

Secondary cell wall integrity sensing

A Transcriptomic Analysis of Xylan Mutants Does Not Support the Existence of A Secondary Cell Wall Integrity System in Arabidopsis.

1 Nuno Faria Blanc^{1†}, Jenny C. Mortimer^{1,2,3†}, Paul Dupree^{1,*}

2 ¹Dept. Biochemistry, University of Cambridge, Cambridge, CB2 1QW, UK

3 ² Joint BioEnergy Institute, Emeryville, CA, 94608, USA

4 ³Biosciences Area, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA

5 [†]This indicates that these authors contributed equally.

6 * **Correspondence:**

7 Paul Dupree

8 p.dupree@bioc.cam.ac.uk

9 **Keywords: xylan, cell wall engineering, cell wall integrity, plant, secondary cell wall,**
10 **biofuels, biomass**

11 *Note to editor: This manuscript was prepared with British English*

12 **Abstract**

13 Yeast have long been known to possess a cell wall integrity (CWI) system, and recently an
14 analogous system has been described for the primary walls of plants (PCWI) that leads to
15 changes in plant growth and cell wall composition. A similar system has been proposed to exist
16 for secondary cell walls (SCWI). However, there is little data to support this. Here, we analysed
17 the stem transcriptome of a set of cell wall biosynthetic mutants in order to investigate whether
18 cell wall damage, in this case caused by aberrant xylan synthesis, activates a signalling cascade
19 or changes in cell wall synthesis gene expression. Our data revealed remarkably few changes to
20 the transcriptome. We hypothesise that this is because cells undergoing secondary cell wall
21 thickening have entered a committed programme leading to cell death, and therefore a SCWI
22 system would have limited impact. The absence of transcriptomic responses to secondary cell
23 wall alterations may facilitate engineering of the secondary cell wall of plants.

24 **1 Introduction**

25 Cell walls are polysaccharide rich matrices which surround the cells of plants, fungi and bacteria.
26 What makes plant cell walls exceptional is their importance to human society. We rely on plant
27 cell walls for dietary fibre, animal feed, building materials, paper, and fuel, amongst other uses.
28 Recent biotechnological advances, such as synthetic biology, combined with a detailed
29 knowledge of cell wall biosynthesis, have raised the possibility of engineering an optimised plant
30 secondary cell wall. For example, we and others have successfully engineered plants cell walls
31 which have biomass designed for more efficient conversion to biofuels (Coleman et al.,
32 2009;Mortimer et al., 2010;Petersen et al., 2012;Smith et al., 2013;Gondolf et al., 2014).

Secondary cell wall integrity sensing

33 Unexpectedly, the engineered plants often grow normally, despite substantial biochemical and
34 structural alterations, suggesting a tolerance for change.

35 Plant cell walls exist as two major types: primary and secondary. The primary wall is a thin layer
36 that forms from the cell plate after mitosis. Since it surrounds growing cells, it must be flexible,
37 enabling cell expansion either isotropically or anisotropically. Secondary cell walls are much
38 thicker, can be lignified, and are laid down on the inside of the primary cell wall in some cell
39 types, at the cessation of growth. The exact composition of these walls varies depending on cell
40 function, tissue type and plant species. Arabidopsis has a primary cell wall composed of
41 cellulose, xyloglucan and pectins, whereas its secondary cell wall is dominated by cellulose and
42 xylan, with small amounts of pectin and mannan. Some secondary cell walls, such as those of the
43 xylem water transport vessels, are extensively lignified after the deposition of the
44 polysaccharides.

45 Due to the importance of the cell wall to an organism, it is perhaps unsurprising that cells might
46 possess a system which detects and repairs damage whilst allowing the dynamic changes
47 required of a growing cell. In yeast, this cell wall integrity system (CWI) has been well
48 characterised. The yeast plasma membrane contains cell surface sensors which have a highly
49 mannosylated serine threonine rich (STR) extracellular domain (Philip and Levin, 2001). These
50 sensors are also thought to be anchored in the cell wall, with the STR acting as a nano-spring to
51 detect stresses and strains between the wall and membrane (Kock et al., 2015). The signal
52 transduction pathway is a mitogen-activated protein kinase (MAPK) cascade acting via the
53 master regulator Rho1p, a small G protein (Levin, 2011). The various outputs include changes in
54 polysaccharide biosynthesis and actin reorganisation.

55 The discovery of a cell wall integrity system in plant primary cell walls (PCWI) is much more
56 recent, although it has been recognised for some time that perturbations to the primary cell wall
57 can lead to compensatory effects. The activation of the PCWI system is linked to the process of
58 control of growth and adaptation of cell wall architecture and composition to varying
59 environments (Voxeur and Hofte 2016). Disruption of primary cell wall cellulose synthase
60 activity is an activator of the PCWI. For example, the *ectopic lignification (eli1)* mutation of
61 *CESA3* or application of the herbicide isoxaben, which targets cellulose biosynthesis, leads to
62 ectopic lignification (Cano-Delgado et al., 2003). *prc1-1*, a mutation in *CESA6* also shows
63 ectopic lignification and callose accumulation (Desprez et al., 2002; Hematy et al., 2007). Virus
64 Induced Gene Silencing (VIGS) of a primary wall CESA in tobacco results in a compensatory
65 increase in homogalacturonan (Burton et al., 2000).

66 Receptor like kinases (RLKs) have emerged as the likely mechanism by which signals are
67 transduced into the plant cell. These proteins have an extracellular ligand-recognition domain, a
68 transmembrane domain and a cytosolic kinase domain. Extracellular activation leads to
69 phosphorylation of downstream signalling components. RLKs form a large gene family (more
70 than 600 in Arabidopsis (Shiu and Bleecker, 2003)) and only a small proportion have been
71 characterised in terms of ligand specificity and function. However, the roles described are
72 extremely varied and include disease resistance, symbiosis, shoot apical meristem (SAM)
73 maintenance and determination of cell fate. THESEUS1 (THE1), a plasma-membrane spanning
74 Ser/Thr RLK, was first identified as a suppressor of the *CESA6* mutant *prc1-1* (Hematy et al.,
75 2007). THE1 was shown to mediate many, although not all, of the downstream outputs which

Secondary cell wall integrity sensing

76 result from inhibition of primary cell wall synthesis. The signal which activates RLKs such as
77 THE1 is also unknown. Another plasma membrane spanning Ser/Thr RLK, WALL
78 ASSOCIATED KINASE1 (WAK1), binds oligogalacturonides (OGs) (Decreux and Messiaen,
79 2005). OGs are oligosaccharide fragments of the pectin homogalacturonan, and are interpreted
80 by the plant as DAMPs (damage associated molecular patterns). Detection of OGs by WAK1
81 leads to downstream activation of defence response genes and changes in growth and
82 development (Brutus et al., 2010).

83 The evidence for a CWI system functioning in the plant secondary cell wall (SCWI) is much less
84 apparent, although it has been widely proposed e.g. (Hamann, 2012; Doblin et al., 2014;
85 Vermerris and Abril, 2014). There have been no clear reports that changes to the secondary cell
86 wall composition induce compensatory changes in these tissues other than those simply due to
87 developmental differences, for example due to dwarfing of the plant. Mutations in the secondary
88 cell wall cellulose synthases (*CESA4*, 7 and 8), unlike the primary wall CESAs, do not result in
89 widespread cell wall composition modifications beyond the directly caused reduction in
90 cellulose. The *irx3* mutant (a point mutation in *CESA7*), has collapsed xylem vessels and less
91 than a fifth of the cellulose content of wild type (WT) stems (Turner and Somerville, 1997), yet
92 continues to make xylan and lignin. Solid-state NMR spectra of *irx3* and WT cell wall material
93 revealed that the difference between the two samples was simply cellulose, indicating there were
94 no observable changes to lignin or hemicellulose that would suggest adaptation to the loss of
95 cellulose (Ha et al., 2002).

96 Plants which have reduced xylan synthase activity such as *irx9*, *irx10* or *irx14* have a secondary
97 cell wall weakness sufficient to result in xylem vessel collapse, Nevertheless, they do not have
98 large changes in wall composition (Brown et al., 2007). The alterations reported are consistent
99 with their stunted development, and do not indicate a compensatory response. Further support for
100 the view that the SCW is amenable to modification comes from the finding that the lignin
101 composition is very plastic and can be altered through metabolic engineering without negative
102 impact on the plant (Wilkerson et al., 2014; Eudes et al., 2015).

103 Perhaps the strongest evidence for a SCWI system comes from lignin biosynthesis mutants.
104 Mutants in lignin biosynthesis which result in a reduction in lignin content, do show evidence of
105 compensatory modifications to the cell wall. For example, silencing of *4-COUMARATE:COA*
106 *LIGASE1 (4CLI)/4CL2* and *CAFFEOYL-COA O-METHYLTRANSFERASE (CCoAOMT)* in
107 Arabidopsis and maize respectively results in an increase in cellulose content (Yang et al.,
108 2011; Li et al., 2013). They show clear changes to transcription (Vanholme et al. 2012).
109 However, this may be because lignification is not always a cell autonomous process.
110 Parenchyma cells surrounding the xylem vessels act as “feeder cells”, providing the monolignol
111 subunits (Petersen et al., 2012; Smith et al., 2013). Evidence for a detection system for a loss of
112 lignification comes from recent work which used a similar approach to that used to identify
113 THE1. A suppressor screen was performed on a dwarf mutant which has reduced *p*-
114 coumaroylshikimate 3'-hydroxylase (3CH) activity, *reduced epidermal fluorescence8-1 (ref8-1)*,
115 and it was shown that interference with two MEDIATOR (MED) complex subunits (MED5a and
116 MED5b) rescues the growth phenotype of *ref8-1* without restoring lignin biosynthesis (Bonawitz
117 et al., 2014). The MED complex is localised in the nucleus, so it is proposed that a currently
118 unknown sensor detects the changed lignin precursor components, and relays that information,
119 via the MED complex, to result in large scale transcriptional changes in the living cells

Secondary cell wall integrity sensing

120 surrounding the fibres and vessels (Bonawitz et al., 2014). Thus, the cells not undergoing
121 secondary cell wall deposition may detect and respond to changes in phenylpropanoid
122 intermediates.

123 Transcription factors (TFs) are key to regulating the development of the secondary cell wall.
124 Since the decision to deposit secondary cell wall usually leads to cell death, plants have a
125 cascade of TFs which control this process. These include VASCULAR-RELATED NAC
126 DOMAIN6 (VND6) and VND7, which directly regulate the expression of the MYB46 TF and
127 some secondary cell wall biosynthetic genes (Kubo et al., 2005; Zhong et al., 2008). A large-scale
128 systems approach has recently identified that this feed-forward loop as one of many involved in
129 regulating secondary cell wall biosynthesis (Taylor-Teeple et al., 2015). As yet, no TFs have
130 been implicated in regulating a SCWI response, in which direction and balance are controlled,
131 rather than turning on the cascade. However, if such a TF or network of TFs exists, we would
132 predict that it is commonly upregulated in secondary cell wall synthesis mutants.

133 Xylan, the dominant secondary cell wall polysaccharide after cellulose, is composed of a (1-4)-
134 β -D-xylose backbone, and depending on species and tissue, it is variously substituted with
135 glucuronic acid, 4-O-methyl-glucuronic acid, arabinose and acetyl groups. In Arabidopsis, IRX9,
136 IRX10 and IRX14 are the key proteins involved in secondary cell wall xylan backbone
137 biosynthesis (Brown et al., 2007; Pena et al., 2007; Brown et al., 2009; Wu et al., 2010). Loss of
138 function mutations in these genes lead to a reduced xylan chain length, although the severity is
139 dependent on which gene is affected (Figure 1). In the absence of these genes, a reduced quantity
140 of defective xylan is made by homologues IRX9L, IRX10L and IRX14L respectively (Brown et
141 al., 2009; Wu et al., 2009; Wu et al. 2010). Arabidopsis secondary cell wall xylan is predominantly
142 composed of (methyl)glucuronoxylan (Figure 1), where GLUCURONIC ACID SUBSTITUTION
143 OF XYLAN1 (GUX1) and GUX2 are responsible for glucuronic acid (GlcA) sidechain addition
144 (Mortimer et al., 2010; Rennie et al., 2012), of which a proportion is subsequently methylated
145 (Urbanowicz et al., 2012) (Figure 1). Since xylan biosynthesis is relatively well understood, we
146 suggest that it provides a good system for in which to test whether Arabidopsis cells which are
147 actively synthesising secondary cell wall, can detect and respond to defects in xylan
148 biosynthesis.

149 In this study, we used a transcriptomics approach to investigate whether we could detect putative
150 components of a SCWI system in Arabidopsis stems. We looked at gene expression in a
151 collection of xylan biosynthetic mutants. The aim of this experiment was threefold. Firstly, we
152 wanted to test whether there is a co-ordinated expression response to xylan defects within the
153 xylan biosynthetic pathway. Secondly, we wanted to identify more general responses to
154 secondary cell wall defects, such as the upregulation of other biosynthetic pathways or
155 transcription factors. Thirdly, we wanted to look for the upregulation of putative SCWI integrity
156 sensors, such as RLKs.

157 **2 Methods**

158 **2.1 Genotypes and growth conditions**

159 The following previously published *Arabidopsis thaliana* T-DNA insertion lines were used:
160 SALK_063763 (*gux1*, At3g18660), GABI-722F09 (*gux2*, At4g33330), SALK_057033 (*irx9*,

Secondary cell wall integrity sensing

161 At2g37090), SALK_046368 (*irx10*, At1g27440), SALK_038212 (*irx14*, At4g36890) and
162 *gux1gux2*. All mutants were in the Columbia-0 (Col-0) ecotype. Seeds were stratified (4°C, 72
163 hours) prior to being grown on soil (Levington M3 compost) in a growth chamber (22°C, 60 %
164 humidity, continuous light). Several plants were initially grown in each pot but these were
165 gradually thinned until only 2 plants remained to ensure all plants were of a similar
166 developmental stage. All lines were genotyped by PCR prior to inclusion in this study, using the
167 relevant published oligonucleotide primers.

168 2.2 Plant material

169 Plants were harvested at the same developmental stage (Boyes et al., 2001), defined as when the
170 inflorescence stem containing an extended silique (1.5 cm) or presented silique fattening. At this
171 point, inflorescence stem height was measured and basal stem with 1/5 of this height was
172 harvested and immediately frozen in liquid nitrogen. Stems (15-20 per genotype) were pooled to
173 form one biological replicate. Samples were harvested at the same time of day (within one hour)
174 to reduce the effects of circadian differences on the data set. Three independently grown
175 biological replicates were collected in this manner.

176 2.3 RNA extraction and microarrays

177 To extract RNA, tissue was ground under liquid N₂ and mixed well with Trizol (1 mL per 100
178 mg of tissue; Invitrogen). Following incubation at room temperature for 2 minutes, chloroform
179 (200 µL) was added and samples were gently mixed for 15 seconds. After centrifugation (12,000
180 x g, 4°C, 15 minutes), the aqueous upper phase was obtained to which 250 µL high salt solution
181 (0.8 M sodium citrate, 1.2 M sodium chloride) and 250 µL isopropanol were added. After mixing
182 and centrifuging the samples, the pellet was isolated and washed with 75% (v/v) and then 100%
183 (v/v) ethanol. After resuspension, samples were incubated at 37°C for 30 minutes with DNase
184 (Promega). Further sample processing was done using the RNeasy kit (Qiagen), according to the
185 manufacturers “RNA Cleanup” protocol. RNA concentration and quality was measured using a
186 BioPhotometer plus (Eppendorf) as well as by agarose gel electrophoresis. Samples were then
187 sent to the Nottingham Arabidopsis Stock Centre (NASC; <http://affymetrix.arabidopsis.info/>) for
188 analysis. Quality control was first performed using an Agilent Bioanalyzer to check the integrity
189 of the RNA. Following preparation of cDNA, the samples were hybridized to the Arabidopsis
190 ATH1 Genome Array (Affymetrix). Raw data can be obtained from NASCarrays, experiment
191 number 668.

192 2.4 Microarray data analysis

193 Data analysis was done using the R-based FlexArray software (Blazejczyk et al., 2007). For the
194 background correction of raw data, Gene Chip – Robust Multi-array Analysis (GC-RMA) was
195 used (Wu and Irizarry, 2004). Arrays were normalised using quantile normalisation (Bolstad et
196 al., 2003) and probeset summarisation used median polish (Berger and Carlon, 2011). Arrays
197 were analysed for significance using the Significance Analysis of Microarrays test (Tusher et al.,
198 2001), and probesets with a p-value ≤ 0.05 and at least 2-fold change of their log values were
199 retained for further analysis. Gene annotations for the probesets were obtained from the
200 Affymetrix website (<http://www.affymetrix.com>) and Gene Ontology (GO) was done using
201 Classification Superviewer tool from the Bio-Analytic Resource for Plant Biology

Secondary cell wall integrity sensing

202 (<http://bar.utoronto.ca>) (Provart and Zhu, 2003). Data is presented as \log_2 (abundance in
203 mutant/abundance in WT), as described in (Vanholme et al., 2012).

204 3. Results and Discussion

205 3.1 Xylan mutants used in this study

206 The following Arabidopsis xylan mutants: *irx9*, *irx10*, *irx14*, *gux1*, *gux2* and *gux1gux2*, along
207 with WT ecotype Col-0 plants (Table 1) were grown alongside each other until they developed
208 their first silique. At this point, basal stems were harvested, and the RNA extracted for
209 microarray analysis. Genotype verification was also performed, and all lines were shown by PCR
210 to be homozygous for the expected T-DNA insertion (Supplemental Figure S1). The effect of
211 each of these mutations on xylan structure, along with their phenotype at the developmental
212 stage used in this study, is shown in Figure 1.

213 The microarray data were normalised (Supplemental Dataset 1) and statistically analysed, as
214 described in the methods, to produce a set of genes which are up- or down-regulated in
215 comparison to WT (Supplemental Dataset 2). The number of genes with a significant change
216 relative to WT was surprisingly small for most of the mutants, and only *irx9* had more than a 1.5
217 % change in its transcriptome. The extent of the change essentially followed the severity of the
218 effect of the mutation on the plants gross morphology and development (Figure 1).

219 We confirmed that the tissue selected for analysis was still actively transcribing secondary cell
220 wall synthesis genes. Expression data revealed that in the WT samples, genes associated with
221 secondary cell wall biosynthesis, such as CESA4/IRX5 and IRX9, were all highly expressed
222 (Supplemental Dataset 1).

223 3.2 Analysis of xylan-related gene expression

224 The microarray data confirmed that, for *irx9*, *irx14*, and *gux1* plants, the corresponding mutant
225 gene had the largest decrease relative to WT (Table 1). However, for *irx10* and *gux2*, whilst
226 transcript levels were reduced, they were still clearly present. This is likely due to the production
227 of non-functional transcripts which can still bind to some of the individual probes in a probeset.
228 For example, *irx10* (SALK_046368) has a T-DNA insertion in the middle of the fourth and final
229 exon. Extraction of the raw probe data (Supplemental Dataset 1) reveals only partially reduced
230 intensity for 5' localised probes, but greatly reduced intensity for 3' localised probes. The GUX2
231 open reading frame (ORF) was originally annotated as two different genes, and hence two
232 different probesets can bind to it (here annotated 3' and 5' according to their location within the
233 cDNA).

234 Next, expression of other xylan-synthesis related genes was explored to test whether a xylan
235 synthesis feedback loop could be detected. For example, we hypothesised that if one gene
236 involved in xylan backbone extension had reduced expression e.g. *IRX9*, resulting in reduced
237 enzyme activity, then a close homologue might show compensatory increased expression e.g.
238 *IRX9L*. Alternatively, if xylan elongation was affected, this could lead to an associated repression
239 of downstream biosynthetic steps, such as GUX or glucuronoxylan methyl transferase (GXMT)
240 activity, which might be apparent at the transcriptional level. Neither of these outcomes was
241 visible in the data (Table 1). In *gux1*, *gux2*, *gux1gux2* and *irx10* essentially no significant

Secondary cell wall integrity sensing

242 differences in transcript levels from each other and from WT were observed (Table 1).
243 Specifically, close homologues of the mutant genes did not show compensatory increases of
244 expression e.g. *IRX10L* in *irx10* or *GUX2* in *gux1*. Whilst *irx9* showed a trend towards slight (but
245 not significant) down regulation of xylan synthetic genes, and *irx14* showed a general (but again,
246 not significant) trend for a small increase, we inferred that the plants were likely not exactly
247 developmentally matched. Indeed, the xylan synthesis pathway is co-ordinately regulated with
248 development of the stem (Brown et al., 2005). Since *irx9* and *irx14* had the largest gross
249 morphological difference compared to WT (Figure 1A), matching the harvest developmental
250 stage was the most challenging for these two genotypes. Overall, these data indicate that loss of
251 xylan synthase activity does not result in altered gene expression of other xylan-related genes.

252 One alternative metabolic strategy to compensate for reduced quantities of enzyme is to boost the
253 substrate pool to increase the rate of activity. Therefore, we analysed expression patterns of
254 genes which have a role in synthesizing the UDP-sugars required for xylan biosynthesis.
255 However, this subset of genes (Supplemental Dataset 3) revealed a very similar pattern to that
256 reported for the xylan genes described above: a general small but significant decrease in *irx9*,
257 and small but significant increase in *irx14*. Other mutants were essentially unaffected.

258 **3.3 Pathways which provide substrates for non-sugar decorations of xylan show evidence** 259 **of feedback in response to the reduced product sink.**

260 Xylan has various non-sugar modifications, including acetylation and methylation which impact
261 the functional properties of the molecule. Mutants with short xylan chains (e.g. *irx9*, *irx10*, *irx14*,
262 *irx15*) have an increased proportion of methylated (MeGlcA) versus unmethylated GlcA
263 decorations, although the combined frequency of GlcA/MeGlcA ([Me]GlcA) substitution along
264 the xylan backbone is maintained (Brown et al., 2007; Brown et al., 2011). This could be due to
265 an active up-regulation of methylation activity to compensate for the shorter xylan chains, by
266 altering the association between xylan and the amphiphilic surface of the lignin (Urbanowicz et
267 al., 2014). Alternatively, it could be a passive effect resulting from the reduced pool of GlcA for
268 the GXMT enzymes to act upon (Brown et al., 2007). Therefore, the expression patterns of the S-
269 adenosylmethionine (SAM) cycle, the pathway responsible for recycling the SAM used for xylan
270 GlcA methylation, and Acetyl-CoA (the substrate for TBL29 and other acetyl transferases)
271 synthesis genes were investigated (Supplemental Dataset 3).

272 It has been previously suggested that the *gxmt* mutants accumulate SAM (Urbanowicz et al.,
273 2012), and therefore we might predict that the *gux* mutants would also accumulate SAM. In fact,
274 many of the SAM synthetases show reduced expression in *irx9* and *gux1gux2* implying that this
275 pathway may respond transcriptionally to the reduced requirement for SAM in the Golgi. Since
276 SAM is also the major methyl-donor for key metabolites, such as the hormone ethylene, vitamin
277 B1 and polyamines (Hesse and Hoefgen, 2003), it is perhaps not surprising that levels are tightly
278 controlled.

279 In the *gux* mutants, which lack GlcA, there is an increased amount of mono-acetylation (Chong
280 et al., 2014) and the growth suppression seen in the xylan acetyl transferase mutant, *tbl29*, can be
281 rescued by overexpression of *GUX1* (Xiong et al., 2015), suggesting that increases in levels of
282 xylan decoration could be beneficial to the cell wall. Acetyl-CoA, the precursor for xylan
283 acetylation is synthesised via 2 independent pathways: from pyruvate in the mitochondria and

Secondary cell wall integrity sensing

284 plastid, and from citrate in the cytosol. The acetyl Co-A is then transported from the cytosol into
285 the Golgi, likely by the RWA proteins (Manabe et al., 2011), where it is proposed to acetylate an
286 intermediate (potentially AXY9 (Schultink et al., 2015)) prior to transfer of the acetyl group to
287 the xylan backbone by the TBL29 acetyltransferase (Xiong et al., 2013;Urbanowicz et al., 2014).
288 In our data, the cytosolic pathway was strongly downregulated, particularly in *irx9*, implying that
289 acetyl Co-A synthesized from citrate is the major source of substrate for xylan acetylation, and
290 that the size of the pool can be detected and used to regulate the cytosolic pool size.

291 **3.4 Biosynthetic genes for non-xylan cell wall polysaccharides are unaffected.**

292 If xylan is altered in the plant, we may predict that an alternative hemicellulose such as mannan
293 would be upregulated to compensate. Indeed, in gymnosperms mannan is the dominant
294 hemicellulose, implying that xylan and mannan could be functionally interchangeable. Previously
295 published biochemical analyses of these xylan mutants have shown that major changes to the cell
296 wall are limited to the xylan itself (Brown et al., 2007;Brown et al., 2009;Mortimer et al., 2010),
297 although it is possible that minor changes were not visible in these analyses. However, in
298 agreement with this, transcriptional data showed no significant changes to genes involved in
299 cellulose, mannan, xyloglucan or pectin synthesis, other than in the pattern recorded above that
300 we have ascribed to developmental issues (decrease in *irx9*, small increase in *irx14*)
301 (Supplemental Dataset 3). Taken together, these results suggest that defects in xylan synthesis do
302 not cause major changes to the expression of genes that are involved in polysaccharide
303 biosynthesis.

304 **3.5 Alternative lignin biosynthetic genes are upregulated in the xylan mutants.**

305 Lignin has previously been shown to be deposited in primary cell walls in response to stress,
306 including in cell wall synthesis mutants (Desprez et al., 2002;Cano-Delgado et al., 2003;Hematy
307 et al., 2007), but not in secondary cell wall synthesis mutants. The exception to this is lignin
308 mutants. Mutations in some lignin biosynthetic genes can result in compensatory upregulation of
309 other parts of the lignin biosynthetic pathway, as well as increases in some matrix
310 polysaccharides, likely pectins (Van Acker et al., 2013). A systems approach to understanding
311 phenolic metabolism has revealed how lignin biosynthesis responds to perturbations (Vanholme
312 et al., 2012). At least eleven enzymes, encoded by multiple genes, have been implicated in
313 monolignol biosynthesis, although only a subset are associated with stem lignin biosynthesis
314 (Table 2, marked in bold (Costa et al., 2003;Goujon et al., 2003;Vanholme et al.,
315 2012;Vanholme et al., 2013)).

316 Here, the gene expression analysis in the xylan synthesis mutants (Table 2) showed trends that
317 appear distinct from that seen for other groups of cell-wall related genes (Supplemental Dataset
318 3). In *irx9*, most of the lignin-related genes were significantly down-regulated (Table 2), which is
319 supported by data showing that *irx9* has an overall decrease in lignin (Petersen et al., 2012).
320 *PAL3*, *CCoAOMT3*, *CAD1* and *CAD3* are the exceptions to this, and were significantly
321 upregulated. None of these isoforms are the dominant stem isoforms, implying that a different
322 subset of the lignin pathway may be activated. The other xylan mutants showed milder
323 alterations, with a general trend towards down-regulation of lignin-related genes, except for
324 *CAD3* in *irx10* and *CCoAMT2* in *irx14*, which were upregulated.

Secondary cell wall integrity sensing

325 A potential answer to why lignin deposition seems to be the exception in terms of responsive
326 2CW modifications comes from recent papers which have explored the spatial and temporal
327 nature of lignin deposition in Arabidopsis (Pesquet et al., 2013; Smith et al., 2013) and poplar
328 (Gorzsás et al., 2011). These papers have provided strong evidence for the “good neighbour
329 hypothesis”, in which it has been proposed that neighbouring, non-lignified cells monolignols to
330 lignifying cells (Hosokawa et al., 2001), at least in some cell types. This evolving view of
331 lignification was recently reviewed by Voxeur and colleagues who proposed two models of
332 lignification: cell autonomous lignification (CAL) and non-cell autonomous lignification
333 (NCAL). In their view of cell wall lignification, the role of CAL and NCAL will depend of cell
334 maturation and type. For example, fibre cells are predominantly lignified by CAL and supported
335 by NCAL, whereas vessels are initially lignified by CAL prior to PCD but NCAL is then key to
336 completion of the lignification process (Voxeur et al., 2015).

337 **3.6 Known secondary cell wall associated transcription factors do not respond to changes** 338 **in xylan content or structure.**

339 Expression changes in secondary-wall related transcription factors (TFs) were also evaluated
340 (Supplemental Dataset 3). The pattern obtained was like that we have shown for multiple other
341 sets of genes i.e. in *irx9*, most of these TFs had reduced expression compared to WT, whilst in
342 *irx14* many were slightly but significantly upregulated. Few changes were seen in the other
343 mutants. Importantly, we did not see the strong upregulation of a TF or network of TFs that
344 might form a SCWI response network (Figure 2).

345 **3.7 Receptor Like Kinases (RLKs) transcripts do not show a consistent response to** 346 **secondary cell wall changes.**

347 As described in the introduction, RLKs are the most likely candidates for detection of a loss of
348 cell wall integrity, although due to their number (~600 in Arabidopsis), they have proven to be
349 difficult to study. Data was extracted for all those RLKs on the ATH1 microarray (Supplemental
350 Dataset 3) and searched for RLKs which showed a significant change in expression in at least
351 two of the xylan mutants. However, none of the genes matched these criteria, including those
352 associated with the PCWI pathways (Table 3). It should be noted that unchanged RLK
353 expression does not exclude the possibility of RLK activation, as is seen for PCWI RLKs, such
354 as THE1.

355 **3.8 Abiotic and biotic stress associated genes are upregulated.**

356 There has been limited research on the wider effect of secondary cell wall modifications on plant
357 physiology. Since changes to xylan structure, when severe enough, have a profound effect on
358 plant growth (as in *irx9*), a more general analysis was performed on the transcriptomics data. The
359 aim was to identify other signalling and response pathways which respond to gross cell wall
360 defects. For example, the shorter xylan chains in *irx9*, *irx10* and *irx14* result, to varying extents,
361 in collapsed xylem vessels. This limits water transport and is likely to induce drought stress,
362 which we predict would be detected by abiotic stress response pathways. It was also recently
363 shown that when plants are exposed to severe abiotic stress, expression of specific secondary cell
364 wall TFs are enhanced. For example, under salt stress, VND7 expression expands to non-stele
365 cells in the root, resulting in an extra strand of metaxylem (Taylor-Teeples et al., 2015).

Secondary cell wall integrity sensing

366 In line with our prediction, gene ontology (GO) analysis of genes upregulated in *irx9* revealed an
367 over-representation of genes associated with abiotic and biotic stress responses (Supplemental
368 Figure S2). This pattern was repeated, although to a lesser extent, in the other *irx* mutants, but
369 not in the *gux* mutants, as might be expected because they do not show collapsed xylem
370 (Supplemental Figure S2). Further experiments are now required to exclude whether the stunting
371 of *irx9* is due to the reduced xylan or poor water transport, or as we consider likely, a response to
372 drought signalling hormones resulting in the suppression of growth. For example, it will be
373 interesting to investigate whether *irx9* dwarfing can be suppressed by an ABA-insensitive (ABI)
374 mutant, such as *abi1* (Koornneef and van der Veen, 1980).

375 Indeed, some cell wall mutants have been reported as showing enhanced resistance to drought
376 e.g. *irx14* (Keppler and Showalter, 2010) and *lew2* (a mutation in *CESA8* (Chen et al., 2005)),
377 but also other abiotic stresses such as cold e.g. *esk1/tbl29* (a xylan acetyltransferase (Xin and
378 Browse, 1998;Urbanowicz et al., 2014)). Furthermore, contrary to intuitive assumptions that
379 modified walls will make plant cells more susceptible to pathogen entry, many cell wall mutants
380 show increased resistance to pathogens e.g. (Manabe et al., 2011;Delgado-Cerezo et al., 2012),
381 as has been reviewed recently (Miedes et al., 2014). When investigated in detail, it was
382 demonstrated that the defence response genes were activated via an abscisic acid (ABA)
383 signalling pathway (Hernandez-Blanco et al., 2007). Whilst many hypotheses have been
384 proposed as to why this occurs, our data supports the possibility that the collapsed vessels
385 “prime” the plants’ abiotic stress response, by simulating drought stress.

386 4 Conclusions

387 In this study, we utilised a collection of xylan mutants to establish whether the plant secondary
388 cell wall has a cell wall integrity (SCWI) pathway whose members or consequences could be
389 identified via transcriptomics. Despite using mutants with mild defects (*gux2*) through to mutants
390 with severe defects (*irx9*), there was no transcriptional evidence of a SCWI pathway (Figure 2).
391 This supports previously published biochemical data from secondary cell wall mutants, where,
392 unlike in primary wall mutants, there was no detection of the compensatory deposition of
393 polysaccharides (Ha et al., 2002;Cano-Delgado et al., 2003;Brown et al., 2007).

394 Transcriptomics does not necessarily reflect the dynamics of the cell, and therefore other
395 approaches such as proteomics may be of use in the future to continue to search for the presence
396 of a SCWI. Additionally, we have only investigated a single species in this study. It may be that
397 perennial or monocotyledonous plants show altered responses to secondary cell wall changes
398 (Tan et al., 2015).

399 The secondary cell wall is still a system about which we know little, despite advances over the
400 last decade. In particular, mechanisms of *in muro* polysaccharide modification and salvage are
401 poorly characterised. We also know little about how the characteristics of the different polymers
402 are combined to produce the cell wall functionality. The identification of further cell wall
403 mutants, along with the application of techniques which allow atomic resolution analysis will
404 provide new insights into the mechanics of cell wall strength.

405 We believe that this data provides encouraging support for cell wall engineering approaches,
406 especially for improved biomass properties, since there is not an intrinsic and sensitive

Secondary cell wall integrity sensing

407 regulatory system to overcome. We predict that as long as the plant is able to maintain form and
408 function, e.g. transpiration flow (Petersen et al., 2012;Eudes et al., 2015;Vargas et al., 2016) by
409 maintaining a functional secondary cell wall, it will be possible to manipulate the cell wall to
410 produce biomaterials to suit different agricultural and biomanufacturing needs. Further work will
411 be required to understand the contributions of interactions between individual wall components
412 to form the assembled structure, to allow the predictive design of a functional secondary cell
413 wall.

414 **5 Conflict of Interest**

415 *The authors declare that the research was conducted in the absence of any commercial or*
416 *financial relationships that could be construed as a potential conflict of interest.*

417 **6 Author Contributions**

418 JCM, NFB and PD designed the experiments and wrote the paper. NFB performed the
419 experiments. NFB and JCM analysed the data.

420 **7 Funding**

421 NFB was supported by a PhD studentship from the Portuguese Foundation for Science and
422 Technology. JCM is supported at the Joint BioEnergy Institute by the Office of Science, Office
423 of Biological and Environmental Research, of the U.S. Department of Energy under Contract
424 No. DE-AC02-05CH11231. PD and JCM were supported by BBSRC grant BB/G016240/1
425 BBSRC Sustainable Energy Centre Cell Wall Sugars Programme (BSBEC).

426 **8 Acknowledgments**

427 The authors would like to thank Xiaolan Yu, who took the photograph of the mutants used in this
428 study.

429 **9 References**

- 430 Berger, F., and Carlon, E. (2011). From hybridization theory to microarray data analysis:
431 performance evaluation. *BMC Bioinformatics* 12, 464.
- 432 Blazejczyk, M., Miron, M., and Nadon, R. (2007). FlexArray: A statistical data analysis software
433 for gene expression microarrays. *Génome Québec, Montreal, Canada, URL*
434 <http://genomequebec.mcgill.ca/FlexArray>.
- 435 Bolstad, B.M., Irizarry, R.A., Astrand, M., and Speed, T.P. (2003). A comparison of
436 normalization methods for high density oligonucleotide array data based on variance and
437 bias. *Bioinformatics* 19, 185-193.
- 438 Bonawitz, N.D., Kim, J.I., Tobimatsu, Y., Ciesielski, P.N., Anderson, N.A., Ximenes, E., Maeda,
439 J., Ralph, J., Donohoe, B.S., Ladisch, M., and Chapple, C. (2014). Disruption of Mediator
440 rescues the stunted growth of a lignin-deficient Arabidopsis mutant. *Nature* 509, 376-
441 380.

Secondary cell wall integrity sensing

- 442 Boyes, D.C., Zayed, A.M., Ascenzi, R., Mccaskill, A.J., Hoffman, N.E., Davis, K.R., and
443 Gorlach, J. (2001). Growth stage-based phenotypic analysis of Arabidopsis: a model for
444 high throughput functional genomics in plants. *Plant Cell* 13, 1499-1510.
- 445 Brown, D.M., Goubet, F., Wong, V.W., Goodacre, R., Stephens, E., Dupree, P., and Turner, S.R.
446 (2007). Comparison of five xylan synthesis mutants reveals new insight into the
447 mechanisms of xylan synthesis. *The Plant Journal* 52, 1154-1168.
- 448 Brown, D.M., Wightman, R., Zhang, Z., Gomez, L.D., Atanassov, I., Bukowski, J.P., Tryfona,
449 T., Mcqueen-Mason, S.J., Dupree, P., and Turner, S. (2011). Arabidopsis genes
450 IRREGULAR XYLEM (IRX15) and IRX15L encode DUF579-containing proteins that
451 are essential for normal xylan deposition in the secondary cell wall. *The Plant Journal*
452 66, 401-413.
- 453 Brown, D.M., Zeef, L.A.H., Ellis, J., Goodacre, R., Tuner, S.R. (2005). Identification of novel
454 genes in Arabidopsis involved in secondary cell wall formation using expression
455 profiling and reverse genetics. *The Plant Cell* 17, 2281-2295.
- 456 Brown, D.M., Zhang, Z., Stephens, E., Dupree, P., and Turner, S.R. (2009). Characterization of
457 IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in
458 Arabidopsis. *The Plant Journal* 57, 732-746.
- 459 Brutus, A., Sicilia, F., Macone, A., Cervone, F., and De Lorenzo, G. (2010). A domain swap
460 approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of
461 oligogalacturonides. *Proceedings of the National Academy of Sciences* 107, 9452-9457.
- 462 Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe,
463 D.C., and Fincher, G.B. (2000). Virus-induced silencing of a plant cellulose synthase
464 gene. *Plant Cell* 12, 691-706.
- 465 Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003). Reduced cellulose
466 synthesis invokes lignification and defense responses in Arabidopsis thaliana. *The Plant*
467 *Journal* 34, 351-362.
- 468 Chen, Z., Hong, X., Zhang, H., Wang, Y., Li, X., Zhu, J.K., and Gong, Z. (2005). Disruption of
469 the cellulose synthase gene, AtCesA8/IRX1, enhances drought and osmotic stress
470 tolerance in Arabidopsis. *The Plant Journal* 43, 273-283.
- 471 Chong, S.L., Virkki, L., Maaheimo, H., Juvonen, M., Derba-Maceluch, M., Koutaniemi, S.,
472 Roach, M., Sundberg, B., Tuomainen, P., Mellerowicz, E.J., and Tenkanen, M. (2014).
473 O-acetylation of glucuronoxylan in Arabidopsis thaliana wild type and its change in
474 xylan biosynthesis mutants. *Glycobiology* 24, 494-506.
- 475 Coleman, H.D., Yan, J., and Mansfield, S.D. (2009). Sucrose synthase affects carbon partitioning
476 to increase cellulose production and altered cell wall ultrastructure. *Proceedings of the*
477 *National Academy of Sciences* 106, 13118-13123.
- 478 Costa, M.A., Collins, R.E., Anterola, A.M., Cochrane, F.C., Davin, L.B., and Lewis, N.G.
479 (2003). An in silico assessment of gene function and organization of the phenylpropanoid
480 pathway metabolic networks in Arabidopsis thaliana and limitations thereof.
481 *Phytochemistry* 64, 1097-1112.

Secondary cell wall integrity sensing

- 482 Decreux, A., and Messiaen, J. (2005). Wall-associated kinase WAK1 interacts with cell wall
483 pectins in a calcium-induced conformation. *Plant Cell Physiology* 46, 268-278.
- 484 Delgado-Cerezo, M., Sanchez-Rodriguez, C., Escudero, V., Miedes, E., Fernandez, P.V., Jorda,
485 L., Hernandez-Blanco, C., Sanchez-Vallet, A., Bednarek, P., Schulze-Lefert, P.,
486 Somerville, S., Estevez, J.M., Persson, S., and Molina, A. (2012). Arabidopsis
487 heterotrimeric G-protein regulates cell wall defense and resistance to necrotrophic fungi.
488 *Molecular Plant* 5, 98-114.
- 489 Desprez, T., Vernhettes, S., Fagard, M., Refregier, G., Desnos, T., Aletti, E., Py, N., Pelletier, S.,
490 and Hofte, H. (2002). Resistance against herbicide isoxaben and cellulose deficiency
491 caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant*
492 *Physiology* 128, 482-490.
- 493 Doblin, M.S., Johnson, K.L., Humphries, J., Newbigin, E.J., and Bacic, A. (2014). Are designer
494 plant cell walls a realistic aspiration or will the plasticity of the plant's metabolism win
495 out? *Current Opinions in Biotechnology* 26, 108-114.
- 496 Eudes, A., Sathitsuksanoh, N., Baidoo, E.E., George, A., Liang, Y., Yang, F., Singh, S.,
497 Keasling, J.D., Simmons, B.A., and Loque, D. (2015). Expression of a bacterial 3-
498 dehydroshikimate dehydratase reduces lignin content and improves biomass
499 saccharification efficiency. *Plant Biotechnol Journal*.
- 500 Gondolf, V.M., Stoppel, R., Ebert, B., Rautengarten, C., Liwanag, A.J., Loque, D., and Scheller,
501 H.V. (2014). A gene stacking approach leads to engineered plants with highly increased
502 galactan levels in Arabidopsis. *BMC Plant Biol* 14, 344.
- 503 Gorzsás, A., Stenlund, H., Persson, P., Trygg, J., and Sundberg, B. (2011). Cell-specific
504 chemotyping and multivariate imaging by combined FT-IR microspectroscopy and
505 orthogonal projections to latent structures (OPLS) analysis reveals the chemical
506 landscape of secondary xylem. *The Plant Journal* 66, 903-914.
- 507 Goujon, T., Sibout, R., Eudes, A., Mackay, J., and Jouanin, L. (2003). Genes involved in the
508 biosynthesis of lignin precursors in Arabidopsis thaliana. *Plant Physiology and*
509 *Biochemistry* 41, 677-687.
- 510 Ha, M.A., Mackinnon, I.M., Sturcova, A., Apperley, D.C., Mccann, M.C., Turner, S.R., and
511 Jarvis, M.C. (2002). Structure of cellulose-deficient secondary cell walls from the irx3
512 mutant of Arabidopsis thaliana. *Phytochemistry* 61, 7-14.
- 513 Hamann, T. (2012). Plant cell wall integrity maintenance as an essential component of biotic
514 stress response mechanisms. *Front in Plant Science* 3, 77.
- 515 Hematy, K., Sado, P.E., Van Tuinen, A., Rochange, S., Desnos, T., Balzergue, S., Pelletier, S.,
516 Renou, J.P., and Hofte, H. (2007). A receptor-like kinase mediates the response of
517 Arabidopsis cells to the inhibition of cellulose synthesis. *Current Biology* 17, 922-931.
- 518 Hernandez-Blanco, C., Feng, D.X., Hu, J., Sanchez-Vallet, A., Deslandes, L., Llorente, F.,
519 Berrocal-Lobo, M., Keller, H., Barlet, X., Sanchez-Rodriguez, C., Anderson, L.K.,
520 Somerville, S., Marco, Y., and Molina, A. (2007). Impairment of cellulose synthases
521 required for Arabidopsis secondary cell wall formation enhances disease resistance. *Plant*
522 *Cell* 19, 890-903.

Secondary cell wall integrity sensing

- 523 Hesse, H., and Hoefgen, R. (2003). Molecular aspects of methionine biosynthesis. *Trends in*
524 *Plant Science* 8, 259-262.
- 525 Hosokawa, M., Suzuki, S., Umezawa, T., and Sato, Y. (2001). Progress of lignification mediated
526 by intercellular transportation of monolignols during tracheary element differentiation of
527 isolated *Zinnia mesophyll* cells. *Plant Cell Physiology* 42, 959-968.
- 528 Keppler, B.D., and Showalter, A.M. (2010). IRX14 and IRX14-LIKE, Two Glycosyl
529 Transferases Involved in Glucuronoxylan Biosynthesis and Drought Tolerance in
530 *Arabidopsis*. *Molecular Plant* 3, 834-841.
- 531 Kock, C., Dufrene, Y.F., and Heinisch, J.J. (2015). Up against the Wall: Is Yeast Cell Wall
532 Integrity Ensured by Mechanosensing in Plasma Membrane Microdomains? *Applied*
533 *Environmental Microbiology* 81, 806-811.
- 534 Koornneef, M., and Van Der Veen, J.H. (1980). Induction and analysis of gibberellin sensitive
535 mutants in *Arabidopsis thaliana* (L.) heynh. *Theoretical and Applied Genetics* 58, 257-
536 263.
- 537 Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T.,
538 Fukuda, H., and Demura, T. (2005). Transcription switches for protoxylem and
539 metaxylem vessel formation. *Genes and Development* 19, 1855-1860.
- 540 Levin, D.E. (2011). Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall
541 integrity signaling pathway. *Genetics* 189, 1145-1175.
- 542 Li, X., Chen, W., Zhao, Y., Xiang, Y., Jiang, H., Zhu, S., and Cheng, B. (2013). Downregulation
543 of caffeoyl-CoA O-methyltransferase (CCoAOMT) by RNA interference leads to
544 reduced lignin production in maize straw. *Genetics and Molecular Biology* 36, 540-546.
- 545 Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., Cherk, C.,
546 Marcus, S.E., Somerville, S., Pauly, M., Knox, J.P., Sakuragi, Y., and Scheller, H.V.
547 (2011). Loss-of-function mutation of REDUCED WALL ACETYLATION2 in
548 *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis*
549 *cinerea*. *Plant Physiology* 155, 1068-1078.
- 550 Miedes, E., Vanholme, R., Boerjan, W., and Molina, A. (2014). The role of the secondary cell
551 wall in plant resistance to pathogens. *Front in Plant Science* 5, 358.
- 552 Mortimer, J., Miles, G., Brown, D., Zhang, Z., Segura, M., Weimar, T., Yu, X., Seffen, K.,
553 Stephens, E., Turner, S., and Dupree, P. (2010). Absence of branches from xylan in
554 *Arabidopsis gux* mutants reveals potential for simplification of lignocellulosic biomass.
555 *Proceedings of the National Academy of Sciences of the United States of America* 107,
556 17409-17414.
- 557 Pena, M.J., Zhong, R.Q., Zhou, G.K., Richardson, E.A., O'Neill, M.A., Darvill, A.G., York,
558 W.S., and Ye, Z.H. (2007). *Arabidopsis* irregular xylem8 and irregular xylem9:
559 Implications for the complexity of glucuronoxylan biosynthesis. *Plant Cell* 19, 549-563.
- 560 Pesquet, E., Zhang, B., Gorzsas, A., Puhakainen, T., Serk, H., Escamez, S., Barbier, O., Gerber,
561 L., Courtois-Moreau, C., Alatalo, E., Paulin, L., Kangasjarvi, J., Sundberg, B., Goffner,
562 D., and Tuominen, H. (2013). Non-cell-autonomous postmortem lignification of
563 tracheary elements in *Zinnia elegans*. *Plant Cell* 25, 1314-1328.

Secondary cell wall integrity sensing

- 564 Petersen, P.D., Lau, J., Ebert, B., Yang, F., Verhertbruggen, Y., Kim, J.S., Varanasi, P.,
565 Suttangkakul, A., Auer, M., Loqué, D., and Scheller, H.V. (2012). Engineering of plants
566 with improved properties as biofuels feedstocks by vessel-specific complementation of
567 xylan biosynthesis mutants. *Biotechnology for Biofuels* 5, 1-19.
- 568 Philip, B., and Levin, D.E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity
569 signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1.
570 *Molecular and Cell Biology* 21, 271-280.
- 571 Provart, N., and Zhu, T. (2003). A browser-based functional classification SuperViewer for
572 Arabidopsis genomics. *Currents in Computational Molecular Biology* 2003, 271-272.
- 573 Rennie, E.A., Hansen, S.F., Baidoo, E.E., Hadi, M.Z., Keasling, J.D., and Scheller, H.V. (2012).
574 Three members of the Arabidopsis glycosyltransferase family 8 are xylan
575 glucuronosyltransferases. *Plant Physiology* 159, 1408-1417.
- 576 Schultink, A., Naylor, D., Dama, M., and Pauly, M. (2015). The Role of the Plant-Specific
577 ALTERED XYLOGLUCAN9 Protein in Arabidopsis Cell Wall Polysaccharide O-
578 Acetylation. *Plant Physiology* 167, 1271-1283.
- 579 Shiu, S.H., and Bleecker, A.B. (2003). Expansion of the receptor-like kinase/Pelle gene family
580 and receptor-like proteins in Arabidopsis. *Plant Physiology* 132, 530-543.
- 581 Smith, R.A., Schuetz, M., Roach, M., Mansfield, S.D., Ellis, B., and Samuels, L. (2013).
582 Neighboring parenchyma cells contribute to Arabidopsis xylem lignification, while
583 lignification of interfascicular fibers is cell autonomous. *Plant Cell* 25, 3988-3999.
- 584 Tan, H.-T., Shirley, N.J., Singh, R.R., Henderson, M., Dhugga, K.S., Mayo, G.M., Fincher, G.B.,
585 and Burton, R.A. (2015). Powerful regulatory systems and post-transcriptional gene
586 silencing resist increases in cellulose content in cell walls of barley. *BMC Plant Biology*
587 15, 1-16.
- 588 Taylor-Teeple, M., Lin, L., De Lucas, M., Turco, G., Toal, T.W., Gaudinier, A., Young, N.F.,
589 Trabucco, G.M., Veling, M.T., Lamothe, R., Handakumbura, P.P., Xiong, G., Wang, C.,
590 Corwin, J., Tsoukalas, A., Zhang, L., Ware, D., Pauly, M., Kliebenstein, D.J., Dehesh,
591 K., Tagkopoulos, I., Breton, G., Pruneda-Paz, J.L., Ahnert, S.E., Kay, S.A., Hazen, S.P.,
592 and Brady, S.M. (2015). An Arabidopsis gene regulatory network for secondary cell wall
593 synthesis. *Nature* 517, 571-575.
- 594 Turner, S.R., and Somerville, C.R. (1997). Collapsed xylem phenotype of Arabidopsis identifies
595 mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell* 9, 689-
596 701.
- 597 Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to
598 the ionizing radiation response. *Proceedings of the National Academy of Sciences* 98,
599 5116-5121.
- 600 Urbanowicz, B.R., Pena, M.J., Moniz, H.A., Moremen, K.W., and York, W.S. (2014). Two
601 Arabidopsis proteins synthesize acetylated xylan in vitro. *The Plant Journal* 80, 197-206.
- 602 Urbanowicz, B.R., Pena, M.J., Ratnaparkhe, S., Avci, U., Backe, J., Steet, H.F., Foston, M., Li,
603 H., O'Neill, M.A., Ragauskas, A.J., Darvill, A.G., Wyman, C., Gilbert, H.J., and York,
604 W.S. (2012). 4-O-methylation of glucuronic acid in Arabidopsis glucuronoxylan is

Secondary cell wall integrity sensing

- 605 catalyzed by a domain of unknown function family 579 protein. *Proceedings of the*
606 *National Academy of Sciences* 109, 14253-14258.
- 607 Van Acker, R., Vanholme, R., Storme, V., Mortimer, J.C., Dupree, P., and Boerjan, W. (2013).
608 Lignin biosynthesis perturbations affect secondary cell wall composition and
609 saccharification yield in *Arabidopsis thaliana*. *Biotechnology for Biofuels* 6, 46.
- 610 Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., Kim, H., Cross, J.,
611 Morreel, K., Araujo, P., Welsh, L., Haustraete, J., McClellan, C., Vanholme, B., Ralph, J.,
612 Simpson, G.G., Halpin, C., and Boerjan, W. (2013). Caffeoyl shikimate esterase (CSE) is
613 an enzyme in the lignin biosynthetic pathway in *Arabidopsis*. *Science* 341, 1103-1106.
- 614 Vanholme, R., Storme, V., Vanholme, B., Sundin, L., Christensen, J.H., Goeminne, G., Halpin,
615 C., Rohde, A., Morreel, K., and Boerjan, W. (2012). A systems biology view of
616 responses to lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell* 24, 3506-3529.
- 617 Vargas, L., Cesarino, I., Vanholme, R., Voorend, W., De Lyra Soriano Saleme, M., Morreel, K.,
618 and Boerjan, W. (2016). Improving total saccharification yield of *Arabidopsis* plants by
619 vessel-specific complementation of caffeoyl shikimate esterase (cse) mutants.
620 *Biotechnology for Biofuels* 9, 139.
- 621 Vermerris, W., and Abril, A. (2014). Enhancing cellulose utilization for fuels and chemicals by
622 genetic modification of plant cell wall architecture. *Current Opinion in Biotechnology*
623 32C, 104-112.
- 624 Voxeur, A., and Hofte, H. (2016). Cell wall integrity signaling in plants: “To grow or not to
625 grow that’s the question”. *Glycobiology* 26, 950-960.
- 626 Voxeur, A., Wang, Y., and Sibout, R. (2015). Lignification: different mechanisms for a versatile
627 polymer. *Current Opinion in Plant Biology* 23, 83-90.
- 628 Wilkerson, C.G., Mansfield, S.D., Lu, F., Withers, S., Park, J.Y., Karlen, S.D., Gonzales-Vigil,
629 E., Padmakshan, D., Unda, F., Rencoret, J., and Ralph, J. (2014). Monolignol ferulate
630 transferase introduces chemically labile linkages into the lignin backbone. *Science* 344,
631 90-93.
- 632 Wu, A.M., Rihouey, C., Seveno, M., Hörnblad, E., Singh, S.K., Matsunaga, T., Ishii, T.,
633 Lerouge, P., Marchant, A. (2009). The *Arabidopsis* IRX10 and IRX10-LIKE
634 glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell
635 wall formation. *The Plant Journal* 57, 718–731.
- 636 Wu, A.M., Hornblad, E., Voxeur, A., Gerber, L., Rihouey, C., Lerouge, P., and Marchant, A.
637 (2010). Analysis of the *Arabidopsis* IRX9/IRX9-L and IRX14/IRX14-L pairs of
638 glycosyltransferase genes reveals critical contributions to biosynthesis of the
639 hemicellulose glucuronoxylan. *Plant Physiology* 153, 542-554.
- 640 Wu, Z., and Irizarry, R.A. (2004). Preprocessing of oligonucleotide array data. *Nature*
641 *Biotechnology* 22, 656-658; author reply 658.
- 642 Xin, Z., and Browse, J. (1998). Eskimo1 mutants of *Arabidopsis* are constitutively freezing-
643 tolerant. *Proceedings of the National Academy of Sciences* 95, 7799-7804.

Secondary cell wall integrity sensing

- 644 Xiong, G., Cheng, K., and Pauly, M. (2013). Xylan O-acetylation impacts xylem development
 645 and enzymatic recalcitrance as indicated by the Arabidopsis mutant *tbl29*. *Molecular*
 646 *Plant* 6, 1373-1375.
- 647 Xiong, G., Dama, M., and Pauly, M. (2015). Glucuronic Acid Moieties on Xylan Are
 648 Functionally Equivalent to O-Acetyl-Substituents. *Molecular Plant* 8, 1119-1121.
- 649 Yang, J., Chen, F., Yu, O., and Beachy, R.N. (2011). Controlled silencing of 4-coumarate:CoA
 650 ligase alters lignocellulose composition without affecting stem growth. *Plant Physiology*
 651 *and Biochemistry* 49, 103-109.
- 652 Zhong, R., Lee, C., Zhou, J., Mccarthy, R.L., and Ye, Z.H. (2008). A battery of transcription
 653 factors involved in the regulation of secondary cell wall biosynthesis in Arabidopsis.
 654 *Plant Cell* 20, 2763-2782.

655

656 10 Figure Legends

657 **Figure 1:** Xylan mutants used in this study. (A) Images of mutant plants used in this study. Scale
 658 bar = 20 mm. (B) Diagram representing the effects of these mutations on xylan structure.
 659 Acetylation and the reducing end oligosaccharide on the xylan molecule is not shown for clarity.
 660 Pentagons = xylose, hexagons = GlcA, triangles and circles = methyl groups. (C) Number of
 661 differentially expressed genes in each genotype compared to WT (fold change < 0.5 or > 2.0, *p*-
 662 value ≤ 0.05).

663 **Figure 2:** Scheme showing a cascade of transcription factors responsible for regulating cellulose
 664 and xylan biosynthesis in Arabidopsis secondary cell walls. Proposed regulatory feedback
 665 pathways which would induce a SCWI response are shown as dotted lines. Our work does not
 666 support their existence, as indicated by the red crosses.

667 11 Tables

668 **Table 1:** Expression of xylan synthesis-related genes represented on the Affymetrix ATH1 gene
 669 chip in relation to WT. Values are shown as log₂(abundance in mutant/abundance in WT).
 670 Values have been shaded red to indicate increased expression with respect to WT and blue to
 671 indicate decreased expression. Values in bold represent genes significantly different from WT.
 672 Grey shaded boxes indicate the mutant gene. GUX2 has two values corresponding to two
 673 probesets (earlier genome annotations assigned this region to two separate ORFs).

674

Gene name	AGI	<i>irx9</i>	<i>irx10</i>	<i>irx14</i>	<i>gux1</i>	<i>gux2</i>	<i>gux1gux2</i>
Reducing End Oligosaccharide							
<i>IRX7/FRA8</i>	AT2G28110	-1.21	-0.1	0.46	-0.05	0.01	-0.26

Secondary cell wall integrity sensing

<i>IRX8</i>	AT5G54690	-1.5	-0.02	0.73	-0.07	-0.05	-0.16
<i>PARVUS</i>	AT1G19300	-2	0	0.75	0.02	0.09	-0.31

Elongation

<i>IRX9</i>	AT2G37090	-7.82	-0.02	0.7	0.06	0.11	0.14
<i>IRX10</i>	AT1G27440	-1.9	-1.29	0.92	0.08	0.14	-0.18
<i>IRX14</i>	AT4G36890	-1.41	-0.25	-6.62	-0.17	0.01	-0.21
<i>IRX9-L</i>	AT1G27600	-0.16	-0.14	-0.28	-0.14	-0.13	-0.21
<i>IRX10-L</i>	AT5G61840	-0.63	-0.16	0.32	-0.04	0.05	-0.09
<i>IRX14-L</i>	AT5G67230	-0.37	-0.02	0.28	-0.2	-0.16	0.09

Substitution

<i>GUX1</i>	AT3G18660	-1.96	-0.19	0.69	-7.26	0.14	-7.18
<i>GUX2 5'/3'</i>	AT4G33330	-1.98/- 1.67	-0.16/- 0.3	1.56/ 1.45	-0.15/- 0.39	-0.73/- 1.25	-0.48/- 1.45
<i>GUX3</i>	AT1G77130	0.06	0.02	-0.38	0.2	0.14	-0.06

Methylation

<i>GXM1</i>	AT1G33800	-2.37	-0.19	0.55	-0.12	-0.14	0
<i>GXM2</i>	AT4G09990	-2.39	-1.3	-0.29	-0.33	-0.16	-0.83
<i>GXM3</i>	AT1G09610	-2.7	-0.28	0.35	-0.05	0.07	-0.34

Acetylation

Secondary cell wall integrity sensing

<i>RWA1</i>	AT5G46340	-1.59	-0.36	0.48	-0.11	0.05	-0.09
<i>RWA3</i>	AT2G34410	-2.22	-0.29	0.39	-0.18	-0.19	-0.32
Others							
<i>IRX15</i>	AT3G50220	-1.56	-0.03	0.88	0.13	0.2	-0.06
<i>IRX15-L</i>	AT5G67210	-1.87	-0.36	0.39	0.03	0.05	-0.16
<i>F8H/IRX7-L</i>	AT5G22940	-0.33	-0.03	0.17	-0.04	-0.02	-0.06
<i>DUF579</i>	AT1G71690	0	0.02	0.02	-0.19	-0.07	-0.14

675

676 **Table 2:** Expression of lignin biosynthetic genes represented on the ATH1 chip in relation to
 677 WT. Values are shown as $\log_2(\text{abundance in mutant}/\text{abundance in WT})$. Values have been
 678 shaded red to indicate increased expression with respect to WT and blue to indicate decreased
 679 expression. Values in bold represent genes significantly different from WT. Gene names/AGI in
 680 bold represent genes which are responsible for the majority of stem lignin biosynthesis.

Gene name	AGI	<i>irx9</i>	<i>irx10</i>	<i>irx14</i>	<i>gux1</i>	<i>gux2</i>	<i>gux1gux2</i>
<i>PAL1</i>	AT2G37040	-1.83	-0.4	-0.1	-0.02	0.04	-0.38
<i>PAL2</i>	AT3G53260	-1.08	-0.36	-0.07	-0.05	-0.02	-0.25
<i>PAL3</i>	AT5G04230	2.21	0.57	-0.94	0.06	-0.01	-0.42
<i>PAL4</i>	AT3G10340	-3.6	-0.72	-0.77	-0.03	0.1	-0.66
<i>C4H</i>	AT2G30490	-1.48	-0.43	-0.22	-0.07	0.01	-0.35
<i>4CL1</i>	AT1G51680	-1.51	-0.38	-0.28	0.01	0.04	-0.38
<i>4CL2</i>	AT3G21240	-0.76	-0.04	0.01	-0.06	0	-0.3
<i>4CL3</i>	AT1G65060	0.04	0	-0.11	-0.14	0.04	-0.02

Secondary cell wall integrity sensing

<i>HCT</i>	AT5G48930	-1.06	-0.31	-0.09	-0.11	-0.09	-0.29
<i>C3H</i>	AT2G40890	-1.83	-0.63	-0.35	-0.15	-0.05	-0.48
<i>CSE</i>	AT1G52760	-1.05	-0.26	-0.16	-0.08	-0.02	-0.27
<i>CCoAOMT1</i>	AT4G34050	-0.94	-0.18	0.14	-0.08	-0.02	-0.16
<i>CCoAOMT2</i>	AT4G26220	-1.42	0.11	1.29	0.06	-0.05	0
<i>CCoAOMT3</i>	AT1G67980	1.7	0.52	0.03	-0.11	-0.07	-0.05
<i>CCoAOMT4</i>	AT1G67990	-0.24	0.1	-0.01	-0.04	0.1	-0.16
<i>CCoAOMT5</i>	AT1G24735	-0.32	-0.4	-0.33	-0.35	-0.39	-0.28
<i>CCR1/IRX4</i>	AT1G15950	-0.76	-0.21	0.22	-0.19	-0.11	-0.21
<i>CCR2</i>	AT1G80820	0.31	-0.32	-0.21	-0.25	-0.42	-0.36
<i>F5H1</i>	AT4G36220	-1.2	-0.44	-0.38	-0.04	0.08	-0.67
<i>F5H2</i>	AT5G04330	-1.64	-0.92	-1.05	-0.35	-0.23	-0.54
<i>COMT1</i>	AT5G54160	-0.89	-0.08	0.25	0.03	0.05	-0.14
<i>COMT2</i>	AT1G21100	-0.17	-0.1	-0.16	-0.2	-0.08	-0.09
<i>COMT5</i>	AT3G53140	-0.09	-0.13	0.04	-0.02	-0.03	-0.12
<i>COMT6</i>	AT5G53810	-0.02	-0.05	-0.02	-0.01	0	-0.03
<i>CAD1</i>	AT1G72680	1.33	0.21	-0.07	-0.09	0.14	0.01
<i>CAD3</i>	AT2G21890	2.37	1.36	-0.03	0.03	0.12	0.13
<i>CAD4</i>	AT3G19450	-2.05	-0.48	0.06	-0.04	-0.05	-0.46
<i>CAD5</i>	AT4G34230	-0.49	-0.08	0.2	-0.06	-0.03	-0.15

Secondary cell wall integrity sensing

<i>CAD6</i>	AT4G37970	-0.16	-0.11	-0.12	-0.11	-0.29	-0.1
<i>CAD7</i>	AT4G37980	-0.04	-0.18	-0.17	-0.11	-0.17	-0.08
<i>CAD8</i>	AT4G37990	0.22	-0.05	-0.11	-0.04	-0.09	0.01
<i>CAD9</i>	AT4G39330	-1.69	-0.4	0.2	0.12	0.26	0.29
<i>LAC4</i>	AT2G38080	-1.78	-0.21	0.21	-0.01	-0.01	-0.43
<i>LAC17</i>	AT5G60020	-2.43	0.32	1.2	0.23	0.14	-0.02

681

682 **Table 3:** Expression of RLK and other genes implicated in the PCWI response in relation to WT.
 683 Values are shown as $\log_2(\text{abundance in mutant}/\text{abundance in WT})$. Values have been shaded red
 684 to indicate increased expression with respect to WT and blue to indicate decreased expression.
 685 Values in bold represent genes significantly different from WT. Note that BZR1 and BZR2 bind
 686 to the same set of oligonucleotide probes.

Gene name	AGI	<i>irx9</i>	<i>irx10</i>	<i>irx14</i>	<i>gux1</i>	<i>gux2</i>	<i>gux1gux2</i>
<i>BAK1</i>	AT4G33430	-0.07	-0.10	0.052	0.024	0.12	0.016
<i>BRI1</i>	AT4G39400	-0.48	-0.20	-0.09	-0.03	-0.08	-0.09
<i>HERK1</i>	AT3G46290	-0.27	0.01	-0.08	0.09	0.00	0.00
<i>HERK2</i>	AT1G30570	-0.11	0.10	0.00	0.16	0.14	0.07
<i>THE</i>	AT5G54380	-0.35	-0.07	0.20	-0.01	-0.16	-0.27
<i>FER</i>	AT3G51550	0.19	0.09	0.07	0.00	-0.02	-0.10
<i>MCA1</i>	AT4G35920	-1.32	-0.13	0.33	-0.16	-0.08	-0.16
<i>BIN2</i>	AT4G18710	0.02	0.04	-0.06	-0.10	-0.05	-0.14
<i>BZR1/BZR2</i>	AT1G75080/ AT1G19350	-0.46	-0.22	-0.11	-0.11	-0.15	-0.05

Secondary cell wall integrity sensing

687

688 **12 Supplemental Data**

689 **Supplemental DataSet 1:** This excel file contains 3 worksheets. (1) All GCRMA normalized
690 data (2) Examples of expression of some secondary cell wall synthesis genes in individual
691 samples, to demonstrate that we harvested material actively transcribing the genes of interest (3)
692 Extracted data for probeset 264493_at for IRX10 (At1g27440), showing the differential response
693 of different oligonucleotide probes in the *irx10* mutant sample, depending on the position of the
694 probe.

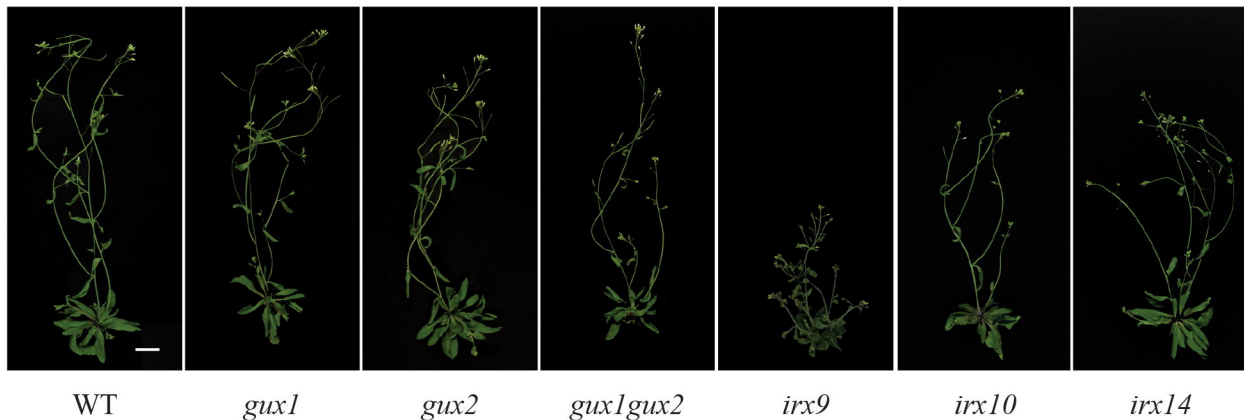
695 **Supplemental Dataset 2:** Lists of genes which are significantly up or down regulated in each
696 mutant.

697 **Supplemental Dataset 3:** Extracted gene expression data for genes known to be involved in the
698 synthesis or regulation of the following secondary cell wall biosynthetic processes: UDP-sugars,
699 S-adenosylmethionine, acetyl Co-A, cellulose, mannan, xyloglucan, pectin, arabinogalactan
700 proteins and transcription factors.

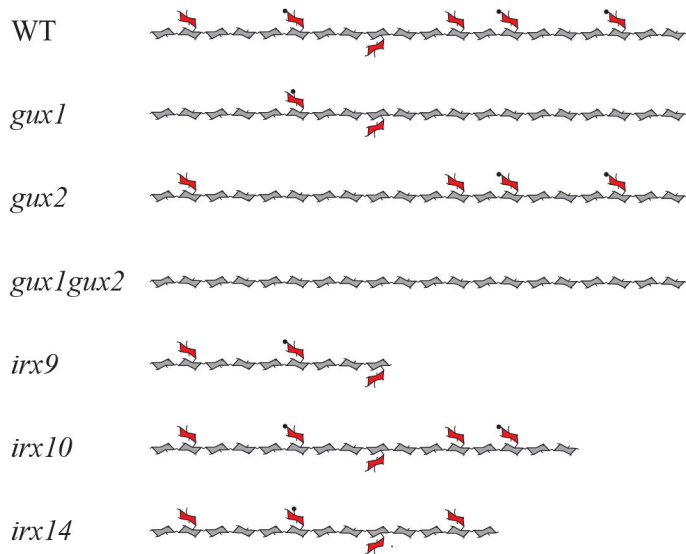
701 **Supplemental Figure 1:** PCR to confirm that all T-DNA lines used in this study were
702 homozygous for the insertion of interest. g/g = amplification of DNA using gene-gene specific
703 primers. g/i = amplification of DNA using gene-insert specific primers, where the insert specific
704 primer is the left border of the T-DNA insertion. Blank = water was used in place of DNA in the
705 PCR reaction.

706 **Supplemental Figure 2:** GO analysis of xylan mutants. (A) *irx9* (B) *irx10* (C) *irx14* (D) *gux1*
707 (E) *gux2* (F) *gux1gux2*. The Classification Superviewer tool from the Bio-Analytic Resource for
708 Plant Biology was used. Data for each category was normalised to the number of times it
709 appeared in the GO analysis divided by the total number of genes assigned to that category.

A



B



C

