

1 Title: Auxin and pectin remodeling interplay during rootlet emergence in white
2 lupin

3 François Jobert ^{1,2}, Alexandre Soriano ¹, Laurent Brottier ¹, Célia Casset ¹, Fanchon Divol ¹, Josip Safran ³,
4 Valérie Lefebvre ³, Jérôme Pelloux ³, Stéphanie Robert ^{2*}, Benjamin Péret ^{1*}.

5 Affiliations:

6 ¹ BPMP, Univ Montpellier, CNRS, INRAE, Supagro, Montpellier, France

7 ² Umeå Plant Science Centre (UPSC), Department of Forest Genetics and Plant Physiology, Swedish
8 University of Agricultural Sciences, Umeå, Sweden

9 ³ UMR INRAE 1158 BioEcoAgro, BIOPI Biologie des Plantes et Innovation, SFR Condorcet FR CNRS 3417,
10 Université de Picardie, Amiens, France

11 *For correspondence: stephanie.robert@slu.se; benjamin.peret@cnrs.fr

12 ORCID:

13 François Jobert: 0000-0002-1939-2214

14 Laurent Brottier: 0000-0002-8584-2199

15 Josip Safran: 0000-0003-0582-7528

16 Valérie Lefebvre: 0000-0003-4482-3810

17 Jérôme Pelloux: 0000-0002-9371-1711

18 Stéphanie Robert: 0000-0002-0013-3239

19 Benjamin Péret: 0000-0003-1336-0796

20 Contributions:

21 F.J. performed most of the experiments and analyzed results. A.S and F.J. analyzed RNAseq data, L.B.
22 designed the binary vector pK7m24GW_CR for hairy root phenotyping experiments, C.C. and F.D.
23 sampled and prepared RNA libraries for sequencing, J.S. generated *in silico* PG models, V.L. performed
24 oligogalacturonide dosages, F.J., J.P. and V.L. analyzed OG dosages, F.J., S.R and B.P. designed the
25 research, F.J, S.R and B.P. wrote the article.

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36

37 Abstract

38 Secondary root emergence is a crucial trait that shapes the plant's underground system. Virtually every
39 developmental step of root primordium morphogenesis is controlled by auxin. However, how the
40 hormone controls cell separation in primordium-overlaying tissues through wall loosening is poorly
41 understood. Here, we took advantage of white lupin and its spectacular cluster root development to
42 assess the contribution of auxin to this process. We show that auxin's positive role on rootlet
43 emergence is associated with an upregulation of cell wall pectin modifying and degrading genes.
44 Downregulation of a pectinolytic enzyme gene expressed in cells surrounding the primordium resulted
45 in delayed emergence. Pectins were demethylesterified in the emergence zone and auxin treatment
46 further enhanced this effect. Additionally, we report specific rhamnogalacturonan-I modifications during
47 cortical cell separation. In conclusion, we propose a model in which auxin has a dual role during rootlet
48 emergence: Firstly, through active pectin demethylesterification and secondly by regulating the
49 expression of cell wall remodeling enzymes.

50

51 Introduction

52 Climate change and extensive use of modern agricultural systems has brought to light alarming issues
53 for crop production. In addition to weather hazards, farmers must deal with impoverished soils while
54 maintaining a sustainable yield. Thus, plants need to be carefully selected based on their ability to cope
55 with specific stresses. White lupin (*Lupinus albus*) is a nitrogen-fixing annual plant from the Leguminosae
56 family that remarkably thrives on phosphorus poor soils. As an adaptation to low phosphate conditions,
57 white lupin grows specialized root structures called cluster roots. These secondary roots almost
58 synchronously develop hundreds of rootlets, defined by their short life and determinate growth in a
59 brush-like structure (Vance *et al.*, 2003). Rootlet morphogenesis in lupin differs from the simplified,
60 however peculiar, lateral root development of *Arabidopsis thaliana*. In contrast to *Arabidopsis*, where
61 the lateral root primordium derives exclusively from mitotically activated pericycle cells, several tissue
62 layers can contribute to the lupin rootlet primordium (Gallardo *et al.*, 2018), which is in common with
63 many angiosperms (Xiao *et al.*, 2019). Rootlet primordia cross these barriers successfully without
64 damaging the outer tissues despite their large number and proximity.

65 During the growth of the rootlet primordium, the surrounding cells are subjected to morphological
66 adjustments including cell division and detachment. Yet, the plant cell wall is an obstacle to the latter,
67 acting as a glue between cells. The cell wall is composed of polysaccharides including cellulose,
68 hemicellulose and pectins together with structural proteins. This dynamic "frame-like" structure
69 provides support and protection but is flexible enough to accommodate the cell fate. The
70 phytohormone auxin is one of the factors regulating wall loosening, notably through the modification of
71 gene expression (Majda *et Robert*, 2018). During *Arabidopsis* lateral root emergence, an auxin maximum
72 is generated by the sequential activation of the auxin efflux carrier PIN-FORMED3 (PIN3) and influx
73 carrier AUXIN TRANSPORTER-LIKE PROTEIN 3 (LAX3) in the primordium-overlaying cells (Swarup *et al.*,

74 2008, Péret *et al.*, 2013). This auxin sink formation induces the expression of the cell wall remodeling
75 enzymes *XTH23/XYLOGLYCAN ENDOTRANSGLYCOSYLASE6 (XTR6)* and *POLYGALACTURONASE INVOLVED*
76 *IN LATERAL ROOT (PGLR)* (Swarup *et al.*, 2008), two carbohydrate-active enzymes affecting wall integrity
77 and reducing cell adhesion. The INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide and its
78 receptor-like kinases HAESA/HAESA-LIKE2 (HAE/HSL2) belong to this auxin-dependent network and
79 regulate the expression of *XTR6* and *PGLR* through a recently identified MITOGEN-ACTIVATED PROTEIN
80 KINASE cascade (Kumpf *et al.*, 2013, Zhu *et al.*, 2019). Cell wall remodeling enzyme activity can be
81 modulated by REACTIVE OXYGEN SPECIES (ROS)-mediated cell wall acidification, as a consequence of the
82 spatial activation of *RESPIRATORY BURST OXIDASE HOMOLOG (RBOH)* genes by auxin (Orman-Liztega *et*
83 *al.*, 2016). In addition, mutants with altered cell wall composition show modified root architecture
84 (Roycewicz and Malamy, 2014) and the mechanical properties of overlaying tissues influence lateral root
85 primordium development (Lucas *et al.*, 2013). A recent report revealed the importance of pectin
86 homogalacturonan methylesterification to regulate pre-branching site formation along the *Arabidopsis*
87 primary root (Wachsman *et al.*, 2020). The authors also suggest that methylesterification in primordium-
88 overlaying cell walls could play a role in facilitating their emergence. However, the modifications of cell
89 wall chemistry and their consequences during lateral root emergence are still unclear.

90 Taking advantage of white lupin cluster roots and their perfectly synchronized rootlet emergence, we
91 assessed the contribution of auxin to cell wall modifications during this process. In this study, we found
92 a positive effect of auxin on rootlet emergence with transcriptomic signatures associated with oxidative
93 stress, transcriptional regulation and cell wall remodeling. Downregulation of an auxin-responsive
94 polygalacturonase gene expressed in the cell layer surrounding the rootlet primordium delayed
95 emergence, suggesting the relevance of localized pectin degradation. Interestingly, homogalacturonans,
96 the known target of polygalacturonases, were strongly demethylesterified in the emergence zone with
97 auxin enhancing this modification. Yet, the methylesterification degree of homogalacturonan did not
98 vary in cortical cells whether challenged by an emerging rootlet primordium or not. Intriguingly,
99 rhamnogalacturonan-I (1,4)- β -D-galactans and extensin glycoproteins showed differential distribution
100 and could be a hallmark of cell separation and/or mechanically challenged cells. In summary, we suggest
101 a model in which auxin acts before rootlet primordium emergence by reducing methylesterification of
102 pectins and thus priming the cell wall for the sequential action of pectin degradation enzymes.

103

104 Results

105 Rootlet primordium emergence is positively regulated by auxin

106 Auxin is a central regulator in lateral root primordium initiation and emergence in higher plants (Du *et*
107 *Scheres*, 2018). In white lupin, the natural auxin indole-3-acetic acid (IAA) has been shown to induce the
108 formation of cluster roots under phosphate deficiency (Neumann *et al.*, 2000, Meng *et al.*, 2012). To
109 understand the precise contribution of auxin in the white lupin rootlet emergence process, we tested
110 the effect of IAA on cluster root development. IAA exogenous application modified the root system
111 architecture quite drastically (Fig. 1a, 1b, Extended Data Fig. 1). Primary and secondary root elongation,
112 including that of specialized cluster roots, was reduced in a dose dependent manner from 1 μ M IAA with
113 a concomitant decrease in rootlet primordium number, while the overall rootlet density was unchanged
114 (Fig. 1b). We assigned the pre-emerged rootlet primordia to seven developmental stages (Gallardo *et*

115 *al.*, 2018). The analysis of rootlet primordium stage distribution revealed that 70% were emerged at the
116 highest concentration of IAA compared to only 30% in the control conditions (Fig. 1c). Accordingly, less
117 primordia were found in stages I to VI in IAA treated plants compared to control plants. Overall, these
118 data suggest that IAA modifies the lupin root architecture by promoting primordium emergence in a
119 dose dependent fashion. To further understand how auxin influences lupin root architecture, we also
120 challenged the plants with naphthylphthalamic acid (NPA) (Abas *et al.*, 2021) and 1-naphthoxyacetic acid
121 (1-NOA) (Lankova *et al.*, 2010), auxin efflux and influx inhibitors respectively (Fig. 1a, Extended Data Fig.
122 1). NPA treatment strongly decreased rootlet number and density (Fig. 1b) and delayed rootlet
123 primordium emergence (Fig. 1c). Inhibition of auxin influx machinery with 1-NOA had a mild negative
124 effect on rootlet number but did not affect rootlet density (Fig. 1b). However, at 25 μ M 1-NOA,
125 emergence was delayed as shown by a higher number of primordia in the intermediate stages V-VI (Fig.
126 1c). Overall, these data demonstrate an essential role of polar auxin transport in rootlet primordium
127 morphogenesis while auxin uptake has a more specific role during primordium emergence. Auxin
128 modulates the expression of a plethora of downstream target genes. To determine the extent of auxin
129 transcriptional responses during rootlet emergence, the *DR5::nlsYFP* synthetic auxin reporter was
130 expressed transiently in lupin hairy roots. A strong activation of *DR5::nlsYFP* expression was found in the
131 pericycle cell layer of cluster roots (Fig. 1d). We also detected a weak *DR5::nlsYFP* signal at the tip of the
132 rootlet primordium starting from stage V. These results suggest that auxin transcriptional responses in
133 secondary root primordia are mostly conserved between lupin and *Arabidopsis*.

134 **Auxin-treated cluster root transcriptome identifies intense cell wall remodeling occurring during** 135 **rootlet emergence**

136 To better understand the effect of auxin on rootlet primordium emergence (Fig. 1c), we performed RNA
137 sequencing on cluster root segments corresponding to the rootlet emergence zone (Fig. 2a). We
138 collected samples treated with IAA at early (30 min, 1 and 2 hours) and late time points (6, 12, 24 and 48
139 hours) after treatment to assess the corresponding auxin-induced transcriptomic regulation. In total,
140 856 differentially expressed genes (DEG) compared to control treatment were detected and classified
141 into seven hierarchical clusters based on their expression patterns (Fig. 2b, Supplementary Table 1).
142 Three expression clusters gathered most of the DEG with 248 (cluster 1), 317 (cluster 2) and 141 (cluster
143 3) DEG and could be defined by distinct transcriptomic signatures (Fig. 2c). The remaining DEG were
144 grouped into four minor clusters showing oscillating responses (Extended Data Fig. 2). We then
145 conducted a gene ontology enrichment analysis to enable the functional interpretation of those clusters
146 (Fig. 2c, Extended data Fig. 2, Supplemental Table 2). Cluster 1 included genes that were slightly
147 upregulated by auxin in the early phase and then downregulated at the late time points. Genes in this
148 cluster were mainly associated with oxidative stress-related processes with the identification of several
149 putative peroxidase encoded genes. Interestingly, ROS production and signaling have been recently
150 proposed as positive regulators of lateral root emergence in *Arabidopsis* (Manzano *et al.*, 2014, Orman-
151 Lizaga *et al.*, 2016). Cluster 2 was assigned to early auxin upregulated genes for which the expression
152 peaked after 30 minutes of treatment and returned gradually to a low level after that (Fig. 2c). Those
153 early-auxin induced genes were associated to processes such as defense responses and DNA-binding
154 transcription factor activity. Cluster 3 differed by its expression and was defined by genes showing a late
155 and steady auxin-induced upregulation starting after two hours of treatment (Fig. 2c). Remarkably, this
156 cluster was enriched in genes with predicted roles in carbohydrate metabolic process, lyase and
157 polygalacturonase activities. This suggests a prominent action of auxin on quick but transient

158 transcriptional responses, rapid modulation of oxidative stress and most importantly a slow but durable
159 cell wall remodeling mechanism occurring during rootlet emergence.

160 **Pectin remodeling genes show specific expression pattern during rootlet emergence**

161 Cell wall properties of primordium-overlying tissues greatly impact the emergence process. Previous
162 studies discovered a large subset of cell wall remodeling genes involved in lateral root emergence in
163 *Arabidopsis*. We established a list of orthologs for important known regulators of cell wall loosening
164 during lateral root morphogenesis including the closest lupin orthologs of *EXPANSIN A1* (*EXPA1* -
165 *At1g69530*, Ramakrishna *et al.*, 2019), *EXPANSIN A17* (*EXPA17* - *At4g01630*, Lee et Kim, 2013),
166 *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6* (*XTR6/XTH23* - *At4g25810*, Swarup *et al.*, 2008),
167 homogalacturonan degrading enzymes *POLYGALACTURONASE LATERAL ROOT* (*PGLR* - *At5g14650*,
168 Kumpf *et al.*, 2013) and *PECTIN LYASE A2* (*PLA2* - *At1g67750*, Swarup *et al.*, 2008) and two
169 homogalacturonan modifying enzymes *PECTIN METHYLESTERASE 3* and *PECTIN METHYLESTERASE*
170 *INHIBITOR 9* (*PME3* - *At3g14310* and *PMEI9* - *At1g62770*, Hocq *et al.*, 2017a) (Extended Data Fig. 3a).
171 Next, we assessed the expression patterns of the selected candidates in lupin hairy roots transformed
172 with promoter::GUS fusion constructs (Extended Data Fig. 3b and Extended Data Fig. 4). Some of the
173 selected candidate genes that were not identified in our transcriptomic dataset displayed overlapping
174 gene expression patterns. *LaXTR1* and *LaPME1* were expressed in the cluster root tip and rootlet
175 primordium, while expression of *LaEXP1* and *LaPMEI1* were restricted to the primordium base and
176 pericycle cells (Extended Data Fig. 3b). Interestingly, *proLaPMEI2::GUS* signal was found in the pericycle,
177 endodermis and inner cortex surrounding rootlet primordia. In order to better understand the link
178 between auxin and cell wall modification during rootlet emergence, we analyzed the expression pattern
179 of additional auxin responsive genes present in cluster 3 (Extended Data Fig. 4). *O-GLYCOSYL*
180 *HYDROLASE FAMILY 17L* (*LaOGH17L*) and *TRICHOME BIREFRINGENCE-LIKE 1* (*LaTBRL1*) were expressed
181 in the cluster root at the base of the rootlet primordium and in the elongation zone (visible in stages VII-
182 VIII). *PECTIN LYASE-LIKE 1* (*LaPLL1*) was expressed specifically in young vascular tissues (stages V –
183 onward). *ProLaPLL2::GUS* displayed a broad signal in the meristematic zone of the cluster root and
184 rootlet primordium. The galactose binding protein *DOMAIN OF UNKNOWN FUNCTION 642* gene
185 (*LaDUF642*) was expressed in the elongation zone but also in the newly formed epidermis at the tip of
186 the cluster root and rootlet primordium (stages V – onward) while *LaEXP2* expression was restricted to
187 the epidermis in the elongation zone. *ProLaPG1::GUS* and *proLaPG2::GUS* activity were found in the
188 vasculature including pericycle cells and *proLaPG2::GUS* was additionally found in the elongation zone of
189 the emerging rootlet primordium (stages VII-VIII).

190 **Auxin responsive *Lupinus albus* *POLYGALACTURONASE3* (*LaPG3*) is expressed in outer cortex cells 191 overlaying rootlet primordium**

192 Despite being expressed at a low basal level, a third *PG* gene was highly responsive to auxin treatment
193 (*LaPG3* – Extended Data Fig. 5). The *LaPG3* protein sequence presents a high percentage of identity
194 (60%) when compared to its closest ortholog in *Arabidopsis*, *POLYGALACTURONASE INVOLVED IN*
195 *LATERAL ROOT* (*AtPGLR*) (Extended Data Fig. 6a). Structural modeling of *LaPG3* showed that it contains a
196 common right-handed β -parallel fold characteristic for pectinases, including fungal endo-PGs and exo-
197 PGs (Extended Data Fig. 6b). Structural alignment with published PG structures (*Pectobacterium*
198 *carotovorum*, *Aspergillus niger* and *Aspergillus aculeatus*) confirmed that *LaPG3* is likely to act as an
199 endo-PG, with conserved amino acid motifs at the active site including Asn211-Thr212-Asp213 (NTD),

200 Asp234-Asp235 (DD), Gly256-His257-Gly258 (GHG) and Arg291-Ile292-Lys293 (RIK) (Extended Data Fig.
201 6c and d). The activity of its promoter was clearly visible in cluster root tips and even more striking in
202 primordium-overlying cells, excluding the endodermis, starting from stages III-IV (Fig. 2d).
203 *ProLaPG3::nlsYFP* transformed roots confirmed the expression of the fluorescent protein in cells that
204 undergo separation (Extended Data Fig. 7a). Interestingly, no signal was found in the mitotically
205 activated endodermis and inner cortex cells. IAA treatment confirmed the responsiveness of *LaPG3* to
206 the hormone, expanding the signal of *proLaPG3::GUS* to the external cortex (Fig. 2d). These results
207 suggest that spatial regulation of auxin responsive *LaPG3* expression is important for rootlet primordium
208 emergence. On top of that, GUS coloration was not found in the epidermis suggesting a different
209 mechanism affecting cell separation in this layer. To understand the functional role of *LaPG3*, we
210 expressed an artificial micro-RNA targeting its transcript under the control of the native promoter
211 (*proLaPG3::amiR-LaPG3*) in lupin hairy roots (Extended Data Fig. 7b). While cluster root length, rootlet
212 number and density were not affected in hairy roots expressing *proLaPG3::amiR-LaPG3* compared to the
213 control *proLaPG3::nlsYFP* (Extended Data Fig. 7c), rootlet primordium emergence was slightly delayed in
214 *proLaPG3::amiR-LaPG3* cluster roots (Fig. 2e). Gene expression analysis showed a 4-fold downregulation
215 of *LaPG3* in *proLaPG3::amiR-LaPG3* cluster roots (Extended Data Fig. 7d). Furthermore, the expression of
216 the auxin-responsive *GRETCHEN-HAGEN3 (GH3)* genes *LaGH3.3*, *LaGH3.5* and *LaGH3.6* were increased
217 in *proLaPG3::amiR-LaPG3* cluster roots, possibly reflecting a feedback between *LaPG3* and auxin
218 homeostasis gene expression (Extended Data Fig. 7d).

219 **Oligogalacturonide profiling reveals auxin-induced pectin remodeling in cluster roots**

220 Homogalacturonans (HG) are secreted into the cell wall as a polymer of galacturonic acids (GalA) which
221 can be methylesterified and/or acetylated. It is suggested that the degree of methylesterification (DM)
222 or acetylation (DA) of the HG plays a role in the availability of substrate for degradation enzymes such as
223 polygalacturonases (Hocq *et al.*, 2017b). To understand the contribution of auxin-mediated changes in
224 HG structure during rootlet emergence, we used an LC-MS/MS oligogalacturonide profiling method to
225 reveal changes in HG composition in our cluster root samples (Voxeur *et al.*, 2019, Hocq *et al.*, 2020). For
226 that purpose, we harvested segments corresponding to the “emergence zone” (as previously done for
227 RNAseq) and the distal part of the cluster root, referred as the “CR tip zone” (Fig. 3a). Using *Aspergillus*
228 PG, we then performed oligogalacturonide fingerprinting on these segments at T0, T24 hours and T48
229 hours after auxin treatment. Most of the oligogalacturonides released from the tip zone displayed a low
230 degree of polymerization ($DP \leq 3$) (Fig. 3b), which slightly accumulated over time in correlation with a
231 decrease in high DP oligogalacturonides ($DP \geq 4$) (Fig. 3b). Principal component analysis revealed a
232 strong effect of auxin treatment in the accumulation of low DP oligogalacturonides (GalA2 and GalA3)
233 compared to mock conditions. Accordingly, oligogalacturonides of high DP ($DP \geq 4$), although weakly
234 represented, were reduced following auxin treatment (Fig. 3b). Those effects were visible after 24 hours
235 of treatment and stable after 48 hours (Fig. 3b). In contrast, oligogalacturonides that were released from
236 the emergence zone differed over the time course. For example, although GalA2 was weakly
237 represented at T0 (< 1% of total oligogalacturonides) its proportion was significantly increased (reaching
238 10%) at T24 (Fig. 3c). In the meantime, high DP oligogalacturonides ($DP \geq 3$) were less abundant in the
239 total released fraction at T24 and T48. However, auxin treatment had only a moderate impact on the DP
240 of total oligogalacturonides released in the emergence zone and we observed a slight increase of GalA3
241 fraction while the GalA4 fraction was diminished (Fig. 3c). We next examined the DM and DA of the
242 oligogalacturonides from the two segments of interest (Fig. 3d, e). In the cluster root tip zone, only slight

243 changes in methylesterification or acetylation degrees occurred during the time course in control
244 conditions, but auxin induced a strong demethylesterification of GalA species at T24 (Fig. 3d). A
245 transient auxin-induced increase in acetylated oligogalacturonides compared to the control was
246 observed at T24 before stabilizing to the control levels at T48 (Fig. 3d). This suggests a robust and early
247 effect of auxin on HG modifications in the first 24 hours following the treatment in the CR tip zone. In
248 the emergence zone, the already low level of released methylesterified oligogalacturonides was stable
249 over time but was further reduced following auxin treatment after 24 hours (Fig. 3e). Remarkably, the
250 proportion of acetylated oligogalacturonides diminished during the control time course but was not
251 affected by auxin (Fig. 3e) suggesting an important relation between early rootlet emergence events and
252 acetylation of HG in control conditions. We briefly summarized our findings in the model shown in Fig.
253 3f. When the plants are grown in control conditions, the tip zone of the cluster root is not subjected to
254 dramatic changes in term of pectin modifications such as HG methylation. A brief variation of
255 acetylation status was observed after 24 hours but stayed stable over time. The DP of
256 oligogalacturonides released was fairly high. However, in the emergence zone in control conditions, we
257 observed a low DM from the beginning of the time-course (T0) while the DA decreased substantially
258 after 24 hours. Likewise, oligogalacturonide DP, which was lower than in the tip zone, decreased after
259 24 hours. When IAA treatment was applied at T0, this affected oligogalacturonide DM, which was
260 dramatically lower after auxin treatment than in the control conditions for both zones. Meanwhile, upon
261 auxin treatment, oligogalacturonide DA remained stable in both zones while DP was reduced in the tip
262 zone similarly to the untreated emergence zone.

263 **Homogalacturonan demethylesterification is homogeneously triggered by auxin in the rootlet** 264 **emergence zone**

265 The spatial regulation of pectin remodeling enzyme activity is known to be essential for organ growth
266 and development ([Levesque-Tremblay et al., 2015](#)). In cluster roots, auxin caused demethylesterification
267 of HG in the tip zone and in the emergence zone (Fig. 3d, e and f). To understand whether the observed
268 changes might be tissue or cell specific, we assessed the pattern of methylesterification of pectins in the
269 emergence zone using a subset of monoclonal antibodies targeting HG of various DM status (Fig. 4).
270 Immunolabeling of un-esterified HG with the LM19 antibody was uniform across rootlet primordium
271 developmental stages and was not affected by auxin treatment (Fig. 4a). In contrast, JIM5, labeling HG
272 stretches with low DM, displayed a strongly increased signal in cortex cells when treated with auxin (Fig.
273 4b). The area directly touching the rootlet primordium was heavily marked, which could be explained by
274 the high number of walls from collapsed cells in this particular location. We also noticed that phloem
275 cells directly below the pericycle cell layer were labeled with JIM5, but this tended to decline in auxin-
276 treated roots. These data suggest that auxin reduces the degree of HG methylesterification. However,
277 pectins seemed to be preponderantly present in fully demethylesterified forms in this zone (Fig. 3e).
278 When looking specifically at highly methylesterified HG using LM20, we observed that the cortex cell
279 three-way junctions were strongly labeled (Fig. 4c). In contrast, weak to no labeling was found in other
280 cell types in the epidermis and the stele. Auxin substantially decreased LM20 labeling homogeneously in
281 all cortical cells. In agreement, JIM7, which recognizes a highly methylesterified HG epitope, showed the
282 same pattern as LM20 with a weaker signal in auxin-treated cluster roots (Fig. 4d). Overall, our results
283 showed a clear positive effect of auxin in the removal of methylester groups from HG regardless of the
284 location (overlying a primordium or not) of the cortical cells.

285 (1,4)- β -D-galactan and extensins/type-I arabinogalactans are differentially distributed in primordium- 286 overlaying cell walls

287 Rhamnogalacturonans-I (RG-I) are the second most abundant polymers in pectins and are composed of
288 a backbone of alternating rhamnose and galacturonic acid with side chains of α -(1,5)-l-arabinans, β -
289 (1,4)-galactans, and type-I arabinogalactans (Caffall et Mohnen, 2009). There is growing evidence that
290 (1,4)- β -D-galactans are involved in cell-cell adhesion in many developmental contexts (Ng et al., 2015,
291 Moore et al., 2014). To determine whether (1,4)- β -D-galactans could play a role in rootlet emergence
292 we examined their distribution using the specific LM5 antibody in cluster root cross sections (Fig. 5). We
293 noticed a significant reduction in the fluorescent signal for LM5 in the primordium-overlying cortical
294 cells from stage IV onward. Interestingly, epidermis inner and shared cell walls were not labeled
295 indicating core cell wall differences between epidermis and cortex tissues. In contrast with HG
296 methylesterification degree, RG-I (1,4)- β -D-galactan reduction was not altered by auxin treatment (Fig.
297 5). Contrarily to the clear depletion of LM5 signal from stage IV onward in the control, we observed an
298 increased distribution of a subset of antibodies targeting putative sugar epitopes from cell wall
299 glycoproteins (Extended Data Fig. 8). Extensin-specific JIM11 (Smallwood et al., 1994) labeling was
300 significantly increased in primordium-overlying cortical cells in latter stages (Extended Data Fig. 8a).
301 The same spatial distribution was observed for JIM93 and JIM94 antibodies for which the epitope is
302 suggested to be found in the arabinogalactan side chain of hydroxyproline-rich glycoproteins (HRGP)
303 (Pattathil et al., 2010, Hall et al., 2013) (Extended Data Fig. 8b, c). Labeling of JIM11, JIM93 and JIM94
304 were highly comparable in the stele, with an unusual labeling of the protoxylem, and in the walls
305 separating the pericycle and endodermis cell layers. These results suggest extensive RG-I remodeling in
306 cortical-overlying cells that are mechanically challenged by the primordium outgrowth.

307

308 Discussion

309 Since more than a century, botanists have been arguing about whether the secondary root emerges
310 purely mechanically or is helped by the digestion of the outer cortical layers or both (Pond, 1908). Using
311 white lupin, Pond elegantly compared the lateral root primordium to a boat cutting through the water,
312 making its way out by pushing away the outer cortical tissues. His microscopic observations determined
313 that separation but not digestion of the outer cortical cells occurs in the late stages, corresponding to
314 stage V onwards (Gallardo et al., 2018). However, previous reports claimed that the lateral root secretes
315 a substance actively digesting the outer cortical cells (Vonhöne, 1880), or that the passage of the
316 primordium may be aided by enzymatic activity of the external tissue (Pfeffer, 1893). Both hypotheses
317 have been put in the spotlight by the discovery in *Arabidopsis*, one century later, of the central action of
318 primordium tip-derived auxin in the activation of cell wall remodeling enzyme genes in the overlying
319 cells (Swarup et al., 2008). Our observation of cluster roots treated with auxin and with auxin transport
320 inhibitors confirmed a positive role of the phytohormone on rootlet initiation and emergence (Fig. 1).
321 IAA treatment modulated the expression of many genes in the cluster root emergence zone (Fig. 2b, c;
322 Extended data Fig. 2; Supplemental Table 2). As the first outcome of auxin stimulus, several genes
323 related to transcriptional regulation were upregulated such as *Lalb_Chr01g0019701* and
324 *Lalb_Chr02g0142291*, the two closest orthologues of *LATERAL ORGAN BOUNDARIES-DOMAIN 29*
325 (*LBD29*) in *Arabidopsis*, a transcriptional activator transducing auxin signals in outer cell layers (Porco et
326 al., 2016), suggesting that early developmental pathways are conserved across species.

327 We also observed in a second transcriptional wave caused by auxin, involving a steady upregulation of
328 cell wall related genes (Fig. 2c, cluster 3), which is a response also found during lateral root development
329 in *Arabidopsis* (Lewis *et al.*, 2013). We showed that in white lupin, an auxin-induced polygalacturonase,
330 named *LaPG3*, is specifically expressed at the location of rootlet primordium emergence, in the outer
331 cortex cell layers (Fig. 2d, Extended Data Fig. 7a). This enzyme shows structural similarities with fungal
332 endo-polygalacturonase (Extended Data Fig. 6) and is the closest lupin orthologue of *AtPGLR*
333 (*At5g14650*, Extended Data Fig. 3a), an acidic polygalacturonase identified as a putative candidate
334 involved in lateral root emergence in *Arabidopsis* (Kumpf *et al.*, 2013, Hocq *et al.*, 2020). Although
335 belonging to a multigenic family (108 members in *L. albus* according to Hufnagel *et al.*, 2020 genome
336 release), amiRNA driven down-regulation of *LaPG3* in lupin hairy roots slightly delayed rootlet
337 emergence (Fig. 2e). A simple explanation for this phenotype would be a reinforced cell wall and
338 stronger cell adhesion in primordium-overlying cells, slowing down the overall process of rootlet
339 emergence. We also noticed the increased expression of auxin responsive *GH3* genes in *proPG3::amiR-*
340 *PG3* cluster roots (Extended Data Fig. 7d). Auxin sensitivity is dampened in tobacco plants expressing
341 fungal endo-PG and treatment with oligogalacturonide elicitors leads to the same effect in *Arabidopsis*
342 (Ferrari *et al.*, 2008, Savatin *et al.*, 2011). Accordingly, we noticed a similar antagonism in the context of
343 our microRNA targeting *LaPG3* transcript. Indeed, a low *LaPG3* expression could reduce
344 oligogalacturonide-eliciting responses, thus alleviating the inhibition of auxin responses. This negative
345 feedback loop between PG and auxin responses could help stabilize the rootlet emergence process by
346 the inactivation of IAA via auxin-amido synthetase GH3.

347 Polygalacturonase activity requires structural modifications of the homogalacturonan pectic backbone
348 and polygalacturonases are mostly active on demethylesterified stretches. Auxin plays an important role
349 in the regulation of pectin methylesterase (PME) and PME inhibitor (PMEI) expression, which in turn
350 modulates the degree of methylesterification in diverse organs. However, auxin action on pectin
351 methylesterification depends on the developmental process at stake, positively regulating the removal
352 of methylester groups in the shoot apical meristem while contrastingly promoting methylesterification
353 during apical hook development (Braybrook et Peaucelle, 2013, Jonsson *et al.*, 2021). We were able to
354 observe the dynamics of homogalacturonan methylesterification in the developing cluster root
355 temporally, during early cluster root development, and spatially, discerning the most distal tip part
356 (meristematic and elongation zone) and the rootlet emergence part (Fig. 3). Auxin triggered
357 demethylesterification in the whole cluster root with the homogalacturonans in the tip zone being
358 substantially more methylesterified than in the emergence zone (Fig. 3d, e, Fig. 4). A low degree of
359 methylesterification was correlated with the release of galacturonic acids with a low degree of
360 polymerization after *Aspergillus aculeatus* endo-PG processing (Fig. 3b – e, Extended data Fig. 7). This
361 could be explained by the preferred affinity of AaPGM2 for demethylesterified galacturonic acid
362 stretches (Safran *et al.*, 2021). Based on its predicted structure and high similarity with fungal endo-PG
363 and *Arabidopsis* PGLR (Pickersgill *et al.*, 1998, Van Santen *et al.*, 1999, Cho *et al.*, 2001, Extended data
364 Fig. 6), *LaPG3* is likely to have a similar mode of action, however our attempts to produce recombinant
365 enzyme for further characterization have failed so far. We hypothesize here that auxin causes pre-
366 processing of the cell walls by regulating enzymes such as PME, allowing cell wall degradation in the
367 emergence zone at specific locations marked by the expression of *LaPG3*. Such a tandem regulation of
368 pectin degradation has been observed during pollen tetrad separation in *Arabidopsis*. *QUARTET1* (*QRT1*)
369 encodes a PME that removes methylester groups, which stimulates *QUARTET3* (*QRT3*), a PG, to cleave
370 homogalacturonans, allowing cell wall loosening (Rhee *et al.*, 2003, Francis *et al.*, 2006). *QRT1* is also

371 specifically expressed at other locations of cell separation in *Arabidopsis*, including lateral root
372 emergence-overlying cells (Francis *et al.*, 2006). An analogous mechanism has been proposed for fruit
373 softening in avocado and tomato (Wakabayashi *et al.*, 2003).

374 The outer cortex is subject to enormous mechanical constraint in the rootlet emergence zone due to the
375 growing primordium. Cell death can be observed in the endodermis and to a lesser extent in the cortex
376 cells in species such as *Arabidopsis* (Escamez *et al.*, 2020). In white lupin, those layers (endodermis and
377 inner cortex) are dividing and become included within the rootlet primordium. Microscopic observations
378 of late-stage primordia (Stage V onwards) indeed show collapsed outer cortex cells but whether cell
379 death occurs remains to be proven (Gallardo *et al.*, 2018). Once the most difficult part of the rootlet's
380 journey has been dealt with by the recruitment of endodermis and inner cortical cell divisions, one can
381 assume that late emergence solely takes place due to the displacement of outer cortex and epidermis
382 cells. The specific expression of LaPG3 in those cells when a primordium grows underneath suggests
383 however that degradation of the pectin-rich middle lamellae is crucial to reduce cell adhesion. Still,
384 LaPG3 expression was not found in epidermis suggesting that cell separation might occur through a
385 different mechanism. This could be explained by a different cell wall composition. Immunolabeling
386 experiments with antibodies targeting homogalacturonan epitopes with diverse degrees of
387 methylesterification confirmed that auxin triggered demethylesterification in the emergence zone.
388 However, we were not able to identify differential distribution of methylesterified and
389 demethylesterified homogalacturonans in cortex cell walls challenged by a primordium or not (Fig. 4).
390 Such a differential distribution of methylesterified pectins was recently reported at the junction
391 between the lateral root primordium and endodermis in the early stages and later between endodermis
392 and cortex in *Arabidopsis* (Waschman *et al.*, 2020). Our observations of homogalactan epitopes were
393 conducted at a late stage of primordium emergence in a region of the cluster root that experiences
394 hundreds of primordia growing in close proximity. The most striking observations of differential cortex
395 cell wall composition were revealed using antibodies that recognize rhamnogalacturonan-I and
396 glycoprotein epitopes (Fig. 5, Extended Data Fig. 8). Rhamnogalacturonans-I are the second most
397 represented component of the pectin matrix. The complex rhamnogalacturonan-I main chain is
398 composed of α -D-galacturonosyl and rhamnosyl residues, with side chains of (1,4)- β -D-galactan and
399 (1,5)- α -L-arabinan residues of various degrees of polymerization. This pectin component is thought to
400 play a role in cell adhesion through crosslinks with cellulose microfibrils (Lin *et al.*, 2015). Notably, a loss
401 of (1,4)- β -D-galactans has been correlated with fruit softening in the ripening process of apple and
402 grapes (Ng. *et al.*, 2015, Moore *et al.*, 2014). It is also worth mentioning that (1,4)- β -D-galactan side
403 chains contribute to the gelation capacity of rhamnogalacturonan-I *in vitro* (Mikshina *et al.*, 2017). In this
404 study, we detected a loss of (1,4)- β -D-galactan epitopes in the primordium-overlying cortical cells, for
405 which the effect was independent of auxin (Fig. 5). The epidermis inner cell wall and the direct
406 subepidermal cell layer were intriguingly not marked, reflecting different pectin composition and
407 possibly mechanical properties of those cells. Nevertheless, it remains to be determined whether (1,4)-
408 β -D-galactan loss in rhamnogalacturonans-I is part of a controlled active process of cell separation or is a
409 passive consequence of cell wall remodeling.

410 The same question needs to be investigated for the visible increase of extensin and HRGP epitope
411 labeling at the location of primordium emergence (Extended Data Fig. 8). Extensins are proteins known
412 to be involved in cell wall relaxation especially in rapidly growing pollen tubes and root hairs (Velazquez
413 *et al.*, 2011, Wang *et al.*, 2018) but their role in cell adhesion is more enigmatic. Nonetheless, a high

414 expression of an extensin coding gene has been correlated to tomato fruit ripening, thereby possibly
415 affecting cell wall cohesion (Ding *et al.*, 2020). JIM11, JIM93 and JIM94 labeled the same tissues in the
416 cluster root emergence zone, but the epitopes are not fully characterized. However, their distribution
417 suggests binding to a highly similar glycan epitope (Pattathil *et al.*, 2010). The strong labeling of JIM11,
418 JIM93 and JIM94 could be due to two possibilities. Firstly, an accumulation of extensins and HRGP in the
419 challenged cortical cell wall. Their accumulation in primordium-overlying cells could lead to cell wall
420 softening and cell separation. Secondly, possible pectin remodeling, suggested here by auxin induced
421 demethylesterification and spatial PG expression during rootlet emergence in addition to the depletion
422 of (1,4)- β -D-galactan residues, could increase the epitope availability. Thus, more extensin and HRGP
423 antibody signal could be caused by increased abundance of the protein and/or by uncovering epitope
424 sites in modified cell walls. This is likely to occur after the auxin-induced changes leading specifically to
425 homogalacturonan modifications (demethylesterification) and degradation (polygalacturonase activity).
426 However, it cannot be excluded that a solely mechanical aspect could trigger those cell wall
427 modifications.

428 Conclusion

429 In this study, we used white lupin (*Lupinus albus*) as a model to understand the contribution of auxin
430 and cell wall remodeling during rootlet emergence because of its striking secondary root developmental
431 phenotype. Auxin accelerates primordium emergence and causes a shift in the transcriptional landscape
432 including the upregulation of cell wall related genes. Among several candidate genes, we identified
433 *LaPG3*, an endo-polygalacturonase gene expressed in the outer cortex and positively regulating rootlet
434 emergence. Additionally, we found that homogalacturonans, the substrate of polygalacturonase, were
435 highly demethylesterified in the emergence zone, an effect amplified by external auxin treatment. In
436 contrast to methylesterified homogalacturonans, we noticed that (1,4)- β -D-galactans from
437 rhamnogalacturonan-I side chains were differentially distributed in mechanically challenged cortex cell
438 wall by the growing primordium. Conversely, we found that extensins and HRGP epitopes were enriched
439 in the walls of those cells. Altogether, we propose a model for auxin-controlled cell separation during
440 secondary root emergence in which auxin triggers homogalacturonan demethylesterification in the
441 cluster root, rendering possible the degradation of pectins in the cortex cells expressing *LaPG3*. The
442 resulting cell wall loosening and loss of cell adhesion are accompanied by depletion of (1,4)- β -D-
443 galactans and accumulation of extensins/arabinogalactan protein.

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452 **List of Figures:**

453 Figure 1: Auxin regulates rootlet development and primordium emergence.

454 **a**, Representative pictures of the cluster roots from nine day-old *L. albus* grown in hydroponics medium
455 after two days supplemented with 0.01% ethanol (control treatment), 10 μ M NPA, 1 μ M IAA, 5 μ M IAA,
456 2.5 μ M 1-NOA or 25 μ M 1-NOA. Bar scale: 1 cm. **b**, Rootlet number, cluster root length and rootlet
457 density in the 4 upper cluster roots in plants treated with 0.01% ethanol (n = 17), 10 μ M NPA (n = 16), 1
458 μ M IAA (n = 19), 5 μ M IAA (n = 19), 2.5 μ M 1-NOA (n = 16) or 25 μ M 1-NOA (n = 16). Statistical
459 significance (compared to control) was computed by the Dunnett multiple comparison test: ****: pVal <
460 0.001, ***: pVal < 0.005, **: pVal < 0.01. **c**, Frequency of primordium stages found in the 4 upper cluster
461 roots in plants treated with 0.01% ethanol (black bars, n = 17), 10 μ M NPA (pink bars, n = 16), 1 μ M IAA
462 (light blue bars, n = 19), 5 μ M IAA (dark blue bars, n = 19), 2.5 μ M 1-NOA (light green bars, n = 16) or 25
463 μ M 1-NOA (dark green bars, n = 16). Statistical significance (compared to control) was computed by the
464 Dunnett multiple comparison test: ****: pVal < 0.001, ***: pVal < 0.005, **: pVal < 0.01, *: pVal < 0.05.
465 **d**, Synthetic auxin response reporter DR5 is active in the pericycle cell layer of the cluster root
466 emergence zone and at the tip of rootlet primordia. Free-hand sections were done immediately after
467 harvesting fresh transformed roots. White dashed lines outline rootlet primordia, white and blue arrows
468 respectively indicate pericycle cell layer and the tip of a primordium/rootlet. Scale bars for longitudinal
469 sections: 50 μ m. Scale bars for cross sections and emerged rootlet tips: 100 μ m.

470 Figure 2: Auxin transcriptome landscape identifies cell wall related genes linked to rootlet emergence.

471 **a**, Auxin RNA sequencing experiment overview. Lupin seeds were germinated in vermiculite for three
472 days before the seedlings ("mohawk" stage) were transferred to hydroponic medium without phosphate
473 (-P) to induce the formation of cluster roots (Day 3). Four days later (Day 7), 1 μ M IAA was added to the
474 hydroponic medium and 1 cm segments of cluster roots 1 cm distant from the primary root,
475 corresponding to the emergence zone, were harvested for RNA extraction and further RNA sequencing
476 at 8 different time points: T0 (just before the treatment), half an hour (T0.5), one hour (T1) and two
477 hours (T2) after the treatment to assess the early auxin transcriptomic responses and six hours (T6), 12
478 hours (T12), one day (T24) and two days (T48) later for the late auxin transcriptomic responses. **b**,
479 Heatmap of white lupin auxin-regulated transcripts. Normalized expression levels are shown as a z-score
480 (See Material and Methods section for further details) and hierarchical clusters of the differentially
481 expressed genes (DEG) are displayed by the colored squares on the right of the heatmap. Expression
482 patterns for each cluster are shown in Extended data Figure 2. **c**, the three largest expression clusters
483 (red, orange and yellow) show distinct gene expression patterns and are enriched in specific gene
484 ontology terms. GO terms of significant importance are shown (red circle: molecular function, MF, green
485 circle: biological process, BP, blue circle: cellular component, CC). **d**, proLaPG3::GUS localization in
486 cluster root tips and at different rootlet primordium development stages in regular hydroponic medium
487 (top) and after 48 hours of 1 μ M IAA treatment (bottom). Scale bars: 100 μ m. **e**, frequency of
488 primordium stages in cluster roots from hairy root composite plants expressing proPG3::amiR-PG3 (n =
489 27 roots) and proPG3::nlsYFP (n = 28) as a control. Statistical significance was computed by the Šídák's
490 multiple comparisons test. *: pVal < 0.05, ****: pVal < 0.001, ns: non-significant.

491 Figure 3: Oligogalacturonide profiling reveals auxin-induced pectin remodeling in cluster roots.

492 **a**, Auxin oligogalacturonide (OG) profiling experiment overview. Lupin seeds germinated in vermiculite
493 for three days before the seedlings ("mohawk" stage) were transferred to hydroponic medium without
494 phosphate (-P) to induce the formation of cluster roots (Day 3). Four days later (Day 7), 1 μ M IAA or
495 0.01% ethanol (Mock) were added to the hydroponic medium and 1 cm segments of cluster roots 1 cm
496 distant from the primary root (hereafter named emergence zone, red color code) and the distal part of
497 the cluster root (CR tip zone, green color code) were harvested for cell wall extraction, digestion with
498 *Aspergillus aculeatus* endo-polygalacturonase M2 (AaPGM2) and OG profiling according to [Voxeur et al.,](#)
499 [2019](#). The samples shown in the pictures (bars: 0.5mm) were dissected and collected at T0 (just before
500 the auxin treatments), and at one day (T24) and two days (T48) after auxin treatments. **b**, Effect of the
501 auxin treatment on GalAx species released in the "CR tip zone" during the time course previously
502 described. Left plot: bi-dimensional plot of principal components calculated by performing PCA of the
503 different OG species relative abundances (grouped by degree of polymerization) for each treatment and
504 time point (3 replicates each). Vectors describe the contribution of the OG species to the biplot. Right
505 plot: OG abundance (% of total OG detected) in the CR tip zone at T0 and 24 and 48 hours after
506 treatment with ethanol (T24 and T48 Mock) or IAA (T24 and T48 IAA). **c**, Effect of the auxin treatment on
507 GalAx species released in the "emergence zone" during the time course previously described. See **b** for
508 descriptions of the plots. **d**, Relative abundance of methylesterified OG (GalAxMemAcn, $x, m \geq 1, n \geq 0$)
509 or acetylated OG (GalAxMemAcn, $x, m \geq 0, n \geq 1$) from the CR tip zone. **e**, Relative abundance of
510 methylesterified OG (GalAxMemAcn, $x, m \geq 1, n \geq 0$) or acetylated OG (GalAxMemAcn, $x, m \geq 0, n \geq 1$)
511 from the emergence zone. **f**, Schematic representation of OG profiling experiment results. Top: control
512 condition. Bottom: IAA-treated condition. Green and red arrows represent the CR tip and emergence
513 zones, respectively. Plots representing the DP, DM and DA status of oligogalacturonides during the time
514 course are displayed above each relevant zone. DP, degree of polymerization; DM, degree of
515 methylesterification; DA, degree of acetylation.

516 Figure 4: Auxin induces HG demethylesterification in cluster root cortical tissues independently of
517 rootlet primordium emergence stage and location.

518 Immunolabeling of the homogalacturonan specific LM19 (**a**), JIM5 (**b**), LM20 (**c**) and JIM7 (**d**) antibodies
519 of cluster root cross-sections 2 days after treatment with 1 μ M IAA or Mock (Ethanol 0.01%). Scale bar:
520 100 μ m. Boxplot represents antibody intensity defined as the ratio of secondary antibody (Alexa 546)
521 signal to autofluorescence signal in the "mechanically unchallenged" cortical tissues (phloem pole) or in
522 that overlaying early (stage I to IV) and late (stage V to VII) rootlet primordia. All data points are
523 displayed, whiskers show minimal and maximal values (**a**, mock: phloem pole: $n = 40$ sections, StI-IV: $n =$
524 32, StV-VII: $n = 8$; IAA: phloem pole: $n = 34$, StI-IV: $n = 27$, StV-VII: $n = 9$; **b**, mock: phloem pole: $n = 36$,
525 StI-IV: $n = 29$, StV-VII: $n = 7$; IAA: phloem pole: $n = 34$, StI-IV: $n = 21$, StV-VII: $n = 13$; **c**, mock: phloem
526 pole: $n = 27$, StI-IV: $n = 21$, StV-VII: $n = 7$; IAA: phloem pole: $n = 46$, StI-IV: $n = 33$, StV-VII: $n = 17$; **d**, mock:
527 phloem pole: $n = 59$, StI-IV: $n = 52$, StV-VII: $n = 7$; IAA: phloem pole: $n = 35$, StI-IV: $n = 26$, StV-VII: $n = 10$).
528 Statistical significance was computed with the Kruskal-Wallis test. ****: $pVal < 0.001$, ***: $pVal < 0.005$,
529 **: $pVal < 0.01$, *: $pVal < 0.05$.

530 Figure 5: Rhamnogalacturonan I (1,4)- β -D-galactan is depleted in rootlet primordium-overlaying cells
531 independently of auxin treatment.

532 Immunohistochemistry with (1,4)- β -D-galactan specific LM5 antibody in cluster root cross-sections 2
533 days after treatment with 1 μ M IAA or Mock (Ethanol 0.01%). Boxplot represents the antibody intensity

534 defined as the ratio of secondary antibody (Alexa 546) signal to autofluorescence signal in the
535 "mechanically unchallenged" cortical tissues (phloem pole) or those overlaying early (stage I to IV) and
536 late (stage V to VII) rootlet primordia. All points are displayed, whiskers show minimal and maximal
537 values (mock: phloem pole: n = 32 sections, StI-IV: n = 22, StV-VII: n = 12; IAA: phloem pole: n = 29, StI-
538 IV: n = 17, StV-VII: n = 14). Statistical significance (compared to "phloem pole" mean intensity ratio mock
539 conditions) was computed with the Kruskal-Wallis test. ***: pVal < 0.005. Scale bars: 100 µm.

540 **List of Extended Data:**

541 Extended Data Fig.1: Representative pictures of nine-day-old *Lupinus albus* grown in hydroponic
542 medium after two days supplemented with 0.01% ethanol (control treatment – two plants), 10 µM NPA,
543 1 µM IAA, 2.5 µM 1-NOA or 25 µM 1-NOA. Scale bar: 5 cm.

544 Extended Data Fig.2: Minor clusters identified in the transcriptome of auxin treated cluster roots.

545 Expression pattern of minor clusters identified in the main Figure 2b (light blue, purple, black and green
546 bars on the heatmap). Bubble plots display gene ontology enrichment analysis with the identified
547 molecular (red), biological process (green) and cellular component (blue) associated with the
548 differentially expressed genes among the clusters.

549 Extended Data Fig.3: Identification of white lupin cell wall related putative orthologues and promoter
550 activity in transformed hairy root.

551 **a**, Phylogenetic tree of cell wall related genes based on protein sequences in *L. albus* (green dot) and
552 *Arabidopsis*. The more distantly related transmembrane amino acid transporter protein GAMMA-
553 AMINOBUTYRIC ACID TRANSPORTER 1 (GAT1, *At1g08230*) was chosen as an outgroup for the analysis. **b**,
554 Representative promoter::GUS pictures for each putative *L. albus* cell wall related gene in cluster root
555 tips and rootlet primordia in the different stages of development described by [Gallardo et al., 2018](#).
556 Cross sections in the emergence zone are also shown. Scale bar: 100 µm.

557 Extended Data Fig.4: Promoter activity of white lupin auxin responsive cell wall genes in transformed
558 hairy root.

559 Representative promoter::GUS pictures for *L. albus* auxin responsive cell wall related genes in cluster
560 root tips and in rootlet primordia in the different stages of development described by [Gallardo et al.,](#)
561 [2018](#). Cross sections in the emergence zone are also shown. Scale bar: 100 µm. Asterisk shows the cross
562 section for *pLaEXP2::GUS* in the elongation zone of the cluster root (not in the emergence zone), where
563 gene expression was found. Expression level of the candidate genes over the course of the auxin
564 transcriptome generated in this study (see Fig. 2) are shown on the right.

565 Extended Data Fig.5: Auxin-responsive *Lupinus albus* POLYGALACTURONASE3 (*LaPG3*) expression.

566 RNAseq expression level of *LaPG3*, an auxin-responsive gene from the expression cluster 3.

567 Extended Data Fig.6: LaPOLYGALACTURONASE3 *in silico* characterization.

568 **a**, Protein alignment of AtPGLR (*AT5g14650*) and LaPG3 (*Lalb_Chr02g0160451*) using clustal omega
569 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>. version 1.2.4). Results are shown using MView, **b**, Surface
570 and ribbon representation of LaPG3 modeled structure (gray) with amino acid of the active site
571 highlighted (blue). **c**, LaPG3 modelled structure (grey) superimposed to the structure of *Pectobacterium*

572 *carotovorum* PG (PDB:1BHE, yellow), *Aspergillus niger* PGII (PDB:1CZF, green) and *Aspergillus acuelatus*
573 PG (PDB:1IA5, purple). **d**, Amino acids of the LaPG3 active site (blue) superimposed onto that of the
574 *Aspergillus acuelatus* active site (yellow).

575 Extended Data Fig.7: Characterization of *LaPOLYGALACTURONASE3* microRNA lines in white lupin hairy
576 roots.

577 **a**, Longitudinal view of proLaPG3::nlsYFP localization in cluster roots during rootlet primordium
578 emergence, stages III-VI. proUBI-mCherry is a transformation internal positive control. White dashed
579 lines outline rootlet primordia. Scale bars: 50 μ m. **b**, Alignment of the artificial microRNA targeting its
580 corresponding *LaPG3* transcript region (*amiR-PG3*). **c**, Rootlet number, cluster root length and rootlet
581 density in hairy root composite plants expressing *proPG3::amiR-PG3* (n = 27 transformed roots) and
582 *proPG3::nlsYFP* (n = 28) as a control. Statistical significance was computed using an unpaired t-test: ns:
583 non-significant. **d**, Relative gene expression of *LaPG3*, *LaGH3.3*, *LaGH3.5* and *LaGH3.6* in *proPG3::amiR-*
584 *PG3* transformed cluster roots compared to *proPG3::nlsYFP*. Gene expression values are relative to the
585 expression in *proPG3::nlsYFP*, for which the value is set to 1. Error bars indicate SEM obtained from
586 three independent biological replicates. Statistical significance was computed using an unpaired t-test:
587 *: pVal < 0.05, **: pVal < 0.01, ***: pVal < 0.005.

588 Extended Data Fig.8: JIM11 and JIM93/JIM94 epitope labeling is increased in rootlet primordium-
589 overlaying cells at late stages.

590 **a**, Immunolabeling of the extensin specific JIM11 antibody of cluster root cross-sections. **b**,
591 Immunolabeling of the type I arabinogalactan chain putative JIM93 antibody of cluster root cross-
592 sections. **c**, Immunolabeling of the type I arabinogalactan chain putative JIM94 antibody of cluster root
593 cross-sections. Boxplots represent antibody intensity defined as the ratio of secondary antibody (Alexa
594 546) signal to autofluorescence signal in the "mechanically unchallenged" cortical tissues (phloem pole)
595 or those overlaying early (stage I to IV) and late (stage V to VII) rootlet primordia. All points are
596 displayed, whiskers show minimal and maximal values (**a**, phloem pole: n = 32, StI-IV: n = 16, StV-VII: n =
597 16; **b**, phloem pole: n = 29, StI-IV: n = 20, StV-VII: n = 11; **c**, phloem pole: n = 19, StI-IV: n = 11, StV-VII: n =
598 8). Statistical significance (compared to "phloem pole" mean intensity ratio) was computed with the
599 Kruskal-Wallis test. ****: pVal < 0.001, **: pVal < 0.01. Scale bars: 100 μ m.

600 **List of Supplemental Tables:**

601 Supplemental Table 1: DEG counts from RNAseq

602 Supplemental Table 2: GO enrichment analysis

603 Supplemental Table 3: Primers list

604 Supplemental Table 4: Antibodies list

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610 **Materials and Methods**

611 **Plant materials and growth conditions**

612 White lupin (*Lupinus albus*) cv. AMIGA (from Florimond Desprez, France) used in this study were
613 germinated in vermiculite for and grown three days under long day conditions (16 h light/8 h dark, 25 °C
614 day/20 °C night, 65% relative humidity and PAR intensity 200 $\mu\text{mol}\cdot\text{m}^2\cdot\text{s}^{-1}$). The three-day-old seedlings
615 were transferred to 1.6 L pots containing a phosphate-free hydroponic solution composed of the
616 following: 400 μM $\text{Ca}(\text{NO}_3)_2$; 200 μM K_2SO_4 ; 54 μM MgSO_4 ; 0.24 μM MnSO_4 ; 0.1 μM ZnSO_4 ; 0.018 μM
617 CuSO_4 ; 2.4 μM H_3BO_3 ; 0.03 μM Na_2MoO_4 ; 10 μM Fe-EDTA. Hydroponic medium was renewed weekly and
618 permanent oxygenation was provided by an air pump.

619 **Chemical treatments and cluster root phenotyping**

620 All chemicals used in this study were applied seven days after germination, at the onset of rootlet
621 primordium development, directly in the hydroponic medium at the desired concentrations. The four
622 upper cluster roots were sampled 48 hours after treatments and cleared in saturated aqueous chloral
623 hydrate solution (250 g in 100 mL water) for two weeks before observation. Cluster root length, rootlet
624 density and developmental stages of primordia were scored using a color camera on Olympus BX61
625 epifluorescence microscope (Tokyo, Japan). For artificial microRNA cluster root characterization, we
626 sampled individual cluster roots (each is an independent transformation event) of 4 to 6 hairy root
627 composite plants one week after hydroponic transfer and screened them under the confocal microscope
628 (Zeiss LSM780) searching for ubiquitous mCherry signal at 561 nm (internal control of transformation).
629 Hairy root experiments were conducted twice. Transformed cluster roots were transferred to chloral
630 hydrate solution for phenotyping or frozen in liquid nitrogen for RNA extraction experiments.
631 Transformed cluster roots showing obvious developmental irregularities such as root fusion or
632 supernumerary xylem poles were excluded from the analysis.

633 **Gene expression analysis**

634 Total RNA was extracted from 150 mg of cluster roots (3 to 4 roots) using the RNeasy Plant Mini Kit
635 (Qiagen, 74904) and treated with the DNA-free DNA Removal Kit (Thermo Fisher Scientific, AM1906). 1
636 μg of total RNA was used for reverse transcription with the Invitrogen SuperScript III Reverse
637 Transcriptase (Thermo Fisher Scientific, 18080093). Quantitative PCR was performed with the
638 SsoAdvanced Universal SYBR Green Supermix (BioRad, 1725271) on a CFX Maestro 96 thermocycler
639 (BioRad). Relative expression was calculated with the delta-delta Ct method, using the validated
640 *LaNORM1* reference gene. Primers are available in Supplementary Table 3.

641 **RNA sequencing**

642 Four independent biological replicates were used to prepare RNA-sequencing libraries with Illumina
643 TruSeq Stranded Total RNA Kit with Ribo-Zero. The libraries were sequenced as 150bp pair-end reads
644 using Illumina HiSeq3000 at Get-PlaGe core facility (INRA, Toulouse, France). A total of 2,110,906,218
645 paired-end reads were generated. The following procedure was applied to each paired read dataset.
646 Cutadapt (version 1.15) was used to remove Illumina Truseq adapters from the sequencing data and to
647 remove bases with a quality score lower than 30, in both 5' and 3' end of the reads. Reads with a length

648 lower than 60 were discarded. The quality checked RNA-seq reads were then used to quantify white
649 lupin transcript abundance using salmon (version 0.12.0) with the options validateMappings, gcBias and
650 seqBias turned on. Data were imported to DESeq2 using tximport and normalized using DESeq2 method.
651 Then normalized counts were extracted for further analyses. Differential expression was performed
652 using the following method: given a gene, if the mean count of the less expressed condition was more
653 than twice inferior to the mean count of the most expressed condition, and if the mean of the most
654 expressed condition was superior to 100, the gene was considered differentially expressed (DE). The DE
655 gene count data for relevant samples were “log regularized”, using the Variance Stabilizing
656 Transformation (vst) function from DESeq2. Then the degPatterns function from the DEGReport package
657 was used to group genes into clusters based on their expression profile using the divisive hierarchical
658 clustering method diana from the cluster package. Gene Ontology (GO) annotation was then performed
659 using the available GO annotation for white lupin that can be found in the first version of the white lupin
660 genome annotation file (<https://www.whitelupin.fr/download.html>). The package goseq was used to
661 find GO enrichment in each of the clusters previously computed. Only enriched GO terms associated
662 with a pvalue < 0.01 were kept.

663 **Phylogenetic trees**

664 Protein sequences from white lupin were retrieved from the white lupin genome database
665 (www.whitelupin.fr – Hufnagel *et al.*, 2020). Sequences producing significant alignments after blastp
666 analysis of full length protein sequences of *Arabidopsis* as queries (Total score > 700; Evalue=0) were
667 considered as putative lupin orthologous proteins for each gene family. Sequences were aligned by
668 MUSCLE and evolutionary history was inferred using the Neighbor-Joining method (bootstrap replication
669 number: 1000). Phylogenetic trees were then exported from the software MEGA X (version 10.0.4).

670 **Promoter GUS cloning**

671 Upstream genomic sequences of white lupin cell wall related genes (primers, gene name and promoter
672 length are listed in Supplemental table 3) were amplified by PCR from *L. albus* cv. AMIGA genomic DNA.
673 The amplified fragments were cloned using the Gateway BP clonase enzyme mix into the pDONR201
674 entry vector (Thermo Fisher Scientific, 11798013) transformed in *E. coli* TOP10, verified by sequencing
675 and recombined into the binary vector pKGWFS7 containing a GFP-GUS fusion (Karimi *et al.*, 2002) using
676 the Gateway LR clonase II enzyme mix (Thermo Fisher Scientific, 11791020).

677 **Artificial microRNA cloning**

678 Artificial microRNA (amiR) targeting *LaPG3* transcripts was designed using the online tool WMD3
679 (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>) and the pRS300 backbone vector according to the
680 protocol of Schwab *et al.*, 2006. The amiR-LaPG3 and the nlsYFP coding sequence (transformation
681 control) were inserted into the entry vector pDONR201 (P1P2 recombination sites) and the promoter of
682 *LaPG3* was inserted into the pDONRP4P1r (sequences listed in Supplemental table 3). The two
683 fragments were recombined into the multisite Gateway vector pK7m24GW_CR, specifically designed for
684 hairy root transient expression and cluster root phenotype analysis in this study, using Gateway LR
685 clonase II enzyme mix. The same procedure was repeated to generate the DR5::nlsYFP construct in
686 pK7m24GW_CR.

687 **Hairy root transformation**

688 *Rhizobium rhizogenes* strain *ARqua-1* was used for hairy root transformation of white lupin. The bacteria
689 were transformed with the expression vector by electroporation. LB agar plates were supplemented
690 with 100 μ M acetosyringone and appropriate antibiotics and inoculated with 200 μ l of liquid bacterial
691 culture. The plates were incubated at 28 °C for 24 h, producing a fresh and dense bacterial lawn used for
692 lupin seedling transformation. Meanwhile, white lupin seeds were surface sterilized 30 min in bleach
693 (Halonet 20%) and washed four times in sterile water. Seed were germinated on half MS medium (pH
694 5.7). Two days after germination, radicles of 1 cm were cut at 0.5 cm from the tip with a sterile scalpel.
695 The wounded part was inoculated with the *Rhizobium rhizogenes* lawn. Inoculated seedlings were
696 placed on square agar plates (0.7 % agar in 1X Hoagland solution) containing 15 μ g/mL Kanamycin.
697 Hoagland medium without phosphate is composed of 200 μ M $MgSO_4$; 400 μ M $Ca(NO_3)_2$; 325 μ M KNO_3 ;
698 100 μ M NH_4Cl ; 10 μ M Na-Fe-EDTA; 9.3 μ M H_3BO_3 ; 1.8 μ M $MnCl_2$; 0.17 μ M $ZnSO_4$; 0.06 μ M $CuSO_4$; 2.3
699 μ M Na_2MoO_4 . The plates were placed in a growth chamber under long day conditions (16 h light/8 h
700 dark, 25 °C day/20 °C night). After seven days, seedlings were transferred to vermiculite in a mini
701 greenhouse. Ten days later, the plants growing hairy roots were transferred to hydroponics (see “Plant
702 materials and growth conditions” section).

703 **GUS Histochemical assay**

704 Cluster roots were harvested from transgenic plants and immediately fixed in ice-cold 90% acetone for
705 30 minutes, washed three times in 0.1 M phosphate buffer (pH 7) and incubated in X-Gluc buffer (0.1%
706 X-Gluc; 50 mM phosphate buffer, pH 7, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide,
707 0.05% Triton X-100) for 1 to 24 hours depending on the construct. X-Gluc buffer was then removed and
708 replaced by saturated aqueous chloral hydrate solution to allow clearing of the tissues.

709 **Microscopic analysis**

710 GUS-stained, *DR5::nlsYFP* and *LaPG3::nlsYFP* transformed cluster roots were embedded in 4% agarose
711 (m/v) and cut with a vibratome to produce thick sections of 70 μ m, (VT1000S, Leica Microsystems). The
712 root sections were mounted on slides in 50% glycerol. Wild type (cv. AMIGA) cluster root thin sections of
713 6 μ m were produced using a microtome (RM2165, Leica Microsystems). They were counterstained for
714 5 min either with 0.05% toluidine blue or with 0.1% ruthenium red in 1X phosphate buffer saline (1X PBS
715 pH 7.4, Sigma Aldrich, P3813). GUS-stained and wild type cluster root sections were observed with a
716 color camera on an Olympus BX61 epifluorescence microscope (Tokyo, Japan) with Camera ProgRes® C5
717 Jenoptik and controlled by ProgRes Capture software (Jenoptik, Jena, Germany). *DR5::nlsYFP* and
718 *LaPG3::nlsYFP* transformed cluster roots were observed with a confocal microscope (Zeiss LSM 780,
719 details in “confocal microscopy” section below).

720 **Immunolabeling experiments**

721 Cross sections of 70 μ m obtained with the vibratome were transferred onto chamber slides (Lab-teak,
722 177402) for immunostaining. They were first rinsed in 0.1 M glycine in 1X PBS and then twice in 1X PBS,
723 each for 10 min. Sections were immersed in blocking buffer containing 5% bovine fetal serum (Sigma
724 Aldrich, A9418) in 1X PBS at 4 °C overnight under gentle agitation. Monoclonal primary antibodies (list in
725 Supplemental table 4) diluted 1/10 in the blocking buffer, were applied overnight at 4 °C under mild
726 agitation. The sections were washed 3 times in 1X PBS for 10 min followed by two hours incubation in
727 the secondary antibody (list in Supplemental table 4) diluted 1/500 in blocking buffer under gentle
728 agitation. Sections were then washed three times in 1X PBS under mild agitation, 10 min each. The

729 chambers were removed, and sections mounted in 50% glycerol prior to observation. Immunolabeling
730 experiments were repeated three times.

731 **Confocal microscopy**

732 Immunostained root sections were imaged on a confocal microscope (Zeiss LSM 780). Autofluorescence
733 observation was performed using an argon laser at 405 nm and secondary antibodies were excited at
734 561 nm. Both were detected at a 566-679 nm window using the same settings (gain, offset, resolution)
735 to allow quantification measurements. For *LaPG3::nlsYFP*, *DR5::nlsYFP* and screening of transformed
736 cluster roots, mCherry internal control and nlsYFP were excited at 561 nm and 514 nm respectively and
737 detected at 583-696 nm and 519-583 nm windows, respectively. Observations were made using Plan-
738 Achromat 10x/0.45 M27 and 20x/0.8 M27 objectives. Image acquisition was performed with the Zeiss
739 ZEN black 2010 software and image analysis was conducted using the ZEN blue 2.3 lite software (Carl
740 Zeiss Microscopy).

741 **Oligogalacturonide characterization and quantification**

742 OG characterization and quantification in cluster root samples from the emergence zone and tip zone
743 were performed using the method published in [Voxeur et al., 2019](#).

744 **Homology modeling**

745 The LaPG3 model was created using I-TASSER software for protein structure and function prediction
746 (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>; [Zhang, 2008](#)) with *Aspergillus aculeatus*
747 rhamnogalacturonase A (PDB: 1RMG, [Pettersen et al., 1997](#)) as the best template. While these enzymes
748 share rather low sequence identity (24.93%), they have high structural homology with estimated RMSD
749 $6.6 \pm 4.0 \text{ \AA}$. The LaPG3 model consisted of 425 AA excluding the 24 AA of the signal peptide. The modeled
750 structure was compared with that of *Pectobacterium carotovorum* PG (PDB:1BHE), *Aspergillus niger* PGII
751 (PDB:1CZF) and *Aspergillus aculeatus* PG (PDB:1IA5). UCSF Chimera (<http://www.cgl.ucsf.edu/chimera/>)
752 was used for creation of graphics ([Pettersen et al., 2004](#)).

753 **Statistical analysis**

754 Statistical analysis for phenotyping experiments and confocal image analysis were performed using
755 GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, California USA).

756 **Data availability**

757 White lupin gene identifiers and full genomic sequences are available on the White Lupin Genome Portal
758 ([Hufnagel et al., 2020](#); www.whitelupin.fr). The RNAseq data have been deposited at NCBI under the
759 temporary name "SUB9968787", bioproject "SAMN20089781".

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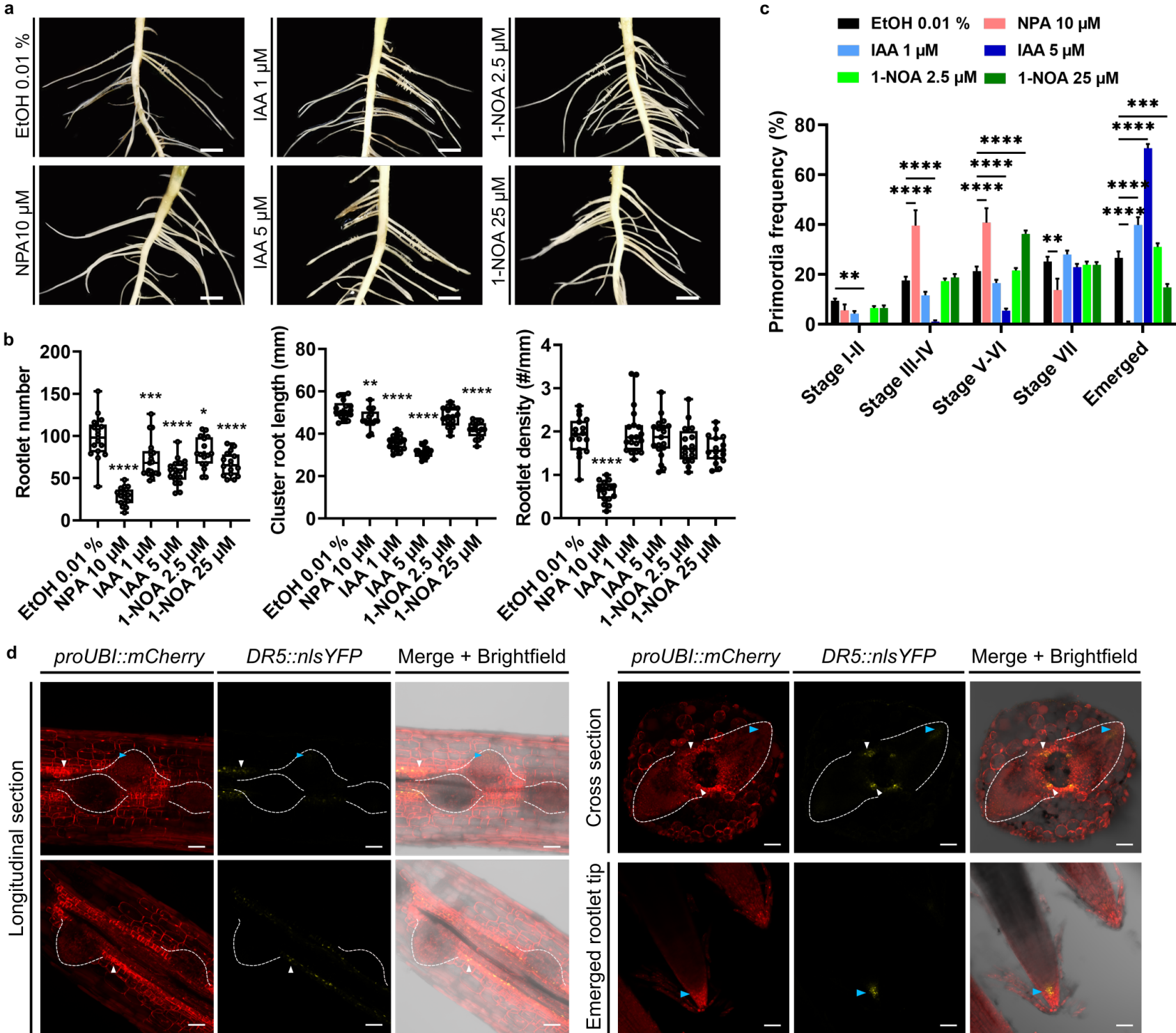


Fig.1: Auxin regulates rootlet development and primordium emergence.

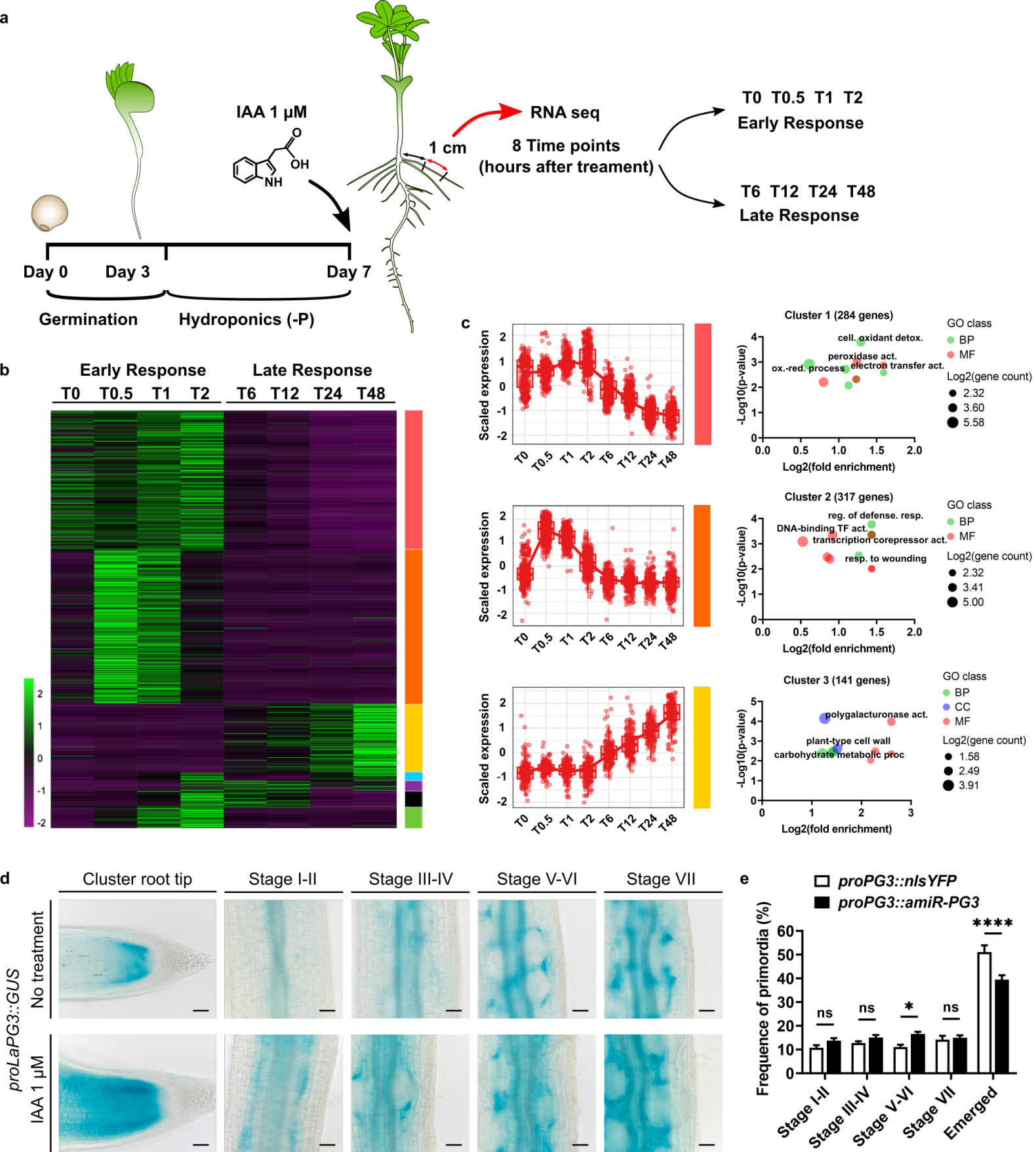


Fig.2: Auxin transcriptome landscape identifies cell wall related genes linked to rootlet emergence.

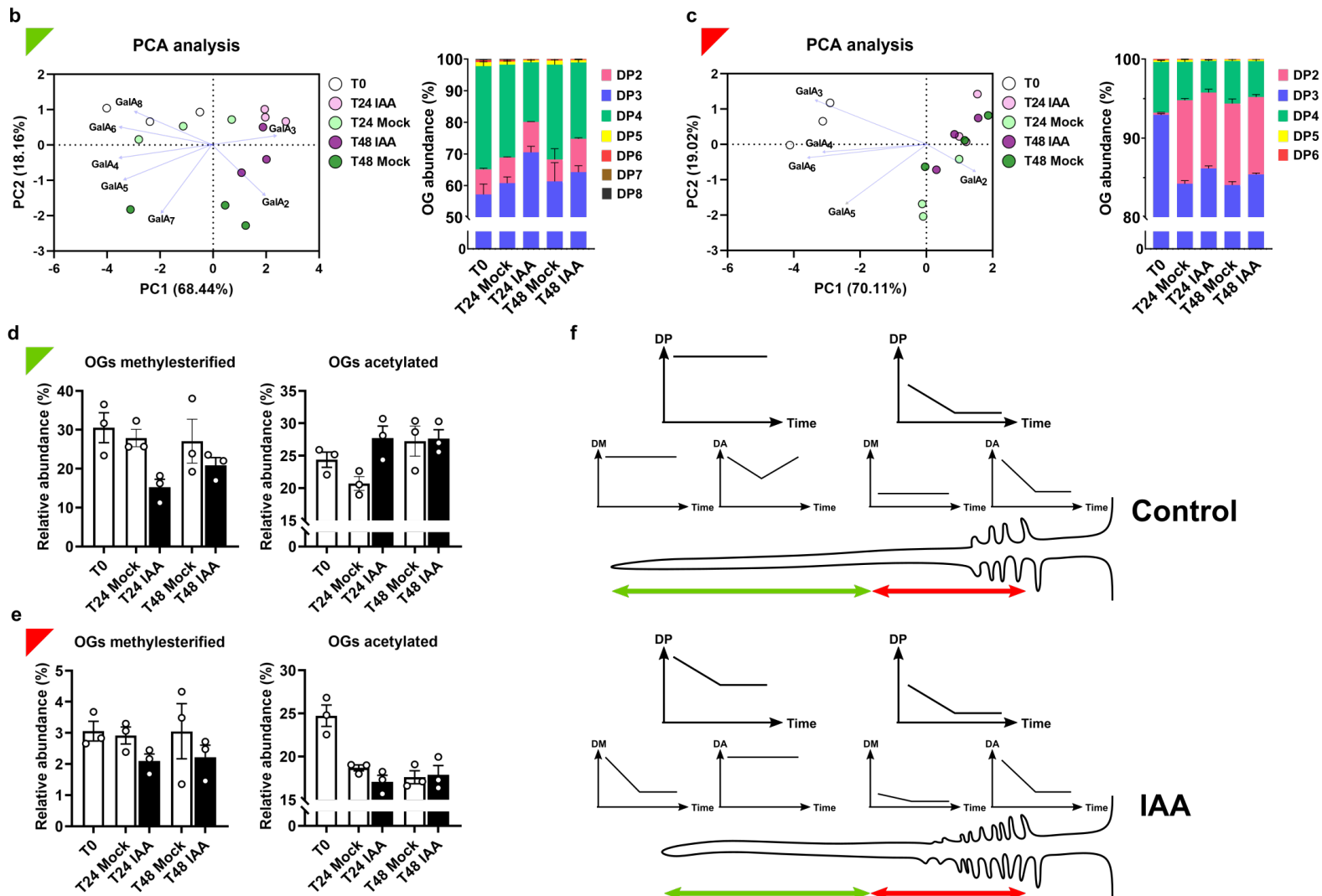
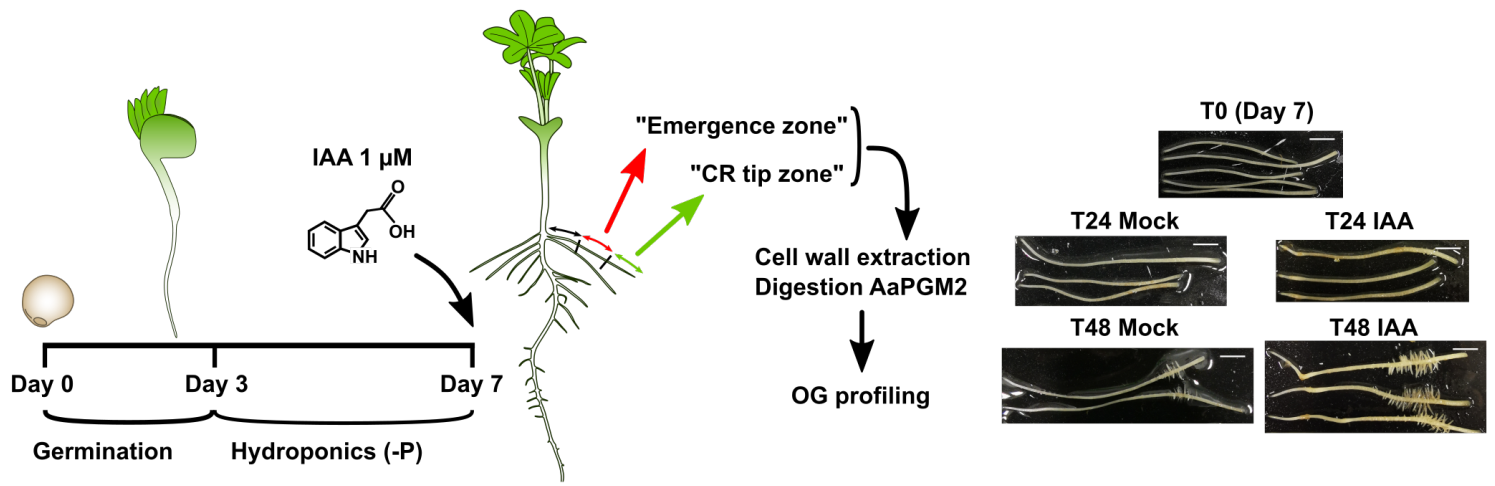


Fig.3: Oligogalacturonides profiling reveals auxin-induced pectin remodeling in cluster roots.

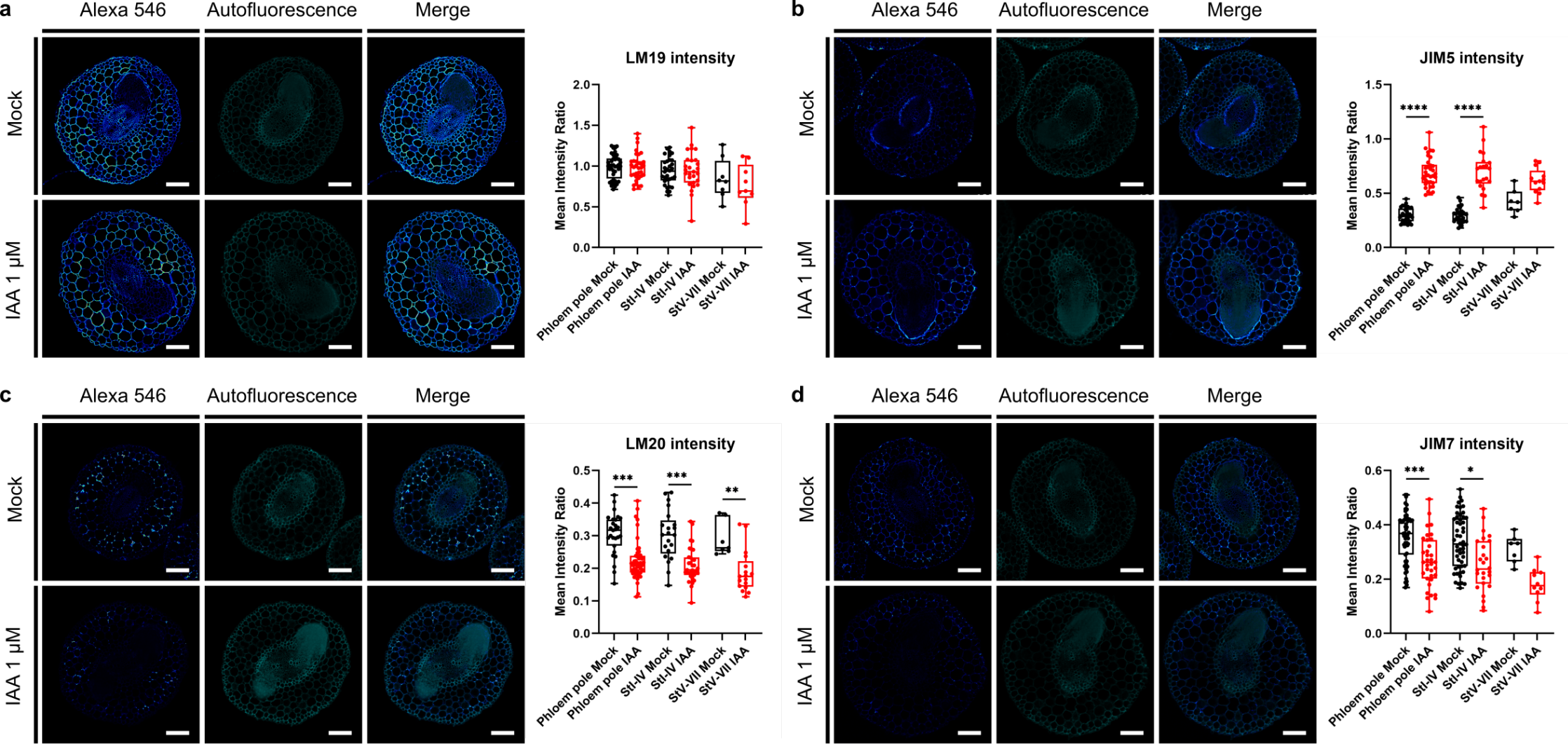


Fig.4: Auxin induces demethylesterification in cluster root cortical tissues independently of rootlet primordia emergence stage and location.

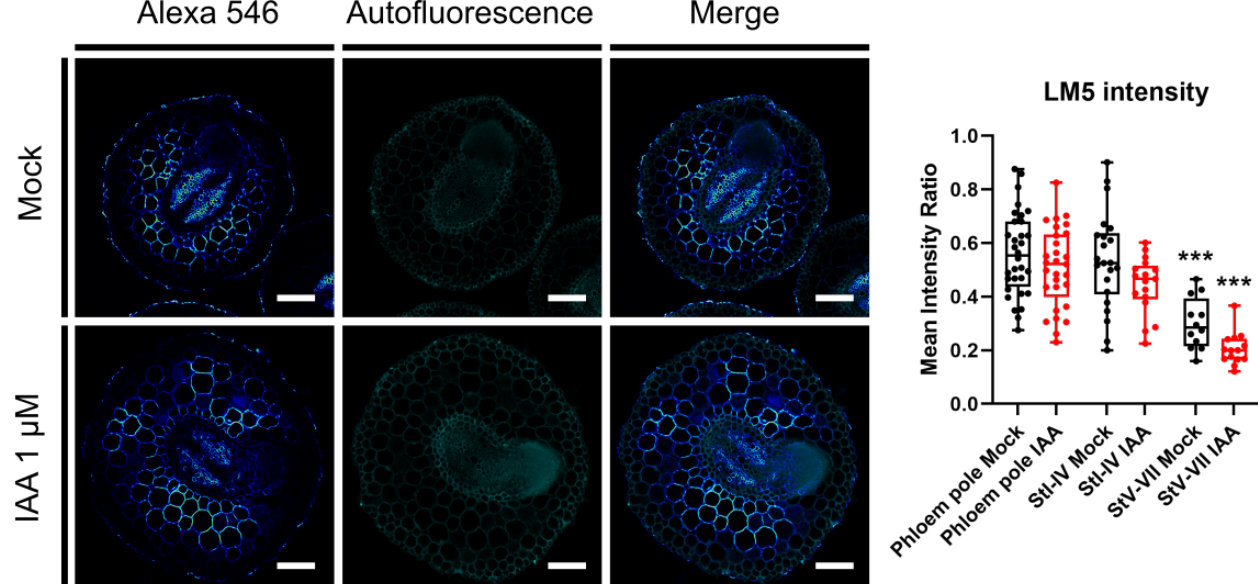


Fig.5: Rhamnogalacturonan I (1,4)- β -D-galactan is depleted in rootlet primordia overlaying cells independently of auxin treatment.