1 **Developmental atlas of white lupin cluster roots**

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3 Cécilia Gallardo, Bárbara Hufnagel, Alexandre Soriano, Fanchon Divol, Laurence Marquès,

4 Patrick Doumas and Benjamin Péret[*]

5

6 BPMP, Univ Montpellier, CNRS, INRAE, Institut Agro, Montpellier, France

7

8 **Correspondence**

9 [*]Corresponding author,

10 tel : (+33)499612859

11 e-mail : benjamin.peret@cnrs.fr

12

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35 **Running title**

36 Developmental atlas of white lupin cluster roots

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38 **Highlight**

39 White lupin cluster roots consist in the formation of numerous rootlets whose development

40 can be divided in 8 stages and involves divisions in the pericycle, endodermis and cortex.

41

42 **Abstract**

43 During the course of evolution, plants have developed various strategies to improve

44 micronutrient acquisition, such as cluster roots. These spectacular structures are dedicated to

45 efficient phosphate remobilization and acquisition. When exposed to Pi-limitation, white

46 lupin forms cluster roots made of dense clusters of short specialized roots, called rootlets.

47 Although the physiological activity of rootlets has been well studied, their development

48 remains poorly described. Here, we provide a developmental atlas of white lupin early rootlet

49 development, using molecular markers derived from the model plant Arabidopsis. We first

50 focused on cell division patterns to determine which cells contribute to the rootlet

51 primordium. Then, we identified homologs of previously described tissue specific genes

52 based on protein sequence analysis and also using detailed transcriptomic data covering

53 rootlet development. This study provides a comprehensive description of the developmental

54 phases of rootlet formation, highlighting that rootlet primordium arises from divisions in

55 pericycle, endodermis and cortex. We describe that rootlet primordium patterning follows

56 eight stages during which tissue differentiation is established progressively.

57

58 **Keywords (6-10)**

59 White lupin, cluster roots, rootlets, root development, patterning, primordium, phosphate.

60

61 **Introduction**

62 Cluster roots (CRs) are considered to be one of the major adaptations to improve plant

63 nutrient acquisition, together with nitrogen-fixing nodules and mycorrhizae (Neumann and

64 Martinoia, 2002). However, CRs differ from nodules and mycorrhizae as they form without

65 the presence of a symbiotic partner (Lamont, 2003). CR bearing species can be found in soils

66 where nutrients are poorly available (Dinkelaker *et al*., 1995; Lambers *et al*., 2003). Plants

67 forming CRs can absorb inorganic phosphate (Pi) at a faster rate than non-forming CRs plants

68 and access to a larger pool of Pi due to increased soil exploration (Vorster and Jooste, 1986).

69 Because this developmental adaptation has a selective advantage, CR arose in a whole range
70 of distant families from Cyperaceae and Restionaceae in monocots to Betulaceae,
71 Casuarinaceae, Cucurbitaceae, Eleagnaceae, Fabaceae, Moraceae, Myricaceae and Proteaceae
72 in dicots (Dinkelaker *et al*., 1995; Lambers *et al*., 2003). Interestingly, most of these families
73 have lost the ability to form mycorrhizae (Oba *et al*., 2001; Delaux *et al*., 2014; Maherali *et*
74 *al*., 2016).

75 CR is an adaptive trait of plants to cope with P-depleted soils (Neumann and Martinoia, 2002;
76 Lambers *et al*., 2015). Because phosphate is mostly concentrated in the topsoil layer, CRs are
77 mainly produced in the upper part of the root system (Lynch and Brown, 2001). Hereafter,
78 CRs will refer to the entire length of the lateral root that comprises at least one cluster of
79 tightly grouped third-order roots termed rootlets (Dinkelaker *et al*., 1995; Skene, 1998). CRs
80 are an exacerbated developmental response that results from massive induction of rootlets and
81 represent a good model to study root adaptive developmental responses to abiotic constraints.

82 White lupin (*Lupinus albus*) is one of only few crops that can form CRs (Fig. 1A-C). It has
83 received most attention for studies on CR morphology and physiology (Johnson *et al*., 1996;
84 Watt and Evans, 1999; Hagström *et al*., 2001; Neumann and Martinoia, 2002; Uhde-Stone *et*
85 *al*., 2003; Cheng *et al*., 2011). White lupin (WL) can form up to 35-45 rootlets per cm
86 (Dinkelaker *et al*., 1989) and secrete massive amount of organic acids and protons
87 (Massonneau *et al*., 2001; Sas *et al*., 2001; Neumann and Martinoia, 2002). Such
88 physiological modifications can increase the Pi availability of soil, resulting in a beneficial
89 effect to other species in mixed cultures (Braum and Helmke, 1995; Cu *et al*., 2005; Li *et al*.,
90 2010). For instance, mixed culture of wheat and white lupin was shown to increase shoot Pi
91 uptake up to 45 % in wheat (Cu *et al*., 2005). This shows how white lupin can be used to
92 improve the poor efficiency of Pi uptake by several crops for a sustainable agriculture
93 perspective.

94 In many angiosperms, secondary roots (or lateral roots) arise from pericycle as described in
95 the model plant *Arabidopsis thaliana* (Laskowski *et al*., 1995; Malamy and Benfey, 1997;
96 Orman-Ligeza *et al*., 2013; Herrbach *et al*., 2014). In some monocots, lateral roots show
97 contribution of dividing endodermal cells including some Poaceae like rice (*Oriza sativa*) and
98 maize (*Zea mays*) (Hochholdinger *et al*., 2004). In dicots, some Legume species also show
99 contribution of division in endodermis and cortex including soybean (*Glycine max*), *Lotus*
100 *japonicus* and alfalfa (*Medicago truncatula*) (Byrne *et al*., 1977; Op den Camp *et al*., 2011;
101 Herrbach *et al*., 2014). In Arabidopsis, it was proposed that lateral root primordium
102 development proceeds through an 8-stage process (Malamy and Benfey, 1997) that can be

103 summed up as a two-step developmental model (Laskowski *et al*., 1995). Early

104 developmental phase of lateral root primordium (stage I-IV) starts with the first division in

105 two adjacent pericycle cells followed by several rounds of divisions to produce a four-layered

106 primordium (Malamy and Benfey, 1997). Then, cells start to differentiate and acquire various

107 identities as tissues organize around a meristematic zone (stage V-VII) (Trinh *et al*., 2018).

108 Transition between these two developmental phases marks the onset of quiescent centre

109 establishment and formation of meristematic initials (Goh *et al*., 2016). With regards to these

110 processes, very little information is available for third-order roots, both in Arabidopsis and

111 lupin. In WL, it was described that xylem pole pericycle cells are recruited to become founder

112 cells (Johnson *et al*., 1996) and a predominant role for auxin was demonstrated (Gallardo *et*

113 *al*., 2019). However, a full description of the entire developmental process of rootlet

114 formation is still missing.

115 In an effort to generate a developmental atlas of rootlet formation in white lupin, we

116 performed an anatomic study to define discrete developmental stages throughout early rootlet

117 development. Since the root structure of WL is more complex than Arabidopsis root, it

118 appeared important to use a set of various molecular markers to identify division and

119 differentiation events. We first used the cell division marker *CYCLIN B1;1* (*CYCB1;1*) from

120 Arabidopsis whose promoter revealed to drive expression in WL dividing cells. This allowed

121 us to identify cell division in pericycle but also in endodermal and cortical cells during rootlet

122 formation. In a second approach, we decided to take advantage of a set of tissular markers and

123 identify their homolog genes in WL genome. We combined sequence analysis to detailed

124 expression profiling to select the best candidates amongst WL highly duplicated gene

125 families. We generated transgenic hairy root plants expressing these tissular markers that

126 show a strong conservation of tissular specificity. Using this approach, we provide a

127 developmental atlas of cell division and differentiation during white lupin rootlet primordium

128 development.

129

130 **Materials and methods**

131 *Plant materials and growth conditions*

132 Seeds of white lupin (*Lupinus albus L. cv. Amiga*) calibrated at 7 mm were used in all

133 experiments. White lupin plants were cultivated in growth chambers under controlled

134 conditions (16 h light / 8 h dark, 25°C day / 20°C night, 65 % relative humidity, and PAR

135 intensity 200 $\mu mol.m^{-2}.s^{-1}$) in hydroponic conditions. The hydroponic solution was modified

136 from (Abdolzadeh *et al*., 2010) without phosphate, and was composed of: $MgSO_4$ 54 μM;

137 $Ca(NO_3)_2$ 400 μM; K_2SO_4 200 μM; Na-Fe-EDTA 10 μM; H_3BO_3 2.4 μM; $MnSO_4$ 0.24 μM;

138 $ZnSO_4$ 0.1 μM; $CuSO_4$ 0.018 μM; Na_2MoO_4 0.03 μM. Plants were grown either in 1,6 L pots

139 or 200 L tanks. Solution was aerated continuously. For plants in pots, the nutrient solution

140 was renewed every 7 days.

141

142 *Molecular cloning*

143 The primers to amplify the promoter sequences of *LaSCR1*, *LaWOL*, *LaPEP*, *LaEXP7* were

144 designed using Primer3plus (http://www.bioinformatics.nl/cgi-

145 bin/primer3plus/primer3plus.cgi). All primer sequences used are summarized in Table S1.

146 Primers were used to amplify at the minimum 1500 bp upstream of the start codon from white

147 lupin genomic DNA. Sizes of all the promoters amplified are summarized in Table S1. AttB1

148 (5'-GGGGCCAAGTTTGTACAAAAAAGCAGGCT-3') and attb2 (5'-

149 CCCCCCACTTTGTACAAGAAAGCTGGGT-3') adapters were added to the primers.

150 Amplified fragments were subsequently cloned into the pDONR221 by Gateway reaction.

151 The promoters were then cloned into the binary plasmid pKGWFS7 containing a GFP-GUS

152 fusion by Gateway cloning.

153

154 *Bacterial strain*

155 *Agrobacterium rhizogenes* strain *ARquaI* was used to perform *hairy root* transformation of

156 white lupin. Bacteria were transformed with the binary plasmid by electroporation.

157 Transformation was confirmed by PCR and sequencing. LB agar plates added with sucrose

158 2%, acetosyringone 100 μM and appropriate antibiotics were inoculated with 200 μL of liquid

159 bacteria culture, and incubated at 28°C for 24 h to get a bacterial lawn. Bacterial lawn was

160 used for white lupin seedling transformation.

161

162 *Hairy root transformation of white lupin*

163 White lupin hairy root transformation was performed as previously described (Uhde-Stone *et*

164 *al*., 2005). White lupin seeds caliber 8 mm were surface sterilized by 4 washes in osmosis

165 water, 30 min sterilization in bleach (Halonet 20%) and washed 6 times in sterile water under

166 sterile conditions. Seed were germinated on half MS medium (pH was adjusted to 5.7). After

167 germination, radicles of 1 cm were cut over 0.5 cm with a sterile scalpel. The radicles were

168 inoculated with the *Agrobacterium rhizogenes* lawn. Fifteen inoculated seedlings were placed

169 on square agar plates (0.7 % agar in 1X Hoagland solution) containing 15 μg.mL^{-1}

170 Kanamycin. Composition of Hoagland medium without phosphate was the following one:

171     $MgSO_4$ 200 µM; $Ca(NO_3)_2$ 400 µM; KNO_3 325 µM; NH_4Cl 100 µM; Na-Fe- EDTA 10 µM;

172   H_3BO_3 9.3 µM; $MnCl_2$ 1.8 µM; $ZnSO_4$ 0.17 µM; $CuSO_4$ 0.06 µM; Na_2MoO_4 2.3 µM. Plates

173   were placed vertically in controlled conditions: 16 h light / 8 h dark, 25°C day / 20°C night,

174   65 % relative humidity, and PAR intensity 200 $\mu mol.m^{-2}.s^{-1}$. After 7 days on plates, 60

175   seedlings were transferred to 12x16.5x5.5 cm trays (20 seedlings per tray) and watered with

176   500 mL pure water. After 12 days, plants presenting hairy roots were transferred to

177   hydroponics in 1.6 L pots containing nutrient solution. Nutrient medium was renewed each

178   week. After 7 days in hydroponic conditions, CRs were sampled on *hairy root* plants.

179

180   *Histochemical analysis*

181   Histochemical staining of β-glucuronidase was performed on CRs from *hairy root* plants.

182   Samples were incubated in a phosphate buffer containing 1 $mg.mL^{-1}$ X-gluc as a substrate (X-

183   Gluc 0.1 %; phosphate buffer 50 mM, pH 7, potassium ferricyanide 2 mM, potassium

184   ferrocyanide 2 mM, Triton X-100 0.05 %). Zone of CRs with non-emerged rootlets were

185   cutted into 4 to 5 sections and fixed (formaldehyde 2%, glutaraldehyde 1%, caffeine solution

186   1%, phosphate buffer at pH 7). Fixation was performed for 2.5 h under agitation at room

187   temperature and then 1.5 h at 4°C.

188

189   *Microscopy analysis*

190   To generate thin sections, roots were progressively dehydrated in ethanol solutions with

191   increased concentrations: 50% (30 min), 70% (30 min), 90% (1 h), 95% (1 h), 100% (1 h),

192   100% (overnight). Samples were impregnated with 50% pure ethanol and 50% resin (v/v) for

193   2 days, then in 100 % resin for 5 days. CRs were embedded in Technovit 7100 resin (Heraeus

194   Kulze, Wehrheim, Germany) according to the manufacturer's recommendations. Thin

195   sections of 10 µm were produced using a microtome (RM2165, Leica Microsystems) and

196   counterstained for 30 min with 0.1% ruthenium red and rinsed two times with ultrapure water.

197   For nuclei visualization, 10 µm thick cluster roots sections were stained for 10 min with 3 µM

198   DAPI solution in PBS buffer at room temperature. After washing root three times with PBS

199   buffer for 5 min, the section were mounted in PBS buffer and analyzed. An argon laser at 405

200   nm provided excitation for DAPI staining. The fluorescence emission signal was detected

201   using a band-pass filter of 420-480 nm for DAPI. Imaging was performed in Montpellier RIO

202   imaging Platform (http://www.mri.cnrs.fr/fr/) with a stereomicroscope (SZX16, Olympus) for

203   macroscopic root images, an epifluorescence microscope with a colour camera (Olympus

204   BX61 with Camera ProgRes®C5 Jenoptik and controlled by ProgRes Capture software) for

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205 thin root section, and a confocal microscope (Leica SP8, Leica Microsystems,

206 https://www.leica-microsystems.com/fr/) for nuclei visualization.

207

208 *Phylogenetic trees*

209 White lupin cDNA-deduced protein sequences were retrieved from the available white lupin

210 genome sequence tool (https://www.whitelupin.fr/). Arabidopsis protein sequences were

211 extracted from TAIR database (https://www.arabidopsis.org) and protein sequences from

212 *Lupinus angustifolius, Medicago truncatula, Cicer arietinum, Glycine max* were downloaded

213 from NCBI database (https://www.ncbi.nlm.nih.gov/guide/proteins/). Phylogenetic trees were

214 constructed using the phylogeny tool NGPhylogeny.fr (https://ngphylogeny.fr/) (Lemoine *et*

215 *al*., 2019). The bootstrap consensus tree was inferred from 500 replicates. Branches

216 corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

217 Sequences were aligned using MUSCLE 3.7 and Gblocks with default parameters were used

218 to eliminate poorly aligned positions and divergent regions. PhyML 3.0 was used to perform

219 phylogenetic inference, using SH-like as aLRT test. Tree visualization was performed through

220 the iTOL v4.4.2 platform (Letunic and Bork, 2019).

221

222 **Results**

223

224 *Describing the developmental stages of rootlet primordium*

225 To provide a detailed description of cluster root development, thin longitudinal sections of 7

226 to 9 day-old lupin seedlings were stained with ruthenium red to observe all developmental

227 stages. Early events of rootlet development could be described in 8 discrete developmental

228 stages (Fig. 1D).

229 **Stage I.** Initiation is the first visible event of CR formation. It consists of two asymmetrical

230 anticlinal divisions in the pericycle (Fig. 1D StIa). Initiation continues with the appearance of

231 divisions in parallel orientation compared to the root axis (Fig. 1D StIb, black arrow). In the

232 longitudinal plane, close to 6 cells show these periclinal divisions. Peripheral cells are not

233 dividing, creating the boundaries of the primordium. In the overlaying endodermis, an

234 increased number of anticlinal divisions is clearly seen as compared to the surrounding

235 endodermal cells. About 8 cells are formed from these divisions.

236 **Stage II.** All pericycle cells participating in the rootlet primordium seem to have divided.

237 These divisions in the pericycle lead to the formation of two layers, named P1 (inner layer)

238 and P2 (outer layer). Following these divisions, cells start to swell and expand in the radial

239   direction. Simultaneously, periclinal divisions occur in the endodermis that divides this layer

240   into E1 (inner layer) and E2 (outer layer) (Fig. 1D StII, black arrow).

241   **Stage III.** A dozen of cells continue to divide in the 4-layered primordium. Anticlinal

242   divisions are seen in the most peripheral cells in the endodermis. At the same time, anticlinal

243   divisions are also seen in the cortex (Fig. 1D StIII, purple arrows).

244   **Stage IV.** A typical dome shaped rootlet primordium begins to form. The dividing cortex

245   comprises 10 cells in length. Four cells at the base of the primordium started to divide

246   between the xylem pole and the P1 inner layer (Fig. 1D StIV, black arrow) giving rise to the

247   future vasculature. Numerous radial, anticlinal and periclinal divisions happen in the tissues

248   derived from pericycle, endodermis and cortex.

249   **Stage V.** Primordium expands radially, pushing the overlaying cortical layers. Cells at the

250   base of the primordium (Fig. 1D StV, black arrow) continue to divide. The number of cells in

251   the overlaying cortex increases to 11 cells in length and are now forming part of the tip of the

252   primordium.

253   **Stage VI.** The rootlet primordium is now strongly deforming the cortical layers around. The

254   stage VI primordium begins to look like a mature root with several layers, although a typical

255   meristematic organization is not clear yet due to the apparent absence of a quiescent centre. In

256   the absence of tissue-specific markers, it is difficult to make strong assumption regarding the

257   nature of each tissue. The presence of elongated cells at the base of the primordium seems to

258   indicate the onset of the vasculature.

259   **Stage VII.** The rootlet primordium is about to cross the last layer of the cluster root, the

260   epidermis. As the primordium enlarges, distinguishing the different tissues inside the growing

261   rootlet becomes challenging due to the high number of cells. Many anticlinal divisions seem

262   to continue in the different layers of the primordium. The vascular tissue seems to be now

263   fully established as many elongated cells are connecting the cluster root stele to the rootlet

264   primordium.

265   **Stage VIII.** No visible difference can be seen in the cellular organization of the primordium

266   but cell elongation pushes the primordium beyond the epidermis and the primordium reaches

267   the rhizosphere.

268

269   *Following cell divisions during rootlet primordium formation*

270   Although many cell divisions can be seen from cell shape and the appearance of extra

271   division planes, the use of a molecular division marker allows to easily monitor mitotic

272   activity and define dividing cells at an early stage. The promoter of the *Arabidopsis thaliana*

273   *CYCB1;1* gene is activated during the G2/M transition (Colón-Carmona *et al*., 1999). We

274   transformed WL roots with a construct containing the Arabidopsis *CYCB1;1* promoter fused

275   to the ß-glucuronidase reporter gene (pAt*CYCB1;1:GUS*). This marker shows expression in

276   the cluster root apex, very similar to the expression described in Arabidopsis, suggesting a

277   conservation of the expression during cell cycle (Supplementary Figure S1A). Similarly,

278   expression is visible at early stages of rootlet initiation and throughout the developmental

279   process up to their emergence (Supplementary Figure S1B-D). We next produced thin cross

280   sections to analyse in detail the mitotic activity in cells of the rootlet primordia. We observed

281   that the first visible event of rootlet initiation is the activation of the pAt*CYCB1;1:GUS*

282   marker in one pericycle cell in front of each xylem pole (Fig. 2A), before division is visible

283   from an anatomical point of view, defining Stage 0. Subsequently, one or more divisions are

284   seen in the pericycle cells (Fig. 2B,C) suggesting that more than one cell participate in the

285   creation of the primordium. Then, divisions are also seen in the endodermis (Fig. 2C) and

286   cortex (Fig. 2D). These divisions seem mainly periclinal and therefore produce extra layers of

287   these tissues. Once the rootlet primordium reaches emergence, several divisions can be seen

288   in what seems to be a fully dividing meristematic zone (Fig. 2E).

289

290   *Expression of tissue-specific markers in CR*

291   Classic histology allows to describe precisely the early developmental stages of rootlet

292   formation and to define discrete stages associated with particular anatomic features. However,

293   cell types cannot be easily identified based solely on their shape during early developmental

294   stages. To which extent pericycle, endodermis and cortex tissues are involved in rootlet

295   initiation and rootlet outgrowth has yet to be determined. Another matter is to determine when

296   tissues are starting to differentiate. To address these questions, a limited number of molecular

297   markers (using the ß-glucuronidase gene as reporter) specifically expressed in different tissue

298   types were tested. The corresponding white lupin genes were chosen (1) based on the protein

299   sequence homology between known molecular markers in the model plant *Arabidopsis*

300   *thaliana* and (2) based on their expression profile in the white lupin transcriptomic dataset.

301   Due to genome triplication, gene families are often larger in white lupin than in Arabidopsis

302   (Hufnagel *et al*., 2020) and expression data helped directing our choice.

303   First, we chose a list of molecular markers based on their tissular expression in *Arabidopsis*

304   *thaliana*: two endodermal markers (WOL, SCR1), one cortical markers (PEP) and one

305   epidermal marker (EXP7) (Sbabou *et al*., 2010; Marquès-Bueno *et al*., 2016; Sevin-Pujol *et*

306   *al*., 2017). Orthologous lupin genes were found by comparing Arabidopsis protein sequences

307   with all the cDNA-deduced protein sequences of WL genome. Comparison was made by

308   multiple comparison alignment by local BLAST on the white lupin database and by

309   generating phylogenetic trees containing the Arabidopsis sequence as well as Legume

310   relatives: *Medicago truncatula*, *Cicer arietinum*, *Lupinus angustifolius* and *Glycine max* (Fig.

311   3). On the other hand, expression profiles of the selected WL genes were checked in a

312   transcriptomic RNAseq database describing the spatial portions of a 5 days-old cluster root

313   and therefore covering the different developmental stages from S0 to S7 (Fig. 4A) (Hufnagel

314   *et al*., 2020). All genes were found to be expressed during rootlet development and genes

315   were discriminated on their level of expression. For each gene family (3 to 4 genes), one gene

316   was found to be more highly expressed that the others and was selected assuming that high

317   gene expression would translate into a nicely visible marker (Fig. 4B-D). As a result, we

318   selected the following WL genes for further analysis: *Lalb_Chr04g0258751* (WOL),

319   *Lalb_Chr19g0123861* (SCR1), *Lalb_Chr11g0065071* (PEP) and *Lalb_Chr09g0324651*

320   (EXP7). Gene sequence and expression data can be found on the White Lupin genome portal

321   (https://www.whitelupin.fr/). The WL promoters (at least 1500 bp upstream start codon) of

322   these genes were cloned upstream the ß-glucuronidase (GUS) sequence and introduced into

323   WL roots via *Agrobacterium rhizogenes*-mediated root transformation (Uhde-Stone *et al*.,

324   2005).

325   To characterize tissue specific expression, their expression pattern was first assessed into

326   secondary-order roots *i.e.* apex of cluster roots. CRs stained for GUS for each marker are

327   shown in Fig. 5, as well as cross sections produced in these lines. The *pLaWOL:GUS* line was

328   expressed in the stele of CR (Fig. 5A-B). Staining was not observed in the elongation zone

329   but was seen in the differentiation zone in the stele (Fig. S2A). A transverse section through

330   the differentiation zone shows GUS staining in stellar tissue along xylem axis and pericycle

331   cells facing the xylem poles (Fig. 5B). Staining in the stele disappears in the primordium

332   patterning zone and became restricted only to pericycle cells and the stele of the primordium.

333   (Fig. S2B). *pLaWOL:GUS* driven expression was observed in the stele of emerged rootlet

334   with a stronger staining in the meristematic zone were QC is expected to locate (Fig. S2C-E).

335   Expression of *pLaSCR1:GUS* in cluster roots matched previous reports (Sbabou *et al*., 2010)

336   and was strongly specific of the endodermis of CR tip (Fig. 5C,D). Promoter expression was

337   also observed early on in rootlet primordia (Fig. S2F,G) and more specifically in the rootlet

338   meristematic zone after their emergence (Fig. S2H-I). Expression of the *pLaPEP:GUS*

339   construct was specific of cortical cells (Fig. 5E,F). Promoter is strongly expressed in the root

340   meristematic zone, and is not expressed in endodermis/cortex initial and the QC (Fig. 5E). A

341   transverse section in the beginning of elongation zone is shown in Fig. 5F showing the

342   specificity of the cortical expression. Expression tends to fade away in the elongation zone in

343   the shootward direction (not shown). Similar pattern for *pLaPEP:GUS* was observed in

344   primordium and emerged rootlet (Fig. S2K-O). Expression of the *pLaEXP7:GUS* construct

345   was absent from the very tip of the CR meristem and started at the onset of the elongation

346   zone (Fig. 5G). A cross section performed in the differentiation zone showed the specificity of

347   the epidermal expression (Fig. 5H). Staining was still present in the epidermal cells in the

348   primordium patterning zone (Fig. S2P) but was absent from primordium (Fig. S2Q) and

349   young rootlets (Fig. S2R,S). Expression resumes in older rootlets when elongation zone is

350   fully active (Fig. S2T). The study of the 4 selected marker genes suggested that 3 of them

351   could be used to further analysis during early rootlet primordium development (since *LaEXP7*

352   shows no expression). We therefore selected *pLaWOL:GUS*, *pLaSCR1:GUS* and

353   *pLaPEP:GUS* to provide a detailed description of rootlet developmental stages and tissue

354   differentiation.

355

356   *Tissue specific marker expression throughout rootlet primordia development*

357   The various stages of rootlet primordia development were studied from the early divisions up

358   to their emergence in order to determine when tissues differentiate and to provide a

359   developmental atlas of rootlet primordia development. We combined thick longitudinal cross

360   sections (Fig. 6) to provide stronger GUS staining together with thin radial cross sections

361   (Fig. 7) for better accuracy. The *pLaWOL:GUS* marker showed strong signal as early as stage

362   II (Fig. 6A) corresponding to 6 pericycle cells in P1/P2 right after the first periclinal division

363   (Fig. 7A). At later stages, the staining pattern becomes limited to a group of cells at the base

364   of the primordium that might be derived from P1 (Fig. 6B,C and Fig. 7B). From stage VI

365   onward, staining is clearly observed in elongated cells at the centre of the primordium (Fig.

366   6D and Fig. 7C). This pattern of expression remains similar up to after emergence

367   corresponding to the newly developed vasculature (Fig. 6E and Fig. 7D). The *pLaSCR1:GUS*

368   endodermal marker expression is first seen in 5 endodermal cells at stage II when these cells

369   appear to divide (Fig. 6F and Fig. 7E). At stage III and IV, staining is not only observed in E1

370   and E2 (Fig. 6G) but also in the overlaying cortical cells (Fig. 7F). At stage VI,

371   *pLaSCR1:GUS* expression starts to be restricted to two inner layers that are surrounding the

372   stele with a typical U-shaped profile (Fig. 6H). A group of cells abutting these two layers also

373   show weak GUS staining at the tip of the rootlet primordium (Fig. 7G). This expression

374   profile remains the same when the rootlet is about to emerge (Fig. 6I,J and Fig. 7H). Marker

11

375   line *pLaPEP:GUS* is not expressed in the first stages of rootlet development but is present in

376   the parental cluster root cortex (Fig. 6K-M and Fig. 7I). At stage V, a faded expression can be

377   observed in the tip of the primordium on thin sections (Fig. 7J). This faint expression profile

378   persists at stage VI (Fig. 7K). After emergence, staining is expressed in 3 to 4 cortical cell

379   layers at the edges of the primordium but is not expressed in younger cortical cell files

380   reminiscent of the expression pattern in cluster roots (Fig. 6N,O, Fig. 7L).

381

Discussion

383   Previous histological work has described cluster root development in white lupin, showing

384   that rootlets seemingly arise from divisions of pericycle cells facing xylem poles (Johnson *et*

385   *al*., 1996; Hagström *et al*., 2001; Watt and Evans, 2003). Here, we provide a detailed

386   anatomical study of rootlet development by taking advantage of molecular markers via

387   genetic transformation of white lupin roots (Uhde-Stone *et al*., 2005). Our study reveals that

388   several tissues divide to participate to the formation of the primordium as shown by the cell

389   cycle marker *pAtCYCB1;1*. As in lateral root formation of most species, divisions in the

390   pericycle cells initiate the formation of new rootlets, but several rounds of divisions in

391   endodermal and cortical cells are also observed. These are reminiscent of lateral root

392   formation in other Legumes such as Medicago (Herrbach *et al*., 2014) and other angiosperms

393   (Torres-Martínez *et al*., 2019). It is not entirely clear to which extent these divisions in the

394   outer tissues contribute to the primordium itself or to a protective layer whose fate remains

395   undescribed in white lupin. The presence of more cell layers than in the model plant

396   Arabidopsis, where most of the real time description and cell lineage work has been

397   performed, suggests that different mechanisms may be involved. These mechanisms may not

398   be solely mechanical (stronger pressure, distinct cell wall remodelling processes) but may

399   also include active cell divisions to accommodate the passage of the rootlet primordium. The

400   existence of a temporary cap-like structure has been reported in other species (Torres-

401   Martínez *et al*., 2019) and the numerous divisions in the endodermis seem to generate such

402   structure as can be seen on a Stage VI rootlet primordium (Fig. 1D). Further cell lineage

403   studies would be needed to clearly demonstrate to which extent these dividing cells from

404   outer layers (endodermis and cortex) participate to the primordium itself and hence to the

405   rootlet formation.

406   Within the rootlet primordium itself, cell division patterns (from the initiation in the pericycle

407   up to the establishment of an organized meristematic zone) is fairly similar to what has been

408   described in other plants and led us to separate its development into 8 stages, similarly to the

409   model plant Arabidopsis (Fig. 8). Although this staging could be seen as somehow artificial

410   due to the numerous extra division events in a multi-layered structure, we believe that it will

411   help comparing similar developmental stages between plant models, especially in the context

412   of transcriptomic analysis similar to the ones already described in Arabidopsis (Voß *et al.*,

413   2015). Interestingly, a meristematic structure is put into place during rootlet development and

414   several divisions occur at the final emergence stage VIII (Fig. 2E) but rootlet development

415   appears to be determinate at later stages (Watt and Evans, 1999). This implies that the

416   primordium meristematic structure, although apparently fully functional at the stages

417   described here, loses its division abilities and fully differentiates later on. Future work should

418   determine at which subsequent stages this switch operates.

419

420   **Acknowledgements**

427

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Tables

Table S1. List of gene names and ID numbers, sequence of primers used for cloning.

Figure legends

Fig. 1. Histological description of the 8 stages of rootlet primordium formation. (A) Whole root system of a 21-day old white lupin in phosphate deficiency conditions harbouring numerous cluster roots. (B) One cluster root harbouring numerous rootlets. (C) Nuclear staining (DAPI) of rootlet primordium during the emergence stage. (D) Longitudinal sections of a cluster root depicting all developmental stages of rootlet development (ruthenium red stained). Last image (StVII) is a cross section. St0: Stage 0. Parental tissues before visible events. StIa: Stage Ia. First asymmetric division in the pericycle. StIb: Stage Ib. First periclinal division in the pericycle and first anticlinal division in the endodermis. StII: Stage II. Formation of a flattened four-layered primordium: two pericycle layers and two endodermal layers. StIII: Stage III. Several rounds of extra anticlinal divisions in the endodermis and first cortical divisions. StIV: Stage IV. Divisions rate increase and start to shape a dome. StV: Stage V. The dome shape is now well established and the rootlet primordium passes through the cortical layers. StVI: Stage VI. The primordium is now half-way through the cortical layers. StVII: Stage VII. The rootlet primordium is about to emerge from the maternal root. p: pericycle, e: endodermis, c: cortex. Arrows pinpoint key division planes. Scale bars: 1 cm (A), 1mm (B), 200 µm (C), 50 µm (D).

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expressing the *pAtCYCB1;1:GUS* marker of the G2/M transition of the cell cycle. (A) Stage 0: one pericycle cell in front of each xylem pole expresses the marker. (B) Close-up view of the earliest observable event of rootlet initiation depicting one cell preparing to divide. (C) Stage II: active divisions in the pericycle and endodermis. (D) Stage IV: numerous divisions occur in the pericycle, endodermis and cortex. (E) Stage VII: just prior to emergence, numerous divisions are seen in what appears to be a fully functional and organized meristem. Scale bars are 100 µm (A) and 50 µm (B-E).

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Fig. 6. Expression of tissue-specific molecular markers during rootlet primordium development on thick longitudinal sections through cluster roots. Images are 80 µm thick sections showing GUS activity in 3 marker lines. (A-E) *pLaWOL:GUS*. (A) StII. Staining is

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Supplementary data

Fig. S1. Expression profile of the *pAtCYCB1;1:GUS* marker in white lupin cluster root is associated with sites with active cell divisions. (A) Cluster root tip shows typical spotted expression of dividing cells in the meristematic region. (B) In the differentiation zone, sites of rootlet formation show expression of the marker at early initiation stages. (C) Later on, cell divisions in the rootlet primordium are also seen. (D) Cell divisions can be seen in the active rootlet primordium upon their emergence. Bars are 350µm.

Fig. S2. Expression profiles of four white lupin promoters in cluster roots. Promoters profiles shown are: *pLaWOL:GUS* (A-E), *pLaSCR1:GUS* (F-J), *pLaPEP:GUS* (K-O) and *pLaEXP7:GUS* (P-T). Whole-mount view of early differentiation zone (A, F, K and P), late differentiation zone with late stages rootlet primordia (B, G, L and Q), rootlet emergence (C, H, M and R), rootlet midgrowth stage (D, I, N and S) and fully grown rootlets (E, J, O and T). Scale bars are 150 µm (column 1-4), 500 µm (column 5).

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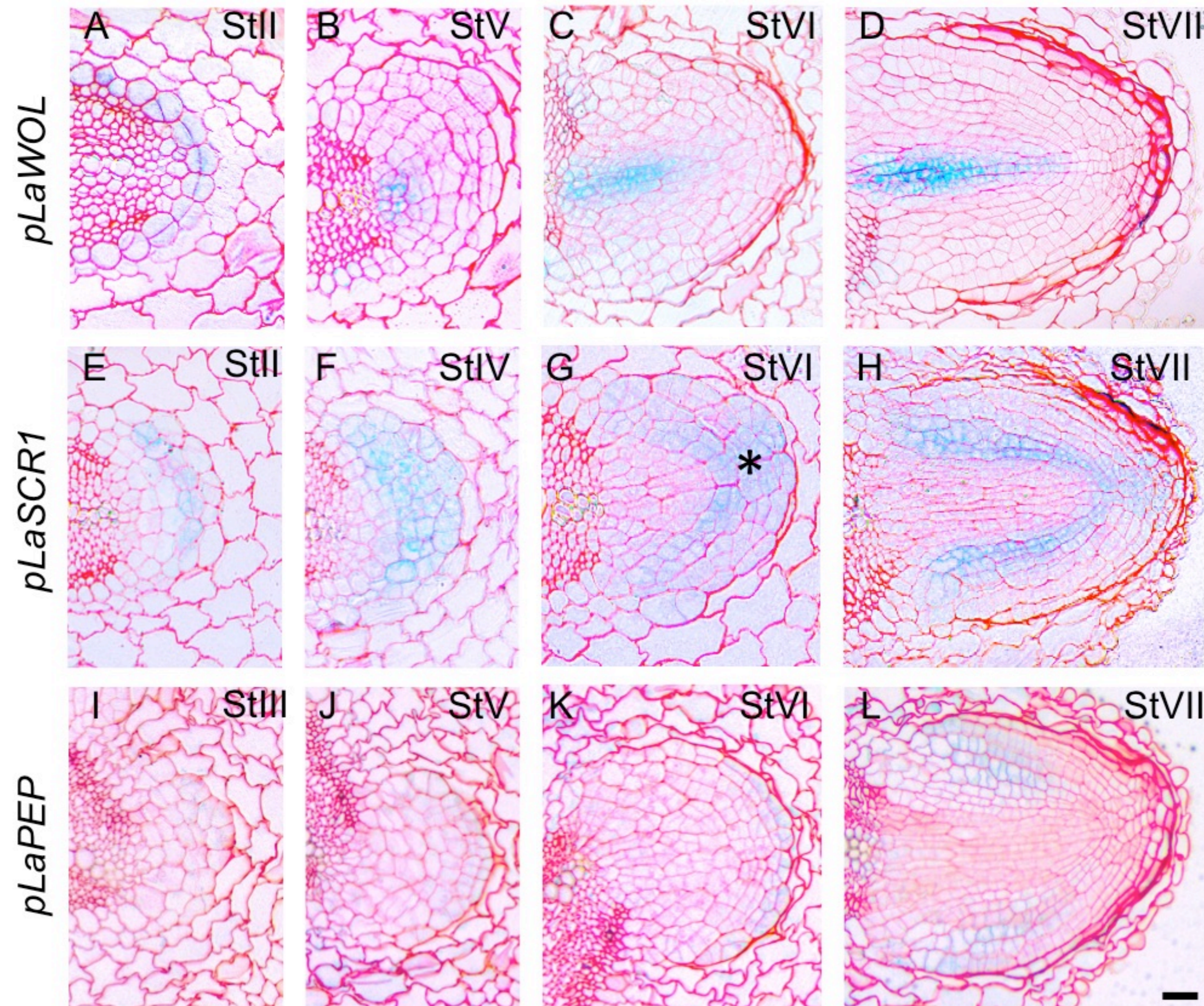
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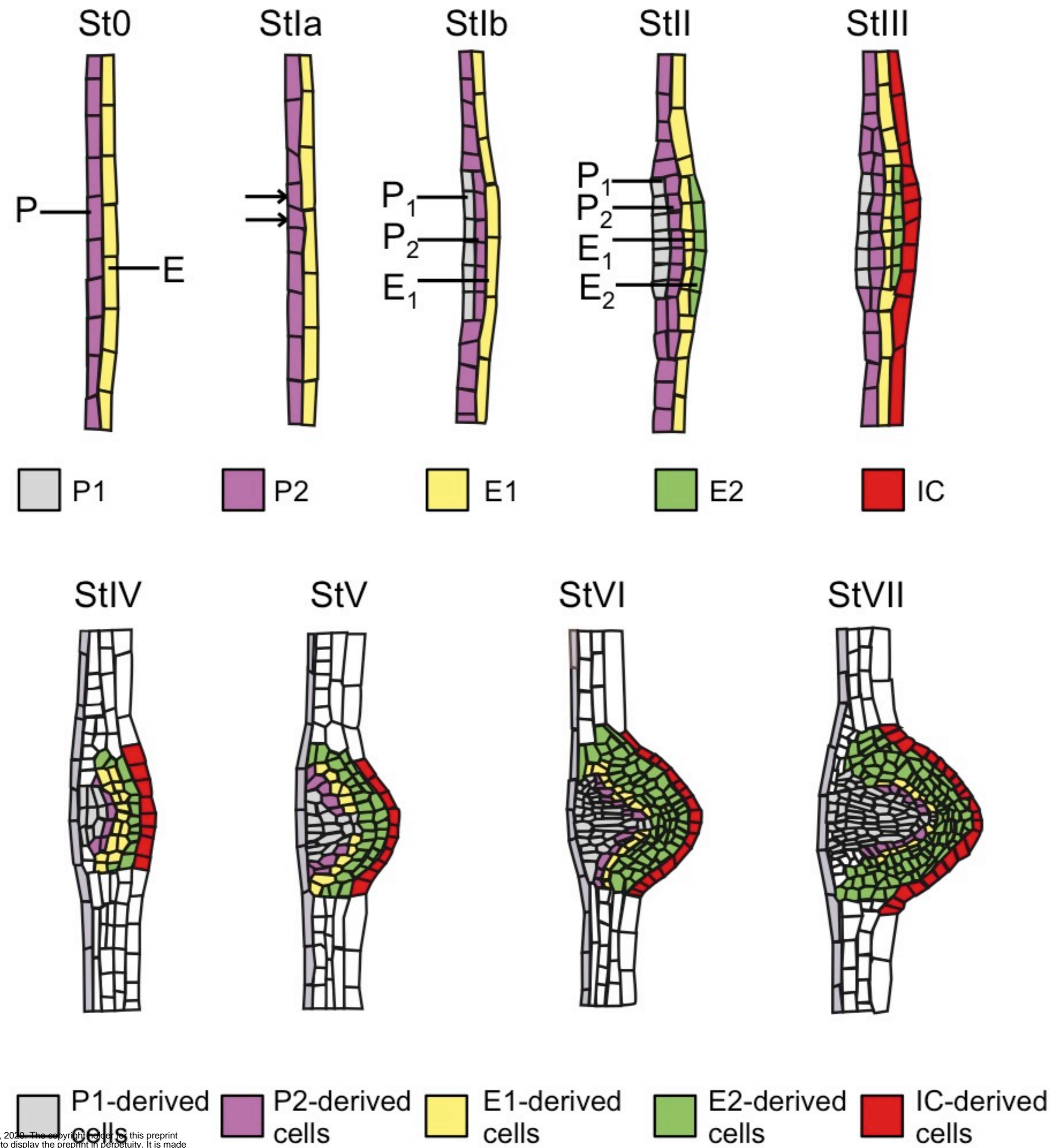
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