulose, lignin and
3 # xylan.

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27 **Abstract:**

28 The woody secondary cell walls of plants are the largest repository of renewable
29 carbon biopolymers on the planet. These walls are made principally from cellulose and
30 hemicelluloses and are impregnated with lignin. Despite their importance as the main
31 load bearing structure for plant growth, as well as their industrial importance as both a
32 material and energy source, the precise arrangement of these constituents within the
33 cell wall is not yet fully understood. We have adapted low temperature scanning
34 electron microscopy (cryo-SEM) for imaging the nanoscale architecture of angiosperm
35 and gymnosperm cell walls in their native hydrated state. Our work confirms that cell
36 wall macrofibrils, cylindrical structures with a diameter exceeding 10 nm, are a
37 common feature of the native hardwood and softwood samples. We have observed
38 these same structures in *Arabidopsis thaliana* secondary cell walls, enabling
39 macrofibrils to be compared between mutant lines that are perturbed in cellulose,
40 hemicellulose and lignin formation. Our analysis indicates that the macrofibrils in
41 Arabidopsis cell walls are composed, at least partially, of cellulose, xylan and lignin.
42 This study is a useful additional approach for investigating the native nanoscale
43 architecture and composition of hardwood and softwood secondary cell walls and
44 demonstrates the applicability of Arabidopsis genetic resources to relate fibril structure
45 with wall composition and biosynthesis.

46 Keywords: SEM, cell walls, macrofibrils, cellulose, xylan, lignin, softwood, hardwood,
47 *Arabidopsis thaliana*

48

49 **Introduction:**

50 The majority of carbon in terrestrial biomass is stored in forests as wood (Ramage et
51 al., 2017, Pan et al., 2011). The current classification system distinguishes two types
52 of timber. Wood from Angiosperm trees is known as hardwood and the wood made by
53 Gymnosperm species is described as softwood (Ramage et al., 2017). Despite
54 significant differences in tissue organisation and chemical composition, both these
55 types of timber are almost entirely formed from plant secondary cell walls – an
56 extracellular matrix made primarily from cellulose, lignin and hemicelluloses
57 (Schweingruber, 2007). Considering the ecological and industrial importance of wood
58 and other cell wall materials, our knowledge of the exact arrangement of these
59 polymers in the cell wall remains poor. A better understanding of the molecular

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60 architecture and ultrastructure of cell walls is needed to describe the complex
61 spatiotemporal deposition pattern of the cell wall polymers. This may contribute to the
62 development of more efficient biofuel feedstocks (Loque et al., 2015), to the
63 improvement in our understanding of novel biomaterials such as nanocellulose (Jarvis,
64 2018), and to applications such as advanced approaches for the use of timber in the
65 construction industry (Ramage et al., 2017)

66 Cellulose is the main constituent of plant cell walls (Pauly and Keegstra, 2008). At the
67 molecular level, cellulose has a simple repeating structure of β-1,4-linked
68 glucopyranosyl residues. These glucan chains coalesce to form a crystalline cellulose
69 microfibril. The exact structure of the microfibril is unknown, however, it has been
70 suggested the elementary microfibril consists of 18 or 24 individual glucan chains
71 (Gonneau et al., 2014, Hill et al., 2014, Turner and Kumar, 2017). Individual cellulose
72 microfibrils associate to form larger order structures known as macrofibrils (Niklas,
73 2004). In plant primary cell walls this close-contact association may be limited to
74 selected parts of microfibril which is proposed to lead to formation of so-called
75 biomechanical hotspots (Cosgrove, 2014). A range of imaging and spectroscopic
76 techniques has been used to investigate cellulose macrofibrils in secondary cell walls,
77 as reviewed by (Purbasha et al., 2009), but due to technical challenges the precise
78 structure in native, unprocessed, hydrated secondary cell walls remains poorly
79 described. Lignin is the main non-polysaccharide component of both hardwood and
80 softwood and is made by coupling of monolignol radicals in secondary cell walls. Three
81 main monolignols exist in plants, which, once turned into chemical radicals by the
82 activity of laccases and peroxidases, can couple in a random manner to form a lignin
83 polymer made from guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units (Ralph et
84 al., 2004). The monolignol composition of hardwood and softwood differs, with the
85 former consisting of predominantly S and G units and the latter being made almost
86 solely from G units (Vanholme et al., 2010). The process of lignification is important
87 for wood mechanical properties. Arabidopsis mutant plants with reduced lignin content
88 or altered monolignol composition often have collapsed xylem vessels and can be
89 severely dwarfed (Bonawitz and Chapple, 2010). Lignin is proposed to associate with
90 cell wall polysaccharides to form the recalcitrant matrix (Terrett and Dupree, 2019).

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91 Xylan and galactoglucomannan are the principal hemicelluloses in hardwood and
92 softwood. Xylan is a polymer of β-1,4-linked xylopyranosyl residues and is the main
93 hemicellulose in hardwood but is also present in softwood (Scheller and Ulvskov,
94 2010). Hardwood and softwood xylans carry α-1–2 linked glucuronic acid (GlcA)
95 branches which can be methylated on carbon 4 leading to formation of 4-O-
96 Methylglucuronic acid (MeGlcA) (Scheller and Ulvskov, 2010). In addition to GlcA and
97 Me GlcA (together, [Me]GlcA) decorations, hardwood xylan hydroxyls are acetylated
98 on carbon 2, carbon 3 or both carbons of the monomer. The softwood xylan, in addition
99 to the MeGlcA branches, carries α-1,3–linked arabinofuranosyl decorations (Scheller
100 and Ulvskov, 2010, Busse-Wicher et al., 2016b). The presence of [Me]GlcA branches
101 on xylan is important for the maintenance of biomass recalcitrance (Lyczakowski et
102 al., 2017) and, together with acetylation in hardwood and arabinose decorations in
103 softwood, these substitutions are mostly distributed with an even pattern on xylosyl
104 units (Bromley et al., 2013, Busse-Wicher et al., 2014, Busse-Wicher et al., 2016b,
105 Martinez-Abad et al., 2017). This so-called ‘compatible’ patterning of xylan
106 substitutions is thought to allow the hydrogen bonding between xylan, in a two-fold
107 screw conformation, and the hydrophilic surface of the cellulose microfibril
108 (BusseWicher et al., 2016a, Simmons et al., 2016, Grantham et al., 2017).
109 Galactoglucomannan (GGM) is the main hemicellulose in softwood (Scheller and
110 Ulvskov, 2010) but is also present in hardwood xylem. GGM has a backbone formed
111 from both β-1,4-linked mannosyl and glucosyl residues with some mannosyl residues
112 substituted by an α-1,6-linked galactosyl branch. The GGM backbone can also be
113 acetylated. The arrangement of mannose and glucose units in softwood GGM is
114 thought to be random, but a recently described regular structure GGM found in
115 Arabidopsis mucilage was proposed to bind to both the hydrophilic and hydrophobic
116 surface of the cellulose microfibril (Yu et al., 2018). *In vitro* studies using TEM and 1D
117 ^{13}C NMR indicate that a range of branched and unbranched mannan and glucomannan
118 structures can interact with bacterial cellulose (Whitney et al., 1998). Softwood GGM
119 is also proposed to interact with the cellulose microfibril (Terashima et al., 2009) and
120 recent evidence demonstrates that it can form covalent linkages with lignin (Nishimura
121 et al., 2018).

122 Although we now have a better understanding of secondary cell wall composition and
123 the nature of the interactions between its main constituents, a picture of the

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124 ultrastructural assembly of wall polymers into a secondary cell wall matrix is not yet
125 complete. Solid state NMR (ssNMR) analysis has been applied extensively to the
126 study of polymer interactions in both primary and secondary walls. This, for example,
127 provided evidence that in dried primary wall samples from Arabidopsis, pectin and
128 xyloglucan may be interacting with the cellulose microfibril (Dick-Perez et al., 2011).
129 Analysis of hydrated secondary cell wall of Arabidopsis with solid state NMR indicated
130 that xylan is likely to interact with the hydrophilic surface of the cellulose microfibril as
131 a two-fold screw (Simmons et al., 2016, Grantham et al., 2017). Recent ssNMR
132 analysis indicates that in dried cell walls of grasses xylan is likely to interact with lignin
133 (Kang et al., 2019). Despite providing excellent insights into the proximity of different
134 cell wall components ssNMR cannot provide information about the assembly of these
135 constituents into higher order structures. Some insights into this process have been
136 achieved with other techniques. This includes application of vibrational
137 microspectroscopy techniques such as FT-IR and RAMAN to study the orientation of
138 cellulose and other cell wall components in the matrix, as reviewed by (Gierlinger,
139 2018). AFM has been applied to the study of cell wall matrix assembly, but the work
140 has been focused on primary cell walls (Cosgrove, 2014) and only recent advances
141 allowed nanoscale resolution imaging of dried spruce secondary cell walls (Casdorff
142 et al., 2017). Moreover, insights into the assembly of cellulose microfibrils in wood
143 walls of conifers (Fernandes et al., 2011) and dicots (Thomas et al., 2014) have been
144 obtained using wide-angle X-ray scattering (WAXS) and small-angle neutron
145 scattering (SANS).

146 In addition to these various approaches, other studies have attempted to use scanning
147 electron microscopy (SEM) to study the structure of plant cell walls. Low temperature
148 SEM (cryo-SEM), in which the sample is rapidly frozen and then maintained cold
149 during imaging, has been used to study the collapse of pine needle tracheid cell walls
150 upon prior dehydration (Cochard et al., 2004) and to visualise the bulging of root hairs
151 in the *kojak* (cellulose synthase-like) mutant (Favery et al., 2001). Additionally, higher
152 magnification cryo-SEM has been used to visualise cell walls of wheat awns (Elbaum
153 et al., 2008). Some awn cell walls exhibit structural differences that are dependent
154 upon the level of hydration and cryo-SEM revealed extensive layering within the wall,
155 however, the technique was not further optimised to investigate individual fibrils. Field
156 emission (FE) SEM techniques were effectively used to study the alignment of

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157 cellulose microfibrils in Arabidopsis hypocotyls (Refregier et al., 2004), roots
158 (Himmelspach et al., 2003) and stems (Fujita et al., 2013). FE-SEM has also been
159 applied to investigate wood structure, including observations of microfibril alignment in
160 fixed cell walls of fir tracheids (Abe et al., 1997) and lignin distribution in spruce
161 tracheids (Fromm et al., 2003). Importantly, FE-SEM analysis of dehydrated pine and
162 poplar wood suggests that secondary cell walls of these species contain macrofibrils
163 – cylindrical fibrillar structures with a diameter of up to 60 nm, which presumably
164 comprise of bundles of elementary cellulose microfibrils (Donaldson, 2007). Moreover,
165 the diameter of these macrofibrils was observed to increase with increasing
166 lignification, suggesting that the macrofibrils may be formed from association of lignin
167 and cell wall polysaccharides. This analysis was extended further to wood from Ginkgo
168 where the FE-SEM was combined with density analysis to propose a model of
169 macrofibril formation based on cellulose, GGM, xylan and lignin interaction (Terashima
170 et al., 2009).

171 It has been suggested that some of the treatments used in preparation of the FE-SEM
172 cell wall samples have little impact on the microfibril alignment and that the technique
173 may provide a true representation of native (unprocessed) cell wall features (Marga et
174 al., 2005). The FE-SEM techniques applied to secondary cell wall samples, however,
175 included additional steps such as (i) fixation and exposure to organic solvents (ii) a
176 thermal treatment that may result in some degree of wall degradation (Fromm et al.,
177 2003) and (iii) a thick coating of heavy metal which may impact upon the resolution
178 (Donaldson, 2007), raising questions about the effect these may have on interpretation
179 of the wall structure. Visualisation of native, hydrated, secondary cell walls with
180 environmental FE-SEM has been challenging and the resolution of obtained images
181 has been low (Donaldson, 2007). We present here a technique for the analysis of
182 native, fully-hydrated, secondary cell wall material from angiosperm and gymnosperm
183 plant species using cryo-SEM. The use of an ultrathin 3 nm platinum film, together with
184 cryo-preservation at high vacuum, enabled us to demonstrate that cell wall macrofibrils
185 are a common feature in all types of native secondary cell wall material analysed.
186 Importantly, we were able to detect the presence of macrofibrils in *Arabidopsis thaliana*
187 vessel secondary cell walls. This allowed us to make use of the readily available cell
188 wall-related genetic resources, revealing Arabidopsis macrofibril diameter to be
189 dependent upon cellulose, xylan and lignin.

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190

191 **Results:**

192 *Softwood and hardwood secondary cell walls contain macrofibrils*

193 In order to investigate and compare the nanoscale architecture of gymnosperm and
194 angiosperm cell walls we analysed stem sections taken from spruce, Ginkgo and
195 poplar using cryo-SEM. Stems were placed in the SEM specimen stub and
196 immediately frozen in nitrogen slush, fractured and then coated with platinum, before
197 being passed in to the SEM chamber for imaging. Nitrogen slush is a suspension of
198 solid nitrogen that enables high freezing rates, greatly reducing the Leidenfrost effect
199 during plunge freezing and thus minimising structural damage (Sansinena et al.,
200 2012). The fine grain size attributed to platinum sputtering allows small and densely
201 packed objects to be resolved. This rapid sample preparation protocol serves to better
202 maintain sample hydration levels and native structures for optimal EM imaging in a
203 high vacuum environment.

204 We first investigated whether our cryo-SEM protocol gave comparable results to the
205 previous FE-SEM analysis of both softwood and hardwood secondary cell walls
206 (Donaldson, 2007). To examine if macrofibrils are found in natively hydrated,
207 nonpretreated cell walls, cryo-SEM imaging was performed on unprocessed, frozen
208 softwood and hardwood samples. For observing gymnosperm cell wall architecture,
209 we first prepared softwood samples from spruce and used a low magnification to see
210 an overview of stem cross-section (Figure 1a) and tracheid structure (Figure 1b). The
211 inner part of the stem cross section was composed of densely packed xylem tracheids,
212 each surrounded by cell walls. To investigate the appearance of the secondary cell
213 walls, higher magnification images of these parts of tracheid cells were acquired. This
214 enabled us to observe that the tracheid cell walls contain fibrous structures which
215 frequently assembled into larger aggregates (Figure 1c and 1d, red arrows). After a
216 further increase in magnification, individual fibrils became resolvable (Figure 1e and
217 1f) and their diameter was found to exceed the 3 nm diameter calculated for a single
218 softwood elementary microfibril (Fernandes et al., 2011). Therefore the observed
219 fibrils, if composed of cellulose, represent a higher order structure that fits the
220 description of a "macrofibril" (Niklas, 2004, Donaldson, 2007). Similarly to spruce stem,
221 sections from another gymnosperm, the Ginkgo, were also observed to contain

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222 macrofibrils (Figure S3). These data show that, in line with previously reported SEM
223 imaging of dried, processed plant material (Donaldson, 2007, Terashima et al., 2009),
224 the native, hydrated cell walls of spruce and Ginkgo also contain macrofibrils.
225 Therefore, these structures may contribute to the higher order assembly of native
226 gymnosperm cell walls.

227 We extended the analysis to the model hardwood species, poplar. Vessels, a distinct
228 cell type of hardwood xylem, were clearly visible using low magnification (Figure 2a
229 and 2b). In addition to the vessels, xylem fibre cells were also observed (Figure 2b;
230 red and yellow arrows for vessels and fibre cells respectively). For some cells we were
231 able to observe spiral thickenings which were preserved during sample preparation
232 and extended above the surface of the fracture plane (Figure 2b). We focused upon
233 the vessel cell walls which showed clearly visible fibrous structures at a vessel-
234 tovessel boundary (Figure 2c). Analysis of vessel cell walls at a higher magnification
235 revealed a clear presence of macrofibril structures, similar to those observed in spruce,
236 in the poplar samples (Figure 2d and 2e). To investigate the dimensions of the
237 macrofibrils we measured their diameter in poplar and spruce (Figure 2f). Our
238 measurements are broadly similar to those reported in a previous study (Donaldson,
239 2007). We carried out comparative analysis of macrofibril diameter between hardwood
240 and softwood by measuring 150 individual macrofibrils in poplar, spruce and Ginkgo.
241 While the diameter of spruce and Ginkgo macrofibrils was not significantly different
242 (Figure S4), the diameter of macrofibrils in poplar secondary cell walls was significantly
243 smaller than that of spruce macrofibrils (Figure 2f). Spruce and Ginkgo were grown in
244 the field while poplar samples were obtained from in vitro grown plants. To control for
245 this difference in growth conditions we also analysed samples from field grown poplar
246 trees. There was no statistically significant difference in the macrofibril diameter
247 between the two poplar samples (Figure S5). For both hardwood and softwood we
248 observed variation in the macrofibril diameter. This may reflect biological differences
249 or may be a result of technical challenges associated with macrofibril width
250 measurement.

251 *Arabidopsis secondary cell walls macrofibrils contain a cellulose scaffold*

252 To further evaluate the nanoscale architecture of plant cell walls and identify possible
253 constituents of the cell wall macrofibrils, the high magnification cryo-SEM imaging was

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254 used to analyse wild type (WT) Arabidopsis secondary cell walls (Figure 3). The initial
255 analysis investigated the structure of WT xylem vessels (Figure 3a and 3b). Sets of
256 vessel bundles were detected and, using higher magnification, fibrous structures
257 similar to those observed in spruce and poplar were also visible in the fractured
258 Arabidopsis material. The width of WT Arabidopsis macrofibrils was comparable to
259 that of poplar macrofibrils but not spruce and suggests Arabidopsis macrofibrils could
260 be used as a good structural model for hardwoods (Figure S4, S5). Despite the use of
261 ultra-thin platinum coating, the use of SEM without the cryo-preservation steps did not
262 allow us to observe the Arabidopsis macrofibrils with good resolution (Figure S6)
263 highlighting the critical importance of sample cryo-preservation to resolve a native cell
264 wall ultrastructure.

265 Based on the data available in the literature we hypothesized that the macrofibrils may
266 be mostly composed of cellulose (Fahlen and Salmen, 2002, Donaldson, 2007). To
267 investigate this, and to understand the nature of these macrofibrils further, we
268 performed a comparative analysis between WT vessel cell walls (Figure 3c) and a
269 commercially available fibrous cellulose standard (Figure 3d) extracted from cotton
270 linters and consisting of 99% pure cellulose (Sczostak, 2009). In this experiment, clear
271 individual fibrils with distinct bright termini were observed in both samples indicating
272 that the vessel wall macrofibrils have a similar appearance to the cellulose fibrils
273 present in this polysaccharide standard. To determine whether these macrofibrils are
274 dependent upon the proper production of cellulose, the morphology of WT Arabidopsis
275 vessel cell walls (Figure 3e and 3g) was compared to that of the *irx3* mutant (Figure 3f
276 and 3h). IRX3 is one of three CESA proteins that make up the secondary wall cellulose
277 synthase complex and *irx3* plants are almost completely devoid of cellulose in their
278 secondary cell walls, but not primary cell walls (Taylor et al., 1999). As previously
279 reported, *irx3* plants had collapsed vessels (Figure S7), since secondary cell wall
280 cellulose contributes to vessel wall strength (Turner & Somerville, 1997). Interestingly,
281 the *irx3* stems lacked the fibrous structures in their vessel secondary cell walls and, in
282 contrast to WT, the *irx3* cell walls were formed from a largely amorphous matrix (Figure
283 3f). It is likely that this matrix is composed of xylan and lignin, which can still be
284 deposited in the secondary cell wall in the absence of IRX3 activity (Takenaka et al.,
285 2018). Some structures resembling the macrofibrils were present in the primary cell

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286 walls of *irx3* plants (Figure S7). Taken together, the data demonstrate macrofibril
287 formation is dependent upon cellulose production.

288 *Reduction in cell wall xylan and lignin, but not in galactoglucomannan content*
289 *decreases the dimensions of Arabidopsis macrofibrils*

290 To investigate the role of xylan in macrofibril formation, cryo-SEM was used to
291 visualise the secondary walls from *irx9, irx10* and *esk1* Arabidopsis plants (Figure 4a,
292 4b and 4c). IRX9 and IRX10 are required for proper xylan synthesis and mutations in
293 the corresponding genes lead to cell wall weakening and collapse of xylem vessels in
294 the Arabidopsis model (Brown et al., 2007, Bauer et al., 2006, Brown et al., 2005). The
295 *irx9* plants have impaired xylan synthesis resulting in a decrease of xylan by more than
296 50% compared to WT (Brown et al., 2007). In *irx10* plants the reduction in xylan
297 content is smaller and does not exceed 20% (Brown et al., 2005). Macrofibrils are
298 clearly observed in *irx9* and *irx10* Arabidopsis (Figure 4a and 4b). However, the
299 median macrofibril diameter between WT and *irx9* cell wall fibres showed a ~30%
300 reduction in the xylan synthesis mutant (Figure 4g). The median macrofibril diameter
301 of *irx10* plants was ~10% smaller than that of WT Arabidopsis (Figure 4g). Although
302 there was a wide variation in macrofibril diameter within each genotype, the difference
303 between the WT macrofibril diameter and the one quantified for the two mutants is
304 statistically significant, suggesting that xylan is incorporated along with cellulose to
305 generate the normal macrofibril size. To investigate the role of xylan-cellulose
306 interaction in the macrofibril formation we assessed the macrofibril size in the *esk1*
307 Arabidopsis mutant (Figure 4c). Mutation in the *ESK1* gene results in reduction of xylan
308 acetylation, but not in a decrease in xylan quantity (Xiong et al., 2013), which leads to
309 changes in xylan [Me]GlcA patterning and loss of interaction between xylan and the
310 hydrophilic surface of the cellulose microfibril (Grantham et al., 2017). In line with the
311 results observed for *irx9* and *irx10* plants the loss of xylan-cellulose interaction caused
312 a reduction in the macrofibril diameter (Figure 4g).

313 Previous work in softwood suggested that lignin (Donaldson, 2007) and
314 galactoglucomannan (GGM) (Terashima et al., 2009) may be involved in macrofibril
315 formation. To investigate the role of these two cell wall components in the maintenance
316 of macrofibril structure we performed imaging of *4cl1* (Figure 4d), *lac4* (Figure 4e) and
317 *csla2/3/9* (Figure 4f) mutant Arabidopsis cell walls. Both 4CL1 and LAC4 are involved

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318 in lignin biosynthesis and plants mutated in genes encoding these enzymes have a
319 30% and 15% reduction in lignin content respectively (Li et al., 2015, Berthet et al.,
320 2011). The median macrofibril diameter for both *4cl1* and *lac4* was significantly smaller
321 than that calculated for WT (Figure 4g). Importantly, the extent of the reduction in
322 macrofibril diameter was in line with the decrease in the lignin content observed for the
323 two mutants, with *4cl1* macrofibrils being ~15% smaller than the WT ones and *lac4*
324 macrofibrils having ~7% reduction in the median diameter. Proteins from the CSLA
325 family are involved in the biosynthesis of a hemicellulose galactoglucomannan and
326 mutations in *csla2/3/9* leads to nearly complete loss of stem GGM in the Arabidopsis
327 model (Goubet et al., 2009). Our quantitative analysis indicates that the diameter of
328 macrofibrils of *csla2/3/9* Arabidopsis was not significantly different to that of the WT
329 plants (Figure 4g).

330

331 **Discussion**

332 The native nanoscale architecture of woody plant secondary cell walls remains poorly
333 understood due to the challenges of keeping the sample hydrated, which is
334 incompatible with some types of techniques. Studies analysing dehydrated and fixed
335 plant cell wall samples with FE-SEM (Donaldson, 2007), together with other work
336 which includes SANS experiments investigating spruce (Fernandes et al., 2011) and
337 bamboo samples (Thomas et al., 2015), suggest there is a higher order arrangement
338 of cellulose microfibrils in plant secondary cell walls. Our work reports the application
339 of a cryo-SEM based analysis technique which, using exclusively samples that have
340 not been dried, heated or chemically processed, indicates that secondary cell wall
341 cellulose microfibrils are likely to come together to form larger macrofibril structures.

342 Our study strongly suggests that these structures, at least in the model plant species
343 *Arabidopsis thaliana,* contain cellulose, xylan and lignin.

344 Previous studies investigated the presence and diameter of macrofibrils in dehydrated
345 softwood samples (Donaldson, 2007). In line with results presented in our work,
346 Donaldson did observe macrofibrils in cell walls of pine tracheids. Moreover, also in
347 agreement with the results presented here (Figure S4), these softwood macrofibrils
348 were larger than those seen in hardwoods. In softwood, in addition to various patterned

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349 types of xylan (Busse-Wicher et al., 2016b, Martinez-Abad et al., 2017), most of which
350 are likely to be compatible with binding to the hydrophilic surface of the cellulose fibril,
351 the cell walls contain large quantities of acetylated GGM (Scheller and Ulvskov, 2010)
352 which may contribute to macrofibril width. Indeed, gymnosperm GGM was proposed
353 to interact with the cellulose microfibril in cell walls of Ginkgo (Terashima et al., 2009).
354 Therefore, the significant difference in macrofibril diameter observed between
355 hardwood and softwood samples may be due to the differences in the cell wall
356 composition. Consequently, we hypothesize that in gymnosperms, GGM, along with
357 xylan, may contribute to the macrofibril size in a way similar to what we observed for
358 xylan in Arabidopsis macrofibrils. With an average diameter ranging between 20 and
359 34 nm, the size of pine macrofibrils measured by Donaldson was somewhat smaller
360 than that measured in spruce wood in the current work. However, these observations
361 are not necessarily inconsistent. Donaldson dehydrated the wood samples prior to the
362 SEM imaging. As the spacing between bundled softwood cellulose microfibrils,
363 estimated to be equal to 3 nm by small angle neutron scattering, is sensitive to wood
364 hydration levels (Fernandes et al., 2011), at least part of the difference in the
365 macrofibril diameter might be due to the changes in the water content within the
366 sample analysed with SEM. Interestingly, Donaldson reported that macrofibrils in dried
367 poplar wood, depending on their position in cell wall, have an average diameter
368 ranging from 14 to 18 nm, which is similar to what was measured for both poplar and
369 Arabidopsis as a part of our study. This observation suggests that the softwood
370 macrofibril size may be more sensitive to drying than the hardwood one. This in turn
371 suggests that, in addition to compositional disparities, hydration could contribute to the
372 differences in softwood and hardwood macrofibril characteristics. In addition to
373 providing scientific insight, this result highlights that imaging of the cryopreserved
374 secondary cell walls offers significant advance over the previously used techniques.

375 Interestingly, similar to a previous report (Donaldson, 2007), we observed that
376 macrofibrils in both hardwood and softwood have a range of diameters. The reasons
377 for this variation in size are not clear. It is possible that the number of individual
378 cellulose microfibrils that come together to form the macrofibril structure in both
379 hardwood and softwood is not constant. This may be regulated by coordinated
380 movement of CesA complexes or their density during cell wall synthesis (Li et al.,
381 2016). It was proposed that the macrofibril diameter is proportional to the degree of

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382 cell wall lignification (Donaldson, 2007), which may also vary between the structures.
383 This hypothesis is supported by our results which indicate that the cell wall lignin
384 content influences macrofibril diameter in Arabidopsis. Variations may also originate
385 from environmental conditions. For example, it was shown that wood density may vary
386 correlatively with climate change (Bouriaud et al., 2005). Although much of this effect
387 is likely to be due to cell size and wall thickness, it can be hypothesized that change
388 in wood density may also originate from compositional changes that impact macrofibril
389 assembly and ultrastructure. It would therefore be relevant to assess macrofibrils of
390 perennial trees with samples spanning several years of growth. We cannot rule out
391 that the width variance may originate from the technical limitations of resolving the
392 macrofibrils by SEM. It will be interesting to see if the emerging He-ion technologies,
393 with an increase in resolution and less dependent upon metal coating, reduce this
394 variance (Joens et al., 2013). The cryo-SEM techniques developed as part of our study
395 offer a significant advantage over the previous investigation (Donaldson, 2007) which
396 applied a thicker coat of chromium (mostly 12 nm) that yield films with coarser grains
397 than the thinner (3 nm) platinum films used in our work. Thus, taking the results
398 described by Donaldson and our technological improvements into consideration, we
399 believe that the variance in the macrofibril width observed in both studies is likely to
400 reflect natural material variation.

401 The prominence of macrofibril structures in Arabidopsis cell walls is a surprising
402 discovery of this study. Previously published results using AFM analysis indicate the
403 presence of some bundled microfibrils in primary cell walls of Arabidopsis but the
404 extent of this bundling is lower than what was observed in primary cell wall samples
405 from other species (Zhang et al., 2016). AFM is not yet technically feasible for analysis
406 of bundling of hydrated secondary cell walls although recent technical advances
407 allowed visualisation of dried spruce wood at a nanometer resolution (Casdorff et al.,
408 2017). The observation of the macrofibrils by cryo-SEM in Arabidopsis allowed us to
409 determine the contribution of cellulose, xylan, lignin and galactoglucomannan to
410 macrofibril formation, thanks to the availability of secondary cell wall related mutants
411 in this model. Macrofibrils were completely absent in vessel cell walls of *irx3* plants,
412 which lack secondary cell wall cellulose, indicating that proper cellulose biosynthesis
413 is required for formation and assembly of secondary cell walls polymers into
414 macrofibrils. In addition, we observed that vessel macrofibril diameter is significantly

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415 decreased in *irx9, irx10* and *esk1* plants, suggesting that xylan may also participate in
416 the correct assembly of such structures. While in *irx9* and *irx10* reduction in macrofibril
417 diameter may be associated with decrease in the xylan content the ~25% reduction in
418 the median macrofibril diameter observed for *esk1* Arabidopsis is harder to explain.
419 Hardwood xylan is proposed to interact with the hydrophilic surface of the cellulose
420 microfibril as a two-fold screw (Simmons et al., 2016, Busse-Wicher et al., 2016a), and
421 this interaction is facilitated by the even pattern of the [Me]GlcA and acetyl branches
422 on the xylan backbone which is lost in *esk1* plants (Grantham et al., 2017). Therefore,
423 the decrease in macrofibril diameter observed in *esk1* Arabidopsis indicates that
424 xylancellulose interaction may have a role in spacing or proper coalescence of
425 microfibrils to form the elementary macrofibril. It is unclear why the macrofibril diameter
426 is reduced in *esk1*, but perhaps fewer elementary fibrils are incorporated into each
427 macrofibril when xylan is not interacting with the hydrophilic surface of the cellulose
428 fibril. This may be different to the effect observed in flax where the absence of xylan
429 may lead to aggregation of glucan chains into larger fibres (Thomas et al., 2013). Such
430 difference may be associated with variations in the stoichiometry of the cellulose
431 synthase complex which were recently reported for angiosperms (Zhang et al., 2018).

432 In addition to implicating xylan in the process of macrofibril formation our results
433 indicate that lignin may contribute to assembly of the structures. As such, our results
434 use genetic assignment to extend previous work which has correlated macrofibril
435 diameter with the degree of wall lignification (Donaldson, 2007). Interestingly, we
436 observed that the macrofibril diameter does not correlate with the cell wall GGM
437 content. This may be associated with low abundance of GGM in angiosperms where
438 the polysaccharide accounts for only up to 5% of the cell wall material (Scheller and
439 Ulvskov, 2010). Alternatively, this result may indicate that in Arabidopsis GGM might
440 be not involved in macrofibril formation. GGM may play a more significant role in the
441 macrofibril assembly in gymnosperms where it accounts for up to 30% of the cell wall
442 material. Importantly, all our conclusions are based on the analysis of native, hydrated,
443 cell wall samples. The assignment of cell wall macrofibril composition, in their native
444 state, would be impossible using techniques such as immunogold due to the
445 pretreatment steps needed before the antibody labelling.

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446 In conclusion, our analysis indicates that vessel cell walls contain fibrous structures
447 composed of cellulose, xylan and lignin. These structures are present in both
448 hardwood and softwood and have a diameter larger than a single cellulose microfibril.
449 Therefore, these structures can be described as cell wall macrofibrils. The reduction
450 in macrofibril diameter observed in *esk1* Arabidopsis suggests that the interaction
451 between xylan and the hydrophilic surface of the cellulose microfibril may be involved
452 in the assembly of these structures. Therefore, this xylan-cellulose interaction may be
453 important for the maintenance of plant cell wall ultrastructure and mechanical
454 properties (Simmons et al., 2016). The techniques developed here and the discovery
455 of the ubiquitous presence of macrofibrils in hardwood and softwood in their native
456 state will contribute to a better understanding of cell wall assembly processes.
457 Furthermore, the ability to resolve macrofibrils in Arabidopsis, along with the
458 availability of genetic resources in this model, will offer the community a valuable tool
459 to further study the complex deposition of secondary cell walls polymers and their role
460 in defining the cell wall ultrastructure. The assembly of cell wall macrofibrils is likely to
461 influence the properties of wood, such as density, which may vary due to different
462 stimuli such as tree fertilisation (Makinen et al., 2002) or environmental changes
463 (Bouriaud et al., 2005). Therefore, we expect that the methodology described here will
464 enable to correlate the native nanoscale features of the cell walls, such as the
465 macrofibril diameter, or a specific macrofibril patterning within the cell wall, with wood
466 properties. Consequently, our approach may be useful to assess this aspect of wood
467 quality at a new level and could benefit numerous industries ranging from building
468 construction, paper manufacturing and biofuel production to generation of novel
469 biomaterials such as nanocrystalline cellulose.

470

471 **Experimental procedures:**

472 *Plant material*

473 *Picea abies,* (spruce) one-year old branch was acquired from 30-50cm tall potted
474 plants grown outdoors purchased from Scotsdale (Great Shelford, Cambridgeshire,
475 UK). *Ginkgo biloba,* (Ginkgo) stem material was obtained from the trees grown at the
476 Cambridge University Botanic Garden. For both spruce and Ginkgo, samples from two
477 individuals were analysed.

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478 Hybrid aspen (*Populus tremula* x *Populus tremuloides*, clone T89), referred to as
479 poplar in the text, was grown *in vitro* (20°C, with a 16-h light, 8-h dark photoperiod,
480 with illumination at 85 microeinstein.$m^{-2}.s^{-1}$) during 76 to 80 days after
481 micropropagation on 1/2MS media with vitamins (Duchefa M0222), 1% sucrose, 0.7%
482 Agar. Samples from three individuals were analysed. For field grown poplar (*Populus*
483 *tremula*), material was obtained from two individuals grown at the Cambridge
484 University Botanic Garden.

485 *Arabidopsis thaliana* (Arabidopsis) Columbia-0 ecotype plants were grown in a cabinet
486 maintained at 21 °C, with a 16-h light, 8-h dark photoperiod. Stem material was
487 collected from 7-week-old plants. Mutant insertion lines described in published work
488 were used in this study. Specifically, *irx3-7* plants (Simmons et al., 2016, Kumar and
489 Turner, 2015), representing a mutant allele of *CESA7*, *irx9-1* (Brown et al., 2005),
490 *irx10-1* (Brown et al., 2005), *esk1-5* (Lefebvre et al., 2011, Grantham et al., 2017),
491 *4cl1-1* (Vanholme et al., 2012), *lac4-2* (Berthet et al., 2011) and *csla2-1csla3-2csla91*
492 (Goubet et al., 2009). Plants were analysed alongside the wild type (WT) material.
493 For each genotype three individuals were analysed.

494 *Cryo-SEM sample preparation and imaging*

495 Fresh stems of 7 week old Arabidopsis plants were prepared for imaging as outlined
496 in Supporting Information Figure S1. Firstly, 1 cm length sections were cut from the
497 bottom part of the stems and mounted vertically in recessed stubs containing a cryo
498 glue preparation consisting of a 3:1 mixture of Tissue-Tec (Scigen Scientific, USA) and
499 Aquadog colloidal graphite (Agar Scientific, Stansted, UK) (see steps 1 to 4 on Figure
500 S1). Stem sections were immediately (within 5 minutes of harvest) plunge frozen in
501 liquid nitrogen slush (step 5 on Figure S1), transferred under vacuum, fractured and
502 then coated with 3 nm of platinum (step 6 on Figure S1) using a PT3010T cryo-
503 apparatus fitted with a film thickness monitor (Quorum Technologies, Lewes, UK). The
504 short time between freezing and harvesting serves to prevent drying of the sample
505 where only the exposed surface, not the fractured face, is expected to exhibit some
506 water loss during the short time it is exposed to air. Finally, fractured stems were
507 imaged using a Zeiss EVO HD15 Scanning Electron Microscope (step 7 on Figure S1)
508 and maintained at -145 °C using a Quorum cryo-stage assembly. The electron source
509 is a Lanthanum Hexaboride HD filament. Images were acquired using a secondary

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510 electron detector and an accelerating voltage of between 5 and 8 kV with a working
511 distance between 4 and 6 mm. Quantification of the width of cell wall macrofibrils was
512 performed using ImageJ software (Schneider et al., 2012). For the measurements of
513 each macrofibril, a line was drawn parallel to the fibril axis. The length of a second line,
514 perpendicular to the fibril axis line and across the width of the macrofibril, was
515 quantified as the macrofibril width (Figure S2). Each fibril width measurement was
516 standardised for the platinum layer applied during the coating process by subtracting
517 the width of the standardised coat from the original measurement. Imaging without the
518 cryo-preservation was performed by visualising hand sectioned platinum coated
519 specimens with the stage maintained at room temperature. For preparation of these
520 samples all freezing steps were omitted.

521

522 *Sampling and statistical analysis*

523 For spruce, Ginkgo and field grown poplar, stem sections were taken from two
524 individual trees and 150 macrofibrils were measured from three tracheids that had
525 each been coated with platinum separately. Imaging of poplar was performed in
526 technical triplicate from three *in vitro* grown trees and 150 poplar macrofibrils were
527 measured from three separately coated vessels as for the gymnosperm samples. For
528 Arabidopsis, cryo-SEM imaging of vessels was carried out on three biological
529 replicates, each from separate individuals. 150 macrofibril diameters were measured
530 across the three individuals.

531 Statistical analysis was performed using packages available with R software (Team,
532 2014). Statistical tests, either Student's T test or ANOVA, used to compare average
533 measurements for samples are defined in Figure legends. The variance between each
534 pairwise combination was estimated to be similar with Levene's test.

535 *Data statement*

536 All data for the quantification of the macrofibril diameter is presented on Figures
537 forming part of the manuscript. Representative images are provided for each
538 genotype/species for which macrofibril diameter data is provided. All images obtained
539 for the different species and genotypes analysed as part of this manuscript are
540 available from Jan J Lyczakowski (jjl55@cam.ac.uk).

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541

542 **Accession numbers**

543 Mutants in the following genes in the Col-0 ecotype were analysed as part of this
544 study:

545 AT5G17420 (*irx3-7*)

546 AT2G37090 (*irx9-1*)

547 AT1G27440 (*irx10-1*)

548 AT3G55990 (*esk1-5*)

549 AT1G51680 (*4cl1-1*)

550 AT2G38080 (*lac4-2*)

551 AT5G22740 (*csla2-1*)

552 AT1G23480 (*csla3-2*) AT5G03760

553 (*csla9-1*)

554

555 **List of abbreviations**

556 1D – one dimensional

557 AFM – atomic force microscopy CesA – Cellulose

558 synthase cryo-SEM – low temperature scanning electron

559 microscopy FE-SEM – field emission scanning electron

560 microscopy

561 FT-IR - Fourier-transform infrared spectroscopy

562 GGM – galactoglucomannan

563 He-ion – Helium ion

564 IRX – irregular xylem

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565 [Me]GlcA – methylated and unmethylated form of glucuronic acid

566 NMR – nuclear magnetic resonance

567 SANS – small angle neutron scattering

568 TEM – transmission electron microscopy

569 WAXS – wide angle x-ray scattering

570

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594

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595 **Author contributions**

596 JJL designed the study, conducted the experiments, analysed the data and wrote the
597 paper. MB performed poplar imaging experiments, analysed the data and wrote the
598 paper. OMT analysed the data and wrote the paper. YH contributed to data analysis
599 and manuscript preparation, RW designed the study, conducted experiments,
600 analysed the data and wrote the paper. PD designed the study and contributed to data
601 analysis and manuscript preparation.

602

603

604

605 **Short legends for supporting information:**

606 Figure S1: Overview of the cryo-SEM procedure.

607 Figure S2: Measurement of macrofibril diameter.

608 Figure S3: Cryo-SEM analysis of Ginkgo cell walls.

609 Figure S4: Comparison of macrofibril diameter in Arabidopsis, poplar, spruce and
610 Ginkgo.

611 Figure S5: Imaging of macrofibrils in field grown poplar.

612 Figure S6: Analysis of native Arabidopsis samples without the cryo-preservation
613 protocol.

614 Figure S7: Cryo-SEM analysis of vessel collapse and primary cell wall cellulose in
615 *irx3* Arabidopsis plants.

616

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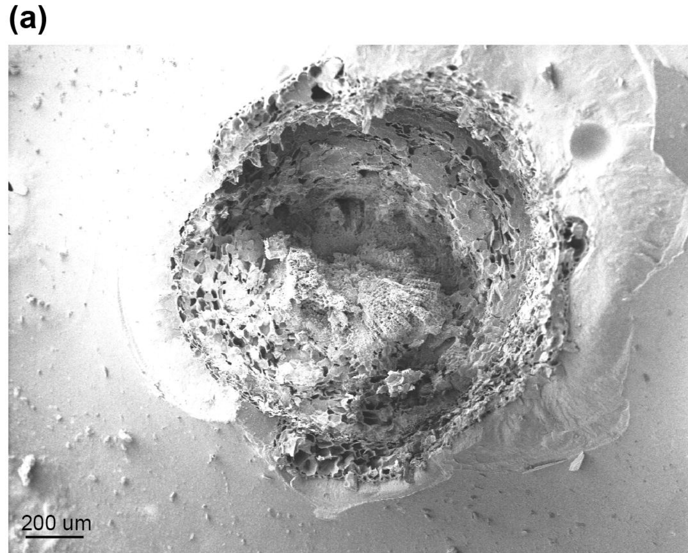
859 **Figure legends**

860 **Figure 1. cryo-SEM analysis of spruce stem sections. (a)** to **(f)** Representative
861 images of stem sections of one-year-old spruce branch at different magnifications. Red
862 arrows indicate tracheids **(b)**, macrofibril bundles **(c** and **d)** and individual macrofibrils
863 **(e** and **f)**. Scale bars are provided for each image.

864 **Figure 2. cryo-SEM analysis of poplar stem sections (a)** to **(e)** Representative
865 images of stem sections of *in vitro* grown poplar trees at different magnifications. Red
866 arrows show vessels **(b)** and macrofibrils **(c** and **e)**. Yellow arrows indicate fibre cells
867 **(b)**. Higher magnification images **(c, d** and **e)** are presented for vessels. Scale bars
868 are provided for each image. **(f)** Diameter of spruce tracheid cell wall fibrils compared
869 to these observed in poplar vessel cell walls. For each bar 150 individual fibrils were
870 measured. Boxplots mark the median and show between 25th and 75th percentile of
871 the data. *** denotes $p \leq 0.00001$ in Student's t-test.

872 **Figure 3. Analysis of Arabidopsis stem sections and fibrous cellulose. (a)** to **(c)**
873 Imaging of WT vessels at increasing magnification **(d)** Imaging of fibrous cellulose
874 standard from cotton linters shows cell wall fibrils with an appearance similar to
875 structures seen *in planta.* **(e)** Imaging of individual vessels in WT plants. **(f)** Imaging of
876 individual vessels in *irx3* plants. **(g)** and **(f)** Macrofibrils are detectable in WT
877 Arabidopsis and are absent in *irx3* secondary cell walls. Red arrows indicate the
878 macrofibril structures throughout the figure. Scale bars are provided for each image.

879 **Figure 4. Analysis of macrofibrils in mutant Arabidopsis plants.** Representative
880 image of **(a)** *irx9*, **(b)** *irx10,* **(c)** *esk1,* **(d)** *4cl1,* **(e)** *lac4* and **(f)** *csla2/3/9* Arabidopsis
881 macrofibrils. Scale bar corresponds to 200 nm on each image. **(g)** Quantification of
882 macrofibril diameter in WT and mutant Arabidopsis plants. N = 150. Boxplots mark a
883 median and show between 25th and 75th percentile of the data. *** denotes $p \leq$
884 0.00001, ** denotes $p \leq 0.0001$, * denotes $p \leq 0.05$ in Tukey test following ANOVA
885 when compared to WT, ns indicates lack of statistically significant difference.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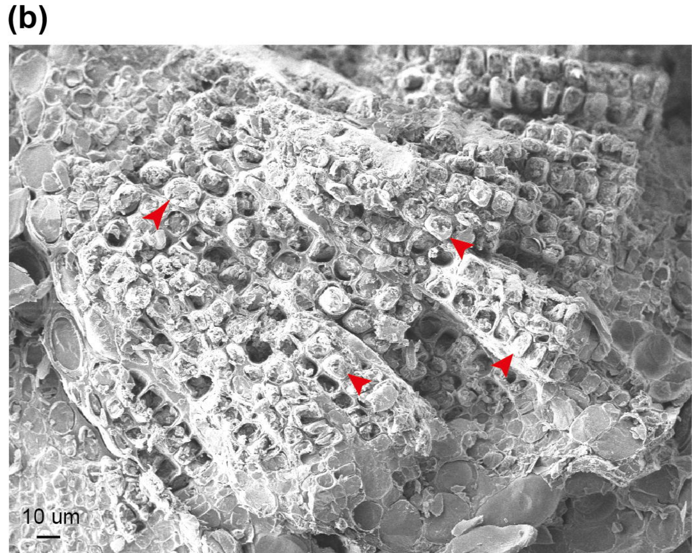
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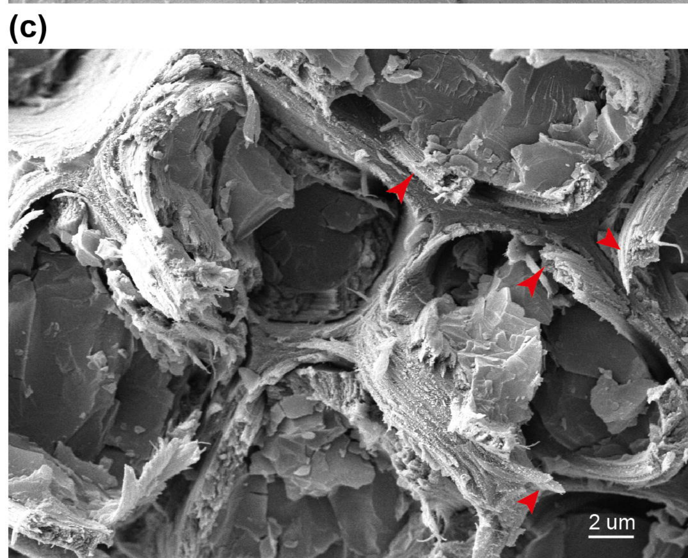
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(d)
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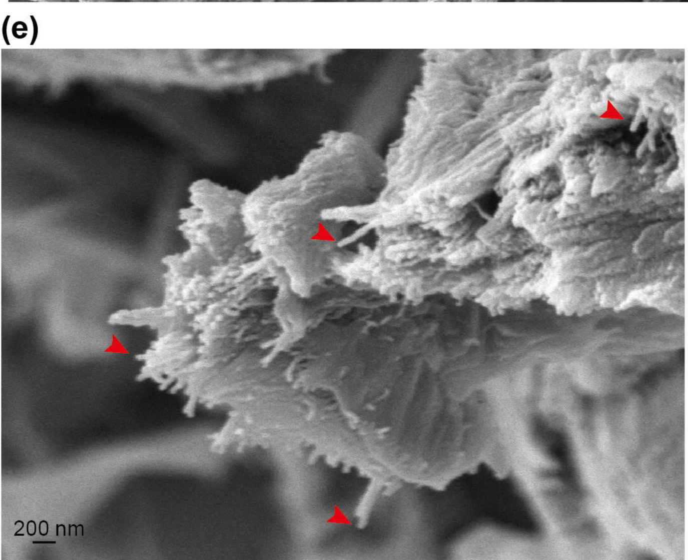
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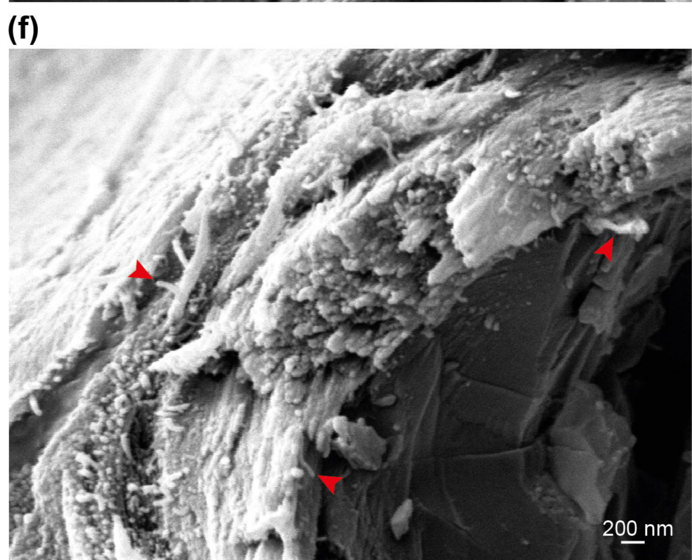
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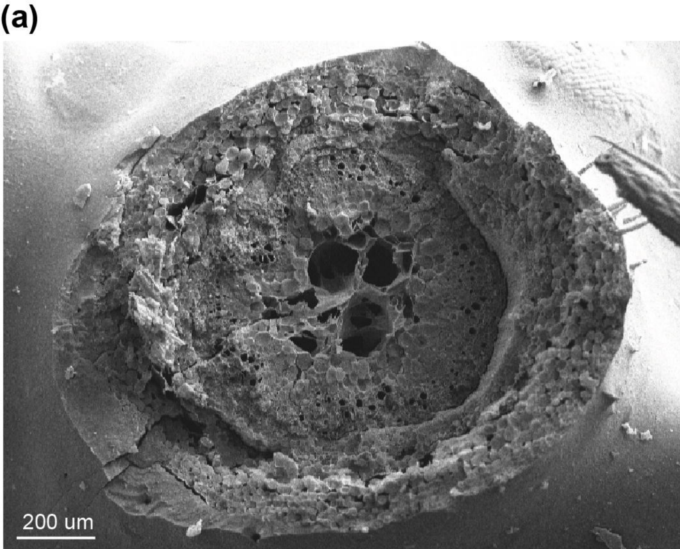
(a)



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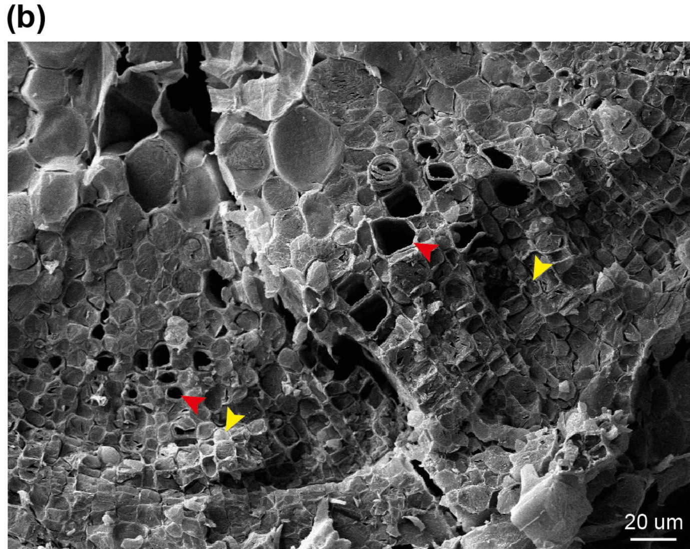

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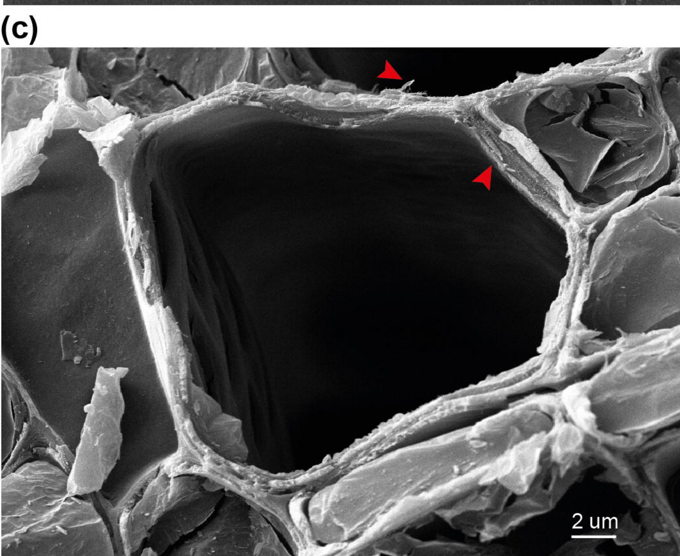

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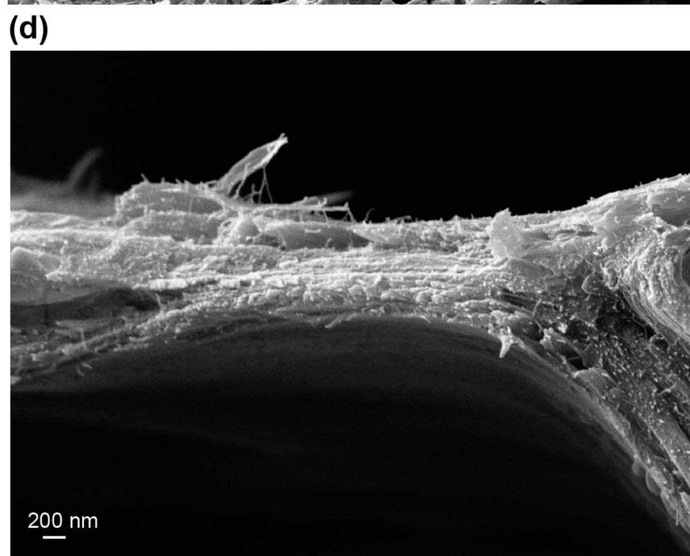

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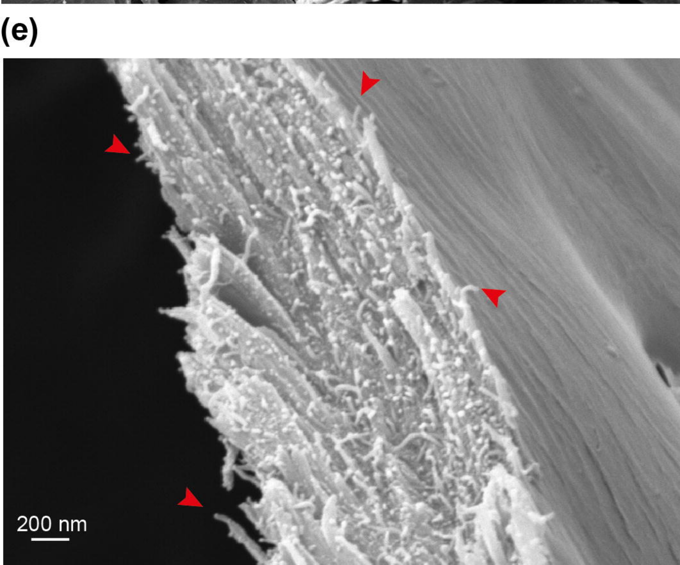

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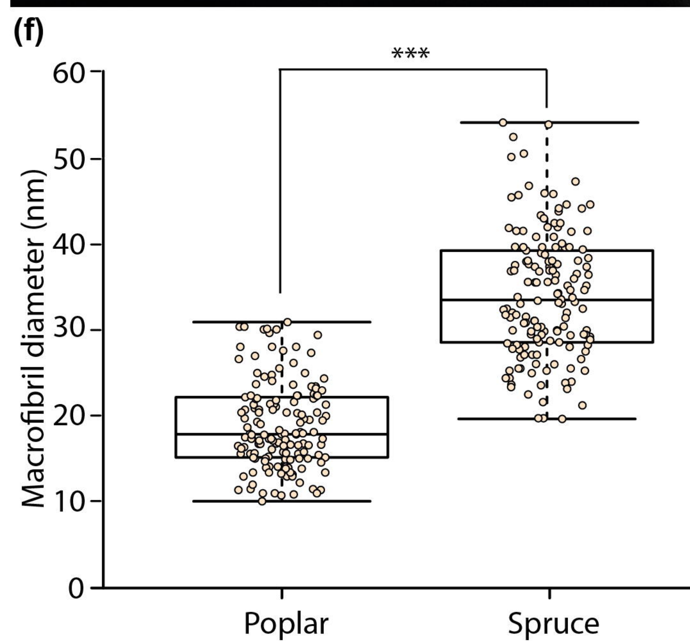

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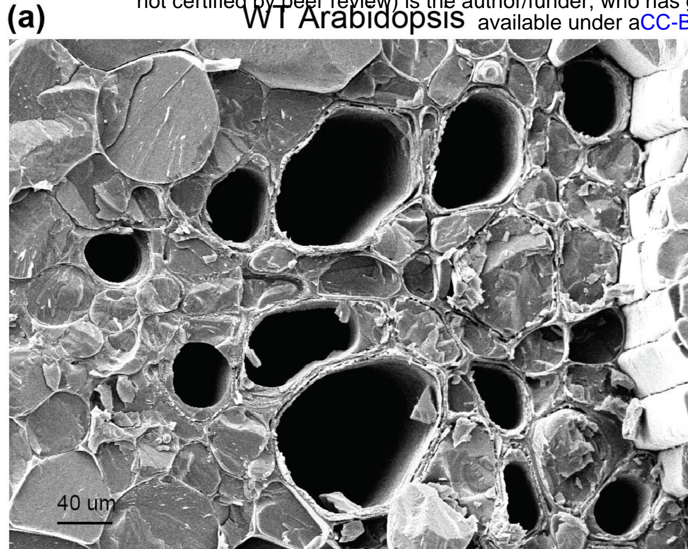

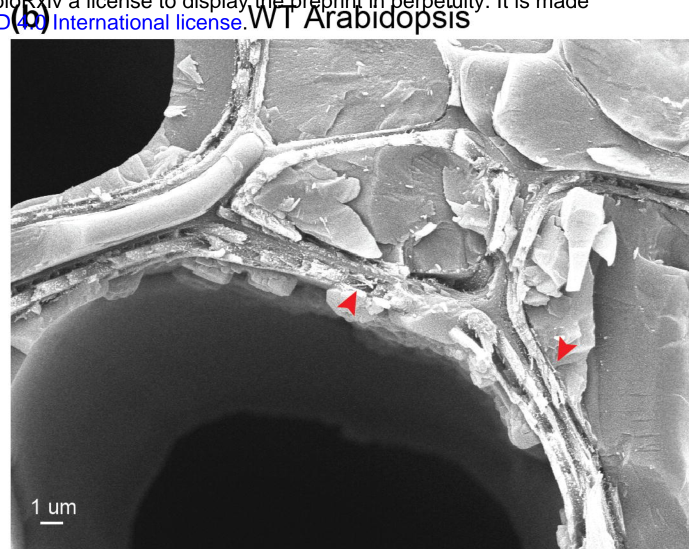

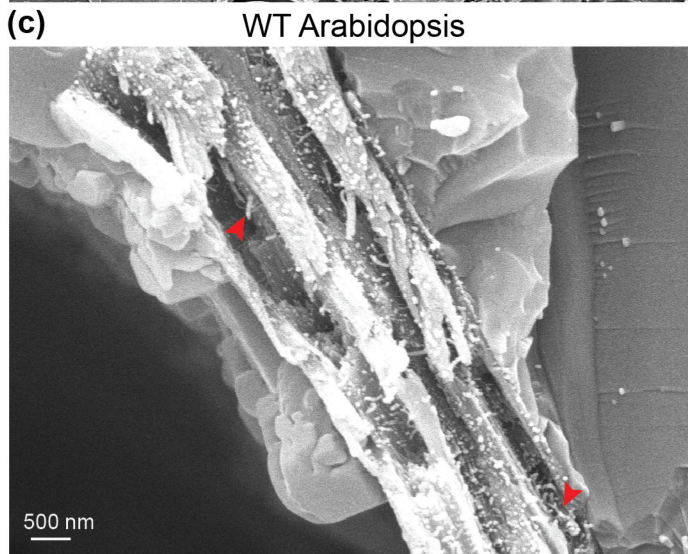

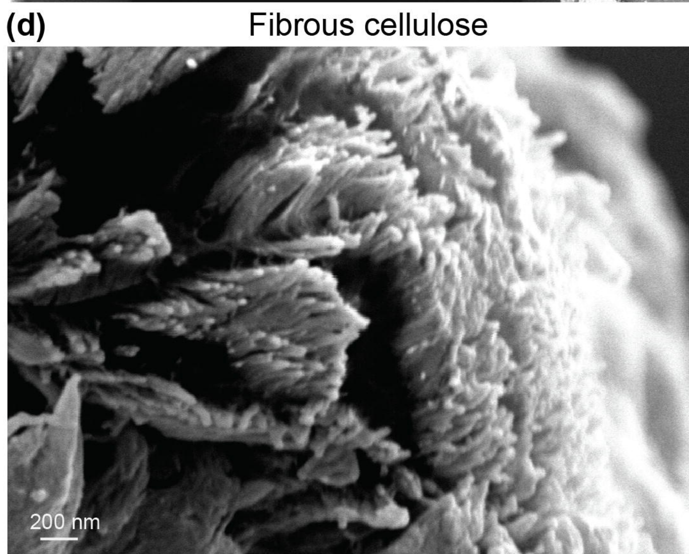

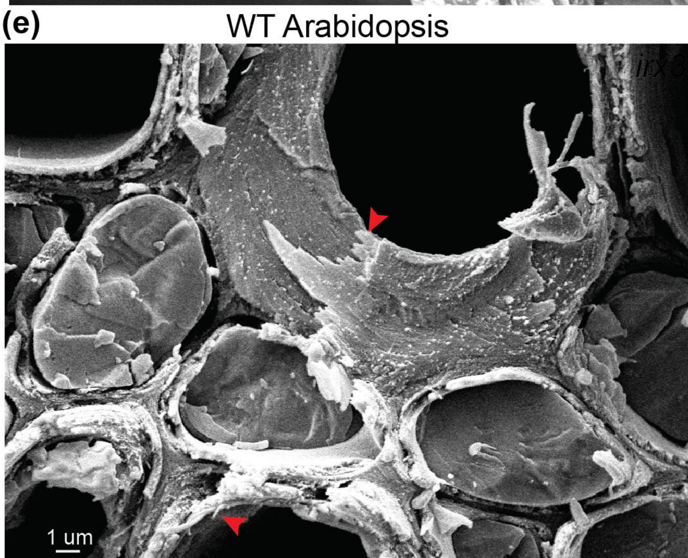

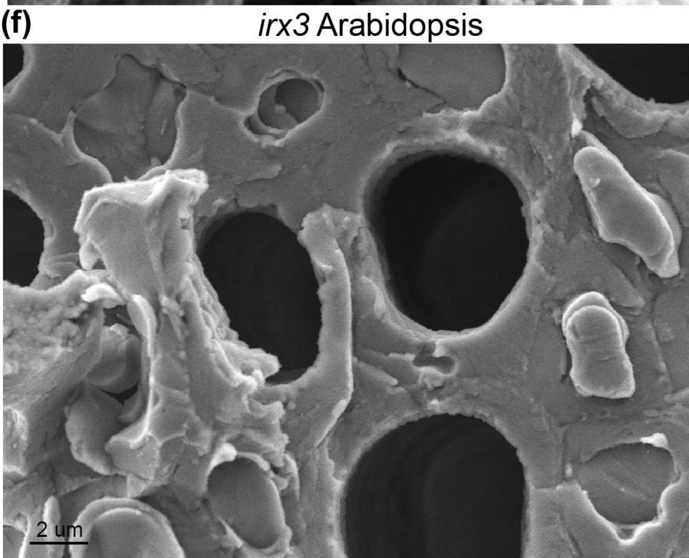

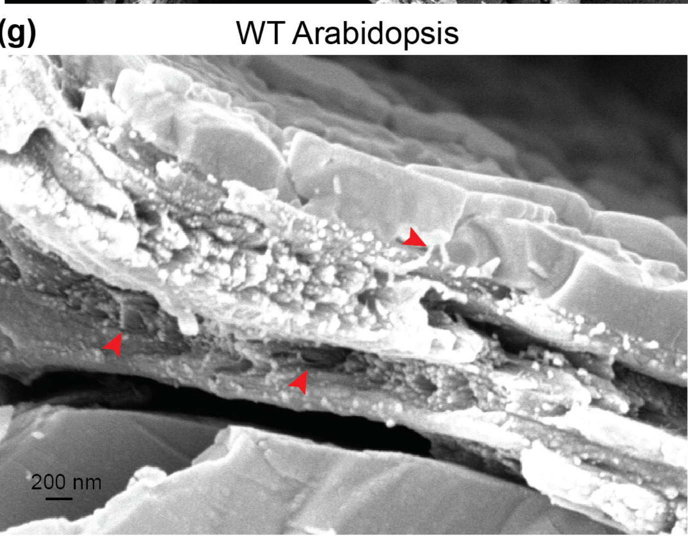

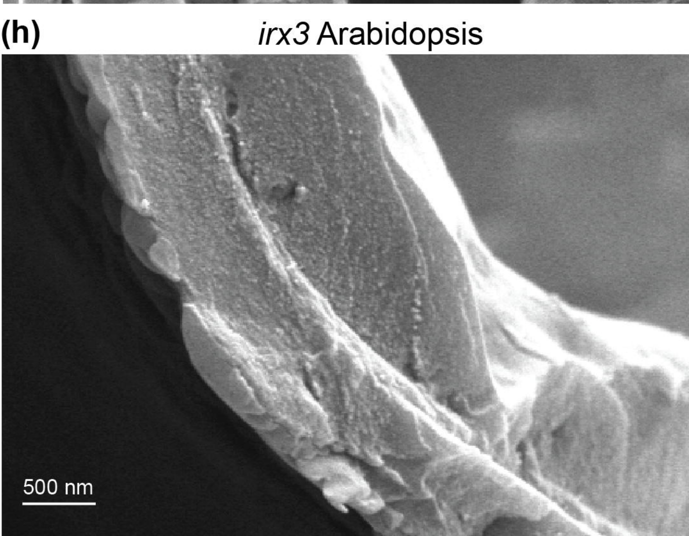

(a) *irx9*

(b) *irx10*

(c) *esk1*

(d) *4cl1*

(e) *lac4*

(f) *csla2/3/9*

(g)

Macrofibril diameter (nm)

30
25
20
15
10
5
0

*** ** *** *** * ns

WT *irx9* *irx10* *esk1* *4cl1* *lac4* *csla2/3/9*

Median macrofibril diameter (nm)	17.5	11.9	15.5	13.6	15.0	16.3	17.3