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2 An even pattern of xylan substitution is critical for interaction with cellulose in plant
3 cell walls

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17 Xylan and cellulose are abundant polysaccharides in vascular plants and essential
18 for secondary cell wall strength. Acetate or glucuronic acid decorations are
19 exclusively found on even-numbered residues in most of the glucuronoxyylan
20 polymer. It has been proposed that this even-specific positioning of the decorations
21 might permit docking of xylan onto the hydrophilic face of a cellulose microfibril¹⁻³.
22 Consequently, xylan adopts a flattened ribbon-like twofold helical screw
23 conformation when bound to cellulose in the cell wall⁴. Here we show that
24 ESKIMO1/XOAT1/TBL29, a xylan-specific O-acetyltransferase, is necessary for
25 generation of the even pattern of acetyl esters on xylan in Arabidopsis. The reduced
26 acetylation in the *esk1* mutant deregulates the position-specific activity of the xylan
27 glucuronosyltransferase GUX1, and so the even pattern of glucuronic acid on the
28 xylan is lost. Solid-state NMR of intact cell walls shows that, without the even-
29 patterned xylan decorations, xylan does not interact normally with cellulose fibrils.
30 We conclude that the even pattern of xylan substitutions seen across vascular plants
31 therefore enables the interaction of xylan with hydrophilic faces of cellulose fibrils,
32 and is essential for development of normal plant secondary cell walls.

33

34 Xylan is the principal hemicellulose in many plant secondary cell walls, and like
35 cellulose is one of the most abundant polysaccharides on Earth^{5,6}. It is thought that
36 xylan hydrogen bonds with cellulose and may be crosslinked to lignin, forming a
37 strong yet flexible composite material⁷. Despite the importance of the molecular
38 architecture of plant cell walls for their material properties and digestibility, we are

39 just beginning to understand some aspects of cellulose microfibril structure and the
40 molecular nature of the interactions of xylan with cellulose^{3,4,8-10}.

41 Xylan is a linear polymer of β -(1,4) linked D-xylosyl (X) residues. Xylan backbone
42 decoration is ubiquitous in vascular plants, but the types of substitution vary. The
43 most common substitutions are glucuronosyl (U) or 4-O-methylglucuronosyl (U^{Me}),
44 arabinosyl (Ara) and acetyl (Ac) groups¹¹. In solution, the molecule is flexible and
45 forms a threefold helical screw^{2,12}. However, we have recently shown using solid-
46 state Nuclear Magnetic Resonance (NMR) that, upon association with cellulose in
47 the cell wall, xylan adopts a twofold helical screw conformation with alternate X
48 residues orientated 180° relative to each other⁴. Cellulose microfibrils have surfaces
49 that are relatively hydrophobic, and also relatively hydrophilic surfaces that can
50 hydrogen bond with water⁸. It is unknown whether xylan binds to the hydrophobic,
51 hydrophilic, or both faces of cellulose fibrils^{4,13}. Random backbone decorations would
52 sterically impede xylan binding in a twofold screw conformation to the hydrophilic
53 surfaces of the fibril, so this mode of binding has been considered unlikely. However,
54 we recently found in vascular plants from gymnosperms to eudicots³, that many
55 xylan molecules could be compatible with this cellulose binding mode, because
56 decorations of U^[Me], Ara and Ac are spaced with an even number of backbone X
57 residues between them¹⁻³. When the even-patterned xylan is flattened into the
58 twofold screw ribbon, all the decorations become oriented along one side of the
59 molecule. This might allow the xylan to dock and form hydrogen bonds with the
60 hydrophilic surfaces of the cellulose microfibrils, forming semicrystalline
61 'xylanocellulose' fibrils, with the decoration facing away from the microfibril^{1-4,14}.

62 Without the substitutions restricted to even X residues, as seen in a minor fraction of
63 eudicot glucuronoxylan and in much of the arabinoxylan of grasses, xylan may

64 nevertheless be able to bind to cellulose on the hydrophobic face of microfibrils^{2,15}.
65 Evidence for the binding of patterned xylan to the hydrophilic face comes from the
66 finding of widespread patterning of xylan, molecular dynamics simulations, and the
67 solid state NMR studies showing the two-fold screw conformation of xylan bound to
68 cellulose. Nevertheless, there are no direct experimental data to support the
69 hypothesis that xylan can bind in this manner to the hydrophilic face. Indeed, it is not
70 known whether the even substitution patterns found in vascular plants are important
71 for allowing the binding of xylan to cellulose. In this work, we show that modifying the
72 even pattern of substitutions prevents normal association with cellulose, providing
73 experimental support for xylan binding largely to the hydrophilic surfaces of cellulose.

74 The presence of the even pattern of xylan substitutions indicates that the
75 biosynthetic machinery is finely regulated to generate precise molecular structures,
76 yet we do not understand how the substitution pattern arises. Two
77 glucuronosyltransferases, GUX1 and GUX2 add α -GlcA (U) decorations onto the 2-
78 OH of around 12% of the X residues in xylan found in Arabidopsis secondary cell
79 walls^{16,17}. These U substitutions can subsequently be methylated to U^{Me} by
80 methyltransferase enzymes¹⁸. GUX1 adds U groups to most of the xylan backbone
81 invariably with an even number of backbone residues between decorations. In
82 contrast, GUX2 adds tightly clustered U decorations with no such even spacing. Both
83 types of U substitution patterns are present within the same molecules¹. These
84 enzymes show preferences in placing U on different positions of short xylan
85 oligosaccharides *in vitro*¹⁹, but it is unknown how GUX1 appears to achieve the
86 remarkable task of placing U up to 20 X backbone residues apart, solely on even-
87 numbered X residues¹.

88 Acetylation is the most abundant xylan decoration in eudicot plants and
89 gnetophytes^{3,11}. These acetyl esters are thought to prevent the xylan from
90 precipitating and may provide a hydrophobic surface for interaction with lignin^{20,21}. In
91 *Arabidopsis*, every second X residue is acetylated on the 2-OH, 3-OH or both 2 and
92 3-OH^{2,22}. Most of the U^[Me] decorations occur on the same X residues as the Ac, i.e.
93 in phase with the acetylation pattern^{2,22,23}. The four Reduced Wall Acetylation
94 (*RWA*)1-4 genes in *Arabidopsis* encode putative Ac-CoA transporters, and so are
95 thought to supply Ac precursors to the Golgi acetyltransferases. In the
96 *rwa1rwa3rwa4* and *rwa1rwa2rwa3* triple mutants, in which one functional RWA
97 protein remains, xylan acetylation is reduced by 20-30%²⁴. Xylan acetylation also
98 requires the action of Trichome Birefringence Like (TBL) family proteins²⁵. The
99 ESKIMO1/XOAT1/TBL29 (ESK1) enzyme has been identified as a xylan-specific O-
100 acetyltransferase²⁶ responsible for adding 50-60% of all xylan acetyl groups²⁵. The
101 *eskimo1* (*esk1*) mutant is dwarfed and shows collapsed xylem vessels, indicating
102 that acetylation is important for xylan function and cell wall strength, although it is not
103 clear why this is the case. A suppressor mutation, *kaktus* (*kak*), rescues the growth
104 phenotype of the *esk1* mutant through increasing xylem vessel lumen area and
105 partially restoring water conductivity, but does not restore acetylation of the xylan
106 chain or wall strength²⁷.

107 To investigate the distribution of residual xylan Ac in the *rwa* and *esk1* mutants,
108 xylan in delignified cell wall alcohol-insoluble residue (AIR) was hydrolysed with a
109 GH10 xylanase. This enzymatic cleavage of xylan is restricted by Ac and U^[Me]
110 decorations, and yields some products with even length, such as X₄Ac₂, diagnostic of
111 the acetylation even pattern². The MALDI-ToF mass spectra of the hydrolysed xylan
112 showed minor differences in digestion products between WT and the *rwa* mutants,

113 but *esk1* xylan was more extensively digested and the X₄Ac₂ product was not
114 detected (Figure S1). Therefore, xylan from *esk1*, like *rwa* xylan, has reduced
115 acetylation, but in contrast to the *rwa* mutants, the even pattern of acetylation is not
116 detected in the *esk1* mutant.

117 Reduction of xylan acetylation leads to increased U^[Me] substitution of xylan,
118 suggesting a link between acetylation and U^[Me] substitution²⁸. As expected, all the
119 reduced acetylation mutants showed increased frequency of U^[Me] decorations
120 (Supplementary Figure 2). Next, the xylan U^[Me] substitution patterns in the *rwa* and
121 *esk1* mutants were determined and compared to WT patterns. Deacetylated WT and
122 mutant xylan was hydrolysed with glucuronoxylanase GH30, which cleaves the xylan
123 backbone one residue towards the reducing end from each glucuronosylated X
124 residue, thus releasing oligosaccharides of a length corresponding to the distance
125 between decorations^{1,29}. Hydrolysis of the WT xylan produced predominantly even
126 degree of polymerisation (DP) oligosaccharides (Figure 1, U^[Me]X DP 6, 8, 10, 12).
127 The *rwa* triple mutants showed similar predominantly evenly spaced U^[Me] patterns.
128 The *rwa1rwa3rwa4* had a higher proportion of DP 6 oligosaccharides and lower
129 proportion of DP 10, 12, consistent with a higher substitution frequency. However,
130 the additional U^[Me] in the xylan of *rwa* mutants does not disrupt the even pattern. In
131 contrast, the *esk1* mutant was devoid of any such even U^[Me] patterning and relatively
132 few oligosaccharides longer than DP12 were seen. Therefore, *rwa* and *esk1* are both
133 acetylation-defective mutants showing increased U^[Me] substitution. However, they
134 show very different alterations to the patterning of the xylan decorations, indicating
135 the ESK1 acetyltransferase is particularly important for generation of the even-
136 patterned U substitutions of xylan.

137 To investigate whether the even pattern of acetylation is also influenced by GUX
138 enzyme activity, the acetylation in *gux1*, *gux2* and *gux1gux2* mutants was studied by
139 MALDI-ToF MS and solution NMR. As expected, the MALDI-ToF mass spectra of
140 the GH10 xylanase hydrolysed xylan showed substantial differences in the
141 proportions of oligosaccharides carrying U between WT and the *gux* mutants
142 (Supplementary Figure 3). However, neutral oligosaccharides with even length
143 diagnostic of the acetylation pattern, such as X₄Ac₂, were abundant in samples from
144 WT and the *gux* mutants². Intact acetylated xylan was analysed by two-dimensional
145 ¹H–¹H nuclear Overhauser effect spectroscopy (NOESY) and ¹³C HSQC NMR
146 spectroscopy to investigate further the acetylation patterns in *esk1* and the *gux*
147 mutants (Supplementary Figure 4). NOEs corresponding to unacetylated X adjacent
148 to acetylated X residues, as previously identified² were observed in the WT, *gux1*,
149 *gux2* and the *gux1gux2* mutants. However, they were largely absent from the *esk1*
150 mutant acetylated xylan, further supporting the view that even-patterned acetylation
151 requires ESK1, but is not substantially affected by GUX enzyme activity.

152 The increased glucuronosylation and the absence of the normal even pattern of U^[Me]
153 on xylan of *esk1* suggests that one or both GUX1 and GUX2 proteins change their U
154 substitution pattern activity on the *esk1* poorly acetylated xylan. To investigate the
155 contribution of each GUX enzyme in the *esk1* mutant, *esk1 gux* double and triple
156 mutants were generated. The *esk1gux1* and *esk1gux2* double mutants grew slowly
157 and were severely dwarfed, and the *esk1gux1gux2* triple mutant was extremely
158 dwarfed (Figure 2). These severe phenotypes indicate there is an important role for
159 both GUX1 and GUX2 enzymes in decorating xylan in *esk1*. To determine the
160 contributions of each enzyme to the xylan decoration, the U^[Me] frequency of the
161 xylan from *esk1* and the *esk1gux* double mutants was determined (Figure 2B). Both

162 the *esk1gux* double mutants showed a reduction in U^[Me] frequency compared to the
163 *esk1* single mutant, indicating that both enzymes contribute to the xylan
164 glucuronosylation in *esk1*. The relative contribution of each GUX enzyme to the total
165 quantity of U^[Me] was similar in *esk1* as it was in WT, with GUX1 providing
166 quantitatively the most U decorations.

167 In WT plants, GUX1 places U decorations solely on even-spaced X residues,
168 whereas GUX2 places decorations with an unpatterned distribution. To determine
169 which of GUX1 or GUX2 produces the abnormal, unpatterned decorations in *esk1*,
170 we analysed the xylan U^[Me] decorations in the *esk1gux1* and *esk1gux2* double
171 mutants by capillary and gel electrophoresis (Figure 2C, Supplementary Figure 5).
172 The pattern of U^[Me] decorations in the *esk1gux1* mutant was similar to that in the
173 *gux1* mutant, consisting largely of DP 5, 6 and 7 oligosaccharides, although a few
174 longer oligosaccharides could be detected. This indicates the GUX2 activity was not
175 greatly altered in its positioning of the U substitutions. However, the pattern of U^[Me]
176 in the *esk1gux2* mutant, in contrast to the *gux2* mutant, did not show the even
177 spacing normally catalysed by GUX1. Therefore, the specific manner in which GUX1
178 decorates xylan is profoundly altered in the *esk1* acetylation defective mutant.

179 According to the proposed model of xylan interaction with cellulose, the abnormal
180 unpatterned xylan in *esk1* should be unable to interact with the hydrophilic face of
181 cellulose, but could nevertheless interact with the hydrophobic face². We studied
182 whether changing the xylan substitution pattern alters xylan interactions with
183 cellulose using solid-state NMR of unprocessed, never-dried stems. To obtain robust
184 plants with a substantial quantity of secondary cell walls for analysis, we grew *esk1*
185 mutants suppressed in the growth phenotype by mutation of the *KAK* gene²⁷. We

186 confirmed that the patterns of xylan substitution in WT and the *esk1* mutant are not
187 altered by the *kak* suppression (Supplementary Figure 6).

188 Xylan is induced to fold as a twofold screw through interaction with cellulose. This
189 interaction and change in conformation leads to a change in the ^{13}C solid-state NMR
190 chemical shift of xylosyl carbon 4 (C4) from the ^{13}C chemical shift of 77.4 ppm
191 corresponding to the threefold screw found in solution to 82.2 ppm corresponding to
192 the twofold screw⁴. A refocussed cross polarisation (CP) INADEQUATE spectrum of
193 *esk1kak* showed that, in contrast to WT, the signal of xylan as a twofold screw was
194 scarcely detectable in this xylan patterning mutant (Figure 3A). In contrast, threefold
195 screw xylan was clearly observed in the mutant. As this CP-INADEQUATE
196 emphasises the more rigid cell wall components, some of the xylan in *esk1* may
197 therefore still interact with cellulose, but with a threefold screw conformation. The
198 more mobile *esk1kak* cell wall components are shown in a direct polarisation
199 INADEQUATE spectrum (Figure 3B). Unlike in the WT⁴, relatively mobile threefold
200 screw xylan is clearly seen in the *esk1kak* cell walls. Thus, the abnormally patterned
201 xylan substitutions in the *esk1* mutant prevent normal interaction of xylan with
202 cellulose and leads to an increase in unbound mobile xylan in the cell wall. The
203 almost complete loss of the cellulose-bound two-fold screw xylan in the mutant
204 suggests most of the xylan in WT plants binds to the hydrophilic face of cellulose in
205 this xylan-substitution even pattern-dependent manner.

206 Our findings indicate ESK1 is essential for generating the even acetylation pattern.
207 Additional TBLs and xylan acetyltransferases may also be involved³⁰. We now also
208 know, since the *esk1* mutant shows disrupted patterns of U, that the
209 glucuronosyltransferase GUX1 generates the even U pattern guided by the ESK1-

210 dependent xylan acetylation. The sites for addition of U are in phase with (multiples
211 of two residues from) patterned acetylated X residues (Figure 4). GUX1 may find
212 gaps in the acetylation pattern, or compete with ESK1 and other TBLs for
213 substitution of appropriate X residues. Starvation of substrate in the *rwa* mutants
214 may lead to an increase in frequency of these gaps, or a slight reduction in the
215 ESK1/TBL activity, and results in an increase in GUX1 activity and even-patterned U
216 substitutions. In the *esk1* mutant, larger regions of unacetylated xylan are available
217 for GUX1 glucuronosyltransferase activity, and GUX1 is unable to maintain the
218 correct even U pattern without the acetylation guidance. There are several aspects
219 of this model that are important areas of future investigation. How ESK1 is required
220 for the Ac pattern generation, the role of other TBLs in acetylation, the subsequent
221 transfer of additional acetate to X residues substituted by U, and the role of putative
222 Golgi xylan acetylsterases remain unresolved³⁰.

223 There is a growing body of evidence that the even-patterned arrangement of xylan
224 decorations is a common feature in all vascular plants³. Since the discovery of the
225 xylan decoration even pattern in *Arabidopsis*¹, it has been unclear what the
226 importance of this is, if any, for xylan function. The pattern was suggested to be an
227 essential feature allowing xylan to interact with hydrophilic surface of cellulose^{2,3}. We
228 have now shown that when the pattern of Ac and U is disrupted in *esk1*, the xylan
229 does not bind in the twofold screw conformation to cellulose (Figure 3). This strongly
230 supports the model of hydrogen bonding of the xylan with the hydrophilic surface of
231 cellulose fibrils, as the even pattern is essential for the docking onto this cellulose
232 surface (Figure 4), but may not be essential for binding to the hydrophobic
233 surfaces^{2,3}. This work therefore provides critical evidence supporting this xylan-
234 cellulose interaction hypothesis, and increases our understanding of the structure of

235 xylanocellulose fibrils. It also demonstrates how such normal interactions may be
236 disrupted, providing strategies to change plant cell walls for improved biorefining and
237 mechanical properties. Whether the loss of normal xylan binding to cellulose affects
238 cellulose synthesis, fibril orientation or fibril aggregation remains to be investigated.

239 The binding of even-patterned xylan to the hydrophilic surfaces of cellulose fibrils in
240 vascular plants could serve many roles. For instance, the modified surface of the
241 xylanocellulose microfibril has greatly reduced H-bond donor capacity compared to
242 the naked cellulose fibril surface. This, and the presence of acetyl esters, may alter
243 the manner of fibril association with water, and could facilitate interactions with the
244 hydrophobic lignin². The lignocellulose assembly would be further strengthened if
245 xylan is crosslinked to lignin via U^[Me]-lignin esters, as proposed³¹. Xylan binding to
246 cellulose improves the mechanical properties of the cell wall, as shown by the fact
247 that the *esk1* plants have collapsed vessels³². This coating of the fibrils may
248 influence cellulose fibril bundling and interaction, perhaps preventing cellulose fibril
249 co-crystallisation (aggregation). Pulp and paper manufacture, biofuel processing,
250 and digestion of feed all involve removal of xylan from cellulose, and so discovery of
251 plants in which xylan is not bound to cellulose may facilitate aspects of these
252 processes^{27,33,34}. This improved understanding of secondary cell wall architecture
253 suggests novel strategies for preparation and application of biomaterials from plant
254 cell walls.

255

256 **Methods**

257 **Plant growth and cell wall preparation**

258 Plants were *Arabidopsis thaliana* Columbia-0 ecotype. The *esk1* ethyl
259 methanesulphonate induced point mutant (*esk1-1*)³⁵ was obtained from Henrik
260 Scheller. T-DNA insertion mutations of ESKIMO1 (*esk1-5*) and KAKTUS (*kak-8*)
261 were used for the NMR experiments²⁷. Plants were grown in compost at 20°C, 100
262 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 16 h light / 8 h dark photoperiod 60% humidity and allowed five to six
263 weeks to mature before harvesting, except the *esk1gux* double and triple mutants,
264 which were grown aseptically in 0.5 x MS (Murashige and Skoog Basal Medium),
265 0.6% (w/v) agar for two weeks. They were then grown in magenta vessels containing
266 the same media for three months prior to harvest. The basal five cm of fresh stems
267 (entire stems for *esk1gux* double mutants) were harvested to make Alcohol Insoluble
268 Residue (AIR) as previously described¹.

269 **PACE and DASH**

270 PACE (Polysaccharide Analysis by Carbohydrate gel Electrophoresis) and DASH
271 (DNA sequencer Assisted Saccharide analysis in High throughput) was performed
272 as previously described^{1,36}. AIR was hydrolysed with *BoGH30*³⁷, *CjGH10B* or
273 *NpGH11A*, kind gifts of Harry Gilbert, Newcastle. Deacetylation was carried out on
274 dried samples by adding 20 μL of 4 M NaOH, incubating for 1 h and neutralising with
275 80 μL of 1 M HCl.

276 **Mass Spectrometry**

277 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass
278 spectrometry (MS) of xylanase digested samples was used to determine the spacing
279 of Ac and U^[Me] groups along the xylan backbone. For CjGH10A hydrolysis,
280 holocellulose was prepared from AIR by peracetic acid delignification, as described
281 previously^{2,38}. The holocellulose was then heat treated at 90 °C for 30 min in 100 mM
282 ammonium acetate buffer, pH 5.5. The sample was centrifuged and the supernatant
283 was discarded. Hydrolysis of the remaining pellet proceeded overnight at room
284 temperature with xylanase CjGH10A (approximately 1 µM). MALDI-ToF MS was
285 performed using a 4700 Proteomics Analyser (Applied Biosystems, USA) as
286 previously described^{2,39}. The acetylated oligosaccharides in aqueous solution were
287 mixed 1:1 (v/v) with 2,5-dihydroxybenzoic acid (DHB, Sigma-Aldrich) matrix (10
288 mg/mL DHB dissolved in 50% MeOH with 0.4 mg/mL Ammonium Sulphate
289 ((NH₄)₂SO₄) to prevent the formation of disodiated adducts⁴⁰.

290 **Solution NMR**

291 Solution NMR of acetylated xylan (prepared by DMSO extraction as described for
292 Mass Spectrometry) was carried out as described ². The NMR data of *gux1gux2*
293 acetylated xylan were reanalysed ².

294 **Solid-State Nuclear Magnetic Resonance**

295 MAS solid-state NMR experiments used ¹³C enriched plants grown and labelled with
296 ¹³CO₂ in a bespoke growth chamber according to Simmons et al⁴. Experiments were
297 performed on a widebore Bruker (Karlsruhe, Germany) AVANCE III 850 MHz solid-
298 state NMR spectrometer operating at 20 T, corresponding to ¹H and ¹³C Larmor
299 frequencies of 850.2 and 213.8 MHz, respectively. Experiments were conducted at

300 room temperature using a 3.2 mm low E field biosolids MAS probe at a MAS
301 frequency of 12.5 kHz \pm 5 Hz. The ^{13}C chemical shift was determined using the
302 carbonyl peak at 177.8 ppm of L-alanine as an external reference with respect to
303 TMS. Two-dimensional double-quantum (DQ) correlation spectra were recorded
304 using the refocused INADEQUATE pulse sequence^{41,42}, which relies upon the use of
305 isotropic, scalar J coupling to obtain through-bond information regarding directly
306 coupled nuclei. Both ^1H to ^{13}C CP, with ramped ^1H amplitude and a contact time of 1
307 ms, and direct polarisation (to emphasise the mobile constituents) versions of the
308 experiment were used to produce the initial transverse magnetization. The ^1H 90°
309 pulse length was 3.5 μs and the ^{13}C 90° and 180° pulse lengths were 4.2 and 8.4 μs ,
310 respectively, with a spin-echo delay of 2.24 ms. SPINAL-64 decoupling⁴³ at a ^1H
311 nutation frequency of 70 kHz during evolution and signal acquisition periods was
312 used throughout. The recycle delay was 1.9 s. The spectral width was 50 kHz in both
313 dimensions with the acquisition time in the indirect dimension (t_1) being 4.2 ms with
314 128 co-added transients for each slice in the CP experiment using the States method
315 for sign discrimination in F_1 and 5.0 ms with 96 co-added transients for each slice in
316 the direct polarisation experiment using the TPPI method for sign discrimination in
317 F_1 . The data obtained were Fourier transformed into 2K (F_2) \times 1K (F_1) points with EM
318 line broadening of 40 Hz in F_2 and squared sine bell in F_1 . All spectra obtained were
319 processed and analysed using Bruker Topspin version 3.2.

320

321 Data availability. Solid state NMR data are available at:

322 <https://doi.org/10.17863/CAM.12996>

323 The authors declare that all other relevant data supporting the findings of this study
324 are available within the article or on request from the corresponding author Paul
325 Dupree (pd101@cam.ac.uk).

326

327 **Keywords**

328 *Arabidopsis thaliana*, xylan, acetylation, glucuronosylation, cellulose interaction,
329 ESKIMO1, acetyltransferase

330

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342 **Author contributions**

343 NJG conducted most of the plant molecular genetic and biochemical experiments,
344 assisted by JWR and MBW. MDT provided *esk1kak* genetic material and supporting
345 information. The solid state NMR experiments were conducted by RD assisted by DI
346 using plants grown by TJS, OMT and JL. Solution NMR was conducted by KS and
347 NJG. Data analysis was conducted by NJG, JWR, OMT, JL, KS, TJS, MBW, SPB,
348 RD, PD. MBW, SPB, RD and PD supervised aspects of the project. The paper was
349 written by NJG, MBW and PD with contributions from all authors.

350

351 Competing interests

352 The authors declare no competing financial interests.

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355

356 **FIGURES**

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362

363 **Figure 1: U^[Me] decoration patterns are disrupted in *esk1* but not *rwa***

364 **acetylation mutants.** Xylan from WT, *rwa1rwa3rwa4*, *rwa1rwa2rwa3* and *esk1*

365 mutants was hydrolysed with glucuronoxylanase GH30 and analysed by DASH

366 capillary electrophoresis (DNA-sequencer-Assisted Saccharide analysis in High-

367 throughput). (a) capillary electrophoresis traces and (b, c) quantification of

368 oligosaccharides showing loss of the predominantly even pattern of U^[Me] spacing in
369 *esk1*. Individual values and means of three independent biological replicates of basal
370 stems from at least five plants, each replicate analysed by three independent
371 hydrolyses, are shown. Significantly different from WT where shown: * $p \leq 0.05$; **
372 $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; by two-tailed t-test. Dagger, background peak.
373

374

375

376 **Figure 2: Both GUX1 and GUX2 contribute to glucuronosylation in the *esk1***
377 **mutant, but GUX1 is deregulated in its patterning activity.** (a) The *esk1* mutant is
378 fertile but dwarfed to approximately 50% of wild type height²⁵. In contrast, both the
379 *esk1gux1* and the *esk1gux2* double mutants are sterile and severely dwarfed. The
380 triple *esk1gux1gux2* mutant did not grow an inflorescence stem. Bars 1 cm. (b) Both
381 GUX1 and GUX2 contribute to xylan glucuronosylation in the *esk1* mutant in similar
382 proportions to WT background. U^[Me] frequencies were measured by DASH capillary
383 electrophoresis of GH11 xylanase hydrolysed xylan. Individual values and means of
384 three independent hydrolyses of a single biological replicate of five plants are shown,
385 and are representative of two independent experiments. * $p \leq 0.05$ in both replicates
386 by two-tailed t-test. ns, not significant (c) DASH capillary electrophoresis analysis of
387 GH30 glucuronoxylanase digested xylan indicates that *esk1gux2* double mutants
388 show clear altered U^[Me] patterning similar to the *esk1* mutant, indicating GUX1 is
389 deregulated in *esk1*. Dagger, a primary cell wall xylan PUX₆ oligosaccharide⁴⁴.

390

391 **Figure 3: Solid-state NMR of WT and *esk1kak* mutant cell walls shows that the**
392 **unpatterned xylan does not bind to cellulose in the twofold helical screw**
393 **conformation found in WT plants.** (a) An overlay is shown of the carbohydrate
394 regions of refocussed CP-INADEQUATE spectra of WT and the *esk1kak* mutant.
395 The Double Quantum (DQ) shift is the sum of the Single Quantum shifts of two
396 bonded (J-coupled) ^{13}C nuclei. Red labelling indicates xylan in the cellulose-bound,
397 twofold screw conformation. The absence of the $\text{Xn}4^{2f}$ - $\text{Xn}5^{2f}$ pair in *esk1kak*
398 indicates twofold screw xylan bound to cellulose is reduced in the mutant. The green
399 labelled xylan in the threefold conformation is substantially more abundant in the
400 *esk1kak* mutant cell walls. (b) A refocussed direct polarisation INADEQUATE
401 spectrum of the *esk1kak* mutant shows that the abnormal, relatively mobile, threefold
402 screw xylan is found in the mutant cell walls. Spectra are representative of data from
403 two independent biological replicates.

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406 **Figure 4: A model of glucuronoxylan substitution pattern generation and its**
407 **consequence for xylan interaction with cellulose.** Xylan is first synthesised by the
408 xylan synthase complex (XSC) in the Golgi apparatus. The even pattern of xylan
409 acetylation on alternate X residues requires the action of ESK1, and perhaps
410 additional enzymes. Next, GUX1 places a U on even-spaced X residues directed by
411 the even pattern of Ac, leading to even-patterned xylan that is compatible with
412 binding to the cellulose hydrophilic surface. GUX2 places a U without maintaining the
413 pattern with other decorations, generating incompatible xylan. After initial acetylation
414 and glucuronosylation, the xylan may be further modified by additional TBL acetyl
415 transferases that place an Ac on the same X that is substituted by a U, and which
416 may generate doubly acetylated X residues. The even pattern of xylan acetylation
417 may also be influenced by acetyl-xylan esterases³⁰. In the *esk1* mutant (right), the
418 absence of the acetylation catalysed by ESK1 results in GUX1 decorating the xylan
419 with U at incorrect positions, and the defective xylan is incompatible with binding to
420 cellulose hydrophilic surfaces.

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