

1 **A single cell view of the transcriptome during lateral root initiation in *Arabidopsis thaliana***

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3 **Short title:** A single cell view of lateral roots

4

5 **One sentence summary:** Single cell RNA sequencing reveals new molecular details about lateral root

6 initiation, including the transcriptional impacts of the primordia on bordering cells.

7

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27 **Abstract**

28 Root architecture is a major determinant of fitness, and is under constant modification in response to  
29 favorable and unfavorable environmental stimuli. Beyond impacts on the primary root, the environment  
30 can alter the position, spacing, density and length of secondary or lateral roots. Lateral root development  
31 is among the best-studied examples of plant organogenesis, yet there are still many unanswered questions  
32 about its earliest steps. Among the challenges faced in capturing these first molecular events is the fact  
33 that this process occurs in a small number of cells with unpredictable timing. Single-cell sequencing  
34 methods afford the opportunity to isolate the specific transcriptional changes occurring in cells  
35 undergoing this fate transition. Using this approach, we successfully captured the transcriptomes of  
36 initiating lateral root primordia, and discovered many previously unreported upregulated genes associated  
37 with this process. We developed a method to selectively repress target gene transcription in the xylem  
38 pole pericycle cells where lateral roots originate, and demonstrated that expression of several of these  
39 targets was required for normal root development. We also discovered novel subpopulations of cells in  
40 the pericycle and endodermal cell files that respond to lateral root initiation, highlighting the coordination  
41 across cell files required for this fate transition.

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45 **Introduction**

46 Plants grow new tissues and organs throughout their lives. To enable this iterative body plan, cells must  
47 maintain mechanisms of organogenesis, proliferation, and differentiation. Iterative growth of plants is  
48 most easily observed above ground, as plants put out new branches, leaves, and flowers, but equally  
49 important is the growth of root systems below the soil. Lateral root development is essential for a plant to  
50 remain stably rooted as well as to obtain essential water and nutrients from its surrounding environment.  
51 Plants use plasticity in their growth patterns to avoid adverse stimuli and take advantage of favorable  
52 ones. In this way, changing root architecture is one of the main mechanisms by which plants can adapt to  
53 changing environmental conditions (Khan et al., 2016).

54

55 Lateral root development proceeds through three discrete stages, specification, initiation, and emergence.  
56 Auxin response exhibits cyclic maxima in the basal meristem with a periodicity of about six hours (De  
57 Smet et al., 2007). During specification, cells in the meristematic zone of the primary root are specified as  
58 competent to form lateral roots if they transit through the basal meristem when auxin response is high (De  
59 Smet et al., 2007). Many other genes oscillate in phase with auxin response in the basal meristem—these  
60 genes may be targets of auxin signaling or independent regulators of specification (Moreno-Risueno et al.,  
61 2010). These competent cells then exhibit a sustained auxin maximum in the differentiated zone of the  
62 root, termed prebranch sites. In *Arabidopsis* and most dicot plants the pericycle cell layer within these  
63 prebranch sites is the cell layer that undergoes lateral root initiation (Beeckman et al., 2001). How this  
64 initial transient auxin response is molecularly translated to the sustained auxin response of prebranch sites  
65 leading to initiation is unknown.

66 The earliest morphological signal of lateral root initiation is the nuclear migration of two longitudinally  
67 adjacent pericycle cells to their shared cell wall. These cells consequently undergo the first anticlinal cell  
68 division that initiates lateral root development. *GATA TRANSCRIPTION FACTOR 23 (GATA23)* is  
69 necessary for this nuclear migration to occur (De Rybel et al., 2010). *LOB DOMAIN-CONTAINING*  
70 *PROTEIN 16 (LBD16)* and *LOB DOMAIN-CONTAINING PROTEIN 29 (LBD29)* are two other  
71 transcription factors shown to play a role in lateral root initiation. These genes are direct targets of *AUXIN*  
72 *RESPONSE FACTOR 7 (ARF7)* and *AUXIN RESPONSE FACTOR 19 (ARF19)* that promote cell division  
73 (Okushima et al., 2007). Mutants of these genes exhibit a loss of lateral root initiation, and overexpression  
74 of LBD16 rescues the *arf7arf19* mutant phenotype which also lacks lateral roots (Goh et al., 2012).  
75 Further cell divisions of specific plane orientations and structural changes of cell files exterior to the  
76 pericycle allows for lateral root emergence. The emergence process is accompanied by strong  
77 upregulation of cell wall remodelers that are also targets of auxin signaling (Lewis et al., 2013;

78 Ramakrishna et al., 2019) and appears to be the easiest stage of lateral root development to arrest, with  
79 many mutants arresting at this stage.

80  
81 Transcriptomic analyses of lateral root development have been a rich resource for determining key  
82 regulators of this developmental process (Vanneste et al., 2005). Careful temporal staging and analyses of  
83 different steps during lateral root formation have led to identification of novel regulators (Voß et al.,  
84 2015) though the complexity of the pathways regulating this process has also become more apparent.  
85 Complicating this analysis is the fact that lateral root development is not cell-autonomous, with many  
86 different cell types playing different roles and activating diverse genetic networks during this process.  
87 Cell sorting analyses on lateral root development have not been done to parse tissue-specific signals,  
88 likely because the regions of the root undergoing this fate transition are prohibitively small for such  
89 analyses. Single-cell RNA-sequencing is an alternative approach to obtain transcriptomes on the level of  
90 individual cells that requires much less tissue compared to cell sorting. In plants, single-cell RNA-  
91 sequencing has been used to characterize several plant tissue types, and single-cell analyses of root  
92 transcriptomes have identified both previously characterized and novel cell type markers (Jean-Baptiste et  
93 al., 2019; Ryu et al., 2019; Shulse et al., 2019; Shahan et al., 2020). To date, single-cell analyses of root  
94 tissue have focused on gene expression in the primary root, transcriptome changes between hair cells and  
95 non-hair cells, endodermal differentiation, and regeneration of the primary root meristem after injury.

96  
97 While initiation of lateral roots is known to be regulated by auxin, only a handful of specific molecular  
98 markers of this fate switch have been identified. One reason for this scarcity of markers may be that for  
99 any given primary root, lateral root initiation only occurs in a very small proportion of xylem pole  
100 pericycle (XPP) cells at near basal meristem, which themselves are a very small proportion of the root  
101 cells (approximately five percent) (Schmidt et al., 2014). Rarity of lateral root fate transition is further  
102 complicated by the pulsatile nature of the auxin signal, making this a highly transient event (Moreno-  
103 Risueno et al., 2010). To counteract these challenges, we microdissected sections of *Arabidopsis* roots  
104 undergoing gravity-induced lateral root initiation, and subjected the resulting protoplasts to single-cell  
105 sequencing. Using this approach, we successfully captured cells from all major cell types of the root  
106 outside the meristem. Through pseudotime analyses found that cells identified as lateral root primordia  
107 (LRP) are transcriptionally derived from those identified as xylem pole pericycle cells (XPP), consistent  
108 with previous morphological analysis (Malamy and Benfey, 1997). Differential gene analyses identified  
109 many previously unreported genes that are upregulated in LRP cells as compared to XPP cells. We  
110 validated the expression patterns of a subset of these genes using fluorescent reporters. In addition, we  
111 developed a CRISPR/dCas9 tool to specifically target the repression of these candidate genes in XPP cells

112 and found that many of these targets shape root architecture. Finally, we were able to harness the single-  
113 cell approach to determine how cells surrounding the developing primordium, specifically endodermal  
114 cells overlaying and pericycle cells flanking LRP<sub>s</sub>, are affected by this fate transition.

115

116

117 **Results**

118 To examine the developmental transition of lateral root initiation, we used gravistimulation to  
119 synchronize the formation of lateral root primordia, and then dissected the region of interest at two time  
120 points and performed single-cell transcriptome analyses. Mechanical or gravitropic bending of primary  
121 roots in *Arabidopsis* causes the accumulation of auxin and the formation of a lateral root at the bend  
122 (**Figure 1A**) (De Smet et al., 2007; Ditengou et al., 2008) In our conditions, wild-type plants have formed  
123 a primordium at either stage I or II by twenty hours after gravistimulation (Guseman et al., 2015) As our  
124 goal was to identify early regulators of lateral root initiation, we analyzed cells twenty hours post-  
125 bending, when initiation has just begun, and eight hours post-bending, where there are no morphological  
126 signs of lateral root development but transcriptome changes have started (Voß et al., 2015). We included  
127 a control treatment group where we did not bend the roots but cut a similar region of the primary root. We  
128 microdissected the root bend regions to maximize our yield of the rare cell types of interest.

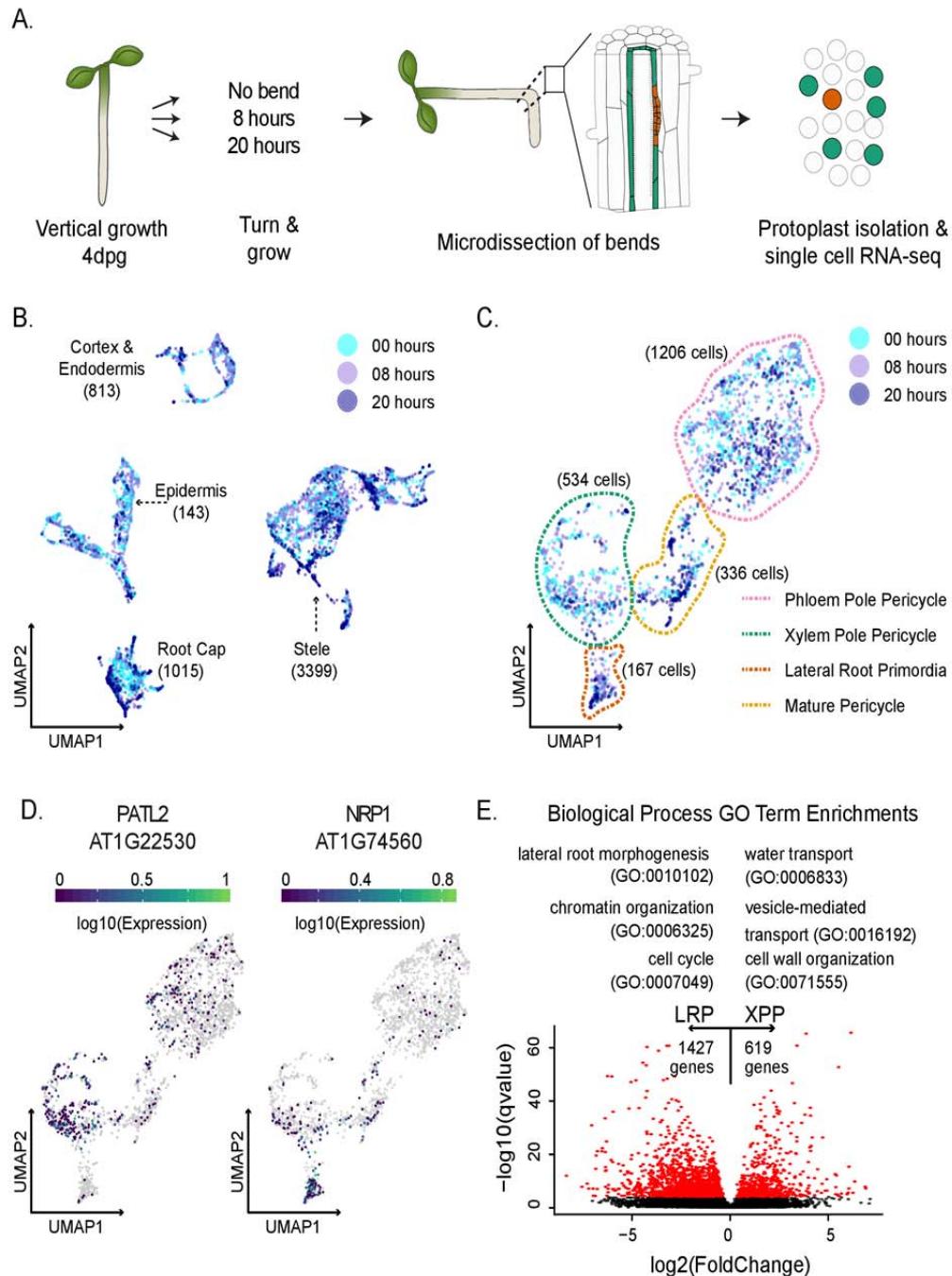
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130 Our experiment yielded 6658 cells with a mean number of 15987 reads per cell and a median of 1383  
131 genes expressed. Of these 6658 cells, 1730 (~26%) cells were from the twenty-hour timepoint, 2443  
132 (~37%) cells were from the eight-hour timepoint, and 2485 (~37%) cells were from the no bend control  
133 (**Supplemental Table 1**). Further analysis was performed using Monocle 3 (Trapnell et al., 2014; Qiu et  
134 al., 2017; Cao et al., 2019), which uses Principal Component Analysis (PCA) and Uniform Manifold  
135 Approximation and Projection (UMAP) to reduce the dimensionality of the dataset and to visualize the  
136 relationships among cellular transcriptomes in a two-dimensional space. Our analysis revealed five  
137 clusters. Using previously defined cell type markers (Brady et al., 2007; Cartwright et al., 2009), each  
138 cluster was assigned a label. Of the 6658 cells, 813 (~12%) cells were classified as cortex & endodermis,  
139 1015 (~15%) cells were classified as columella/root cap, 1431 (~22%) cells were classified as epidermis,  
140 and 3399 (~51%) cells were classified as stele (**Figure 1B, Supplemental Figure 1A**). These results  
141 show that the microdissection was successful in capturing a representative sample of stele cells, as the  
142 proportion of these cells in our data was similar to that determined by imaging analyses (Shulse et al.,  
143 2019).

144

145 **Xylem pole pericycle cells are precursors of mature pericycle and lateral root primordia cells.**

146 To better understand the relationships between vasculature cell types, we re-clustered all cells identified  
147 as belonging to the stele. Of the 3399 stele cells, 216 cells were labeled phloem, 242 cells were labeled  
148 xylem, 1206 cells were labeled phloem pole pericycle (PPP), 534 cells were labeled XPP, 336 cells were  
149 labeled mature pericycle, 167 cells were LRP, and 698 cells were too ambiguous in their gene expression  
150 to assign a label (**Supplemental Figure 1B, Supplemental Figure 1C, Supplemental Table 1**). A



**Figure 1: Analysis of lateral root initiation using single-cell RNA-seqencing.** A. Experimental design: *Arabidopsis thaliana* seedlings were grown vertically for 4 days post-germination (dpg) then rotated (or marked, in case of control) and grown for an additional eight or twenty hours. Protoplasts were prepared from microdissected root sections for single-cell RNA sequencing. B. UMAP of all 6658 cells colored by experiment. Cell type identities were assigned to each partition based on a set of marker genes. The cell type identities are indicated on the UMAP with the number of cells corresponding to each cell type. C. UMAP of pericycle specific cells, such as lateral root primordia (LRP), mature pericycle (MP), phloem pole pericycle (PPP), and xylem pole pericycle cells (XPP) colored by experiment. D. Expression pericycle-specific UMAPs of two DEGs between LRP and XPP. E. Scatter plot of the log<sub>2</sub> fold change of average gene expression between XPP and LRP and the Mann Whitney qvalue. DEGs are colored in red. A selection of GO Terms associated with DEGs are shown above the plot.

152 (Figure 1C). A cell developmental trajectory connected the pericycle sub-clusters and recapitulated the  
153 known developmental relationship between these cell types. Trajectories initiating either from the XPP or  
154 the PPP converged to mature pericycle but only the XPP (and not the PPP) population branched out  
155 toward the LRP, confirming that our results were faithfully recapitulating the known exclusive  
156 relationship of XPP to LRP cells (Malamy and Benfey, 1997). The XPP and the PPP cells are expected to  
157 differentiate at a similar time, whereas LRP and mature pericycle cells should form later. Our dataset  
158 confirmed this developmental progression, as approximately twenty percent of the XPP and PPP cell  
159 clusters were from the twenty-hour timepoint whereas approximately fifty percent of the LRP and mature  
160 pericycle clusters were from this later timepoint. (Supplemental Figure 1D).

161  
162 We next attempted to identify genes expressed as XPP cells transitioned to LRP cells. To do this, we  
163 carried out a pseudotime analysis originating from the XPP population and connecting to either the LRP  
164 or the mature pericycle (Supplemental Figure 2A, Supplemental Figure 2B). Using a false discovery  
165 rate (FDR) cutoff of 0.1, 1892 genes were identified as changing in at least one of the trajectories. Of  
166 these genes, 878 genes were specific to the XPP to mature pericycle trajectory, 504 genes were specific to  
167 the XPP to LRP trajectory, and 510 genes were observed in both trajectories (Supplemental Figure 2C,  
168 Supplemental Figure 2D). As XPP cells develop, expression of genes found in both trajectories  
169 decreased and most were not expressed at high levels in the LRP cells or the mature pericycle cells.  
170 Genes uniquely upregulated across the XPP to the LRP trajectory were overrepresented with transcription  
171 factors including the well-known markers of initiation *LBD16* and *LBD29* (Okushima et al., 2007) as well  
172 as *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)*, *CYTOKININ RESPONSE FACTOR 2 (CRF2)* and  
173 *PUCHI*, all of which have previously been shown to play a role or be expressed in lateral root  
174 development (Hu and Xu, 2016; Jeon et al., 2016; Goh et al., 2019) (Supplemental Figure 2E). Other  
175 genes unique to this trajectory were regulatory kinases like *MUSTACHES (MUS)*, *MAP KINASE KINASE*  
176 *6 (MKK6)*, and *RGF INSENSITIVE 5 (RGIS5)*, which have also been shown to regulate lateral root  
177 development (Zeng et al., 2011; Ou et al., 2016; Xun et al., 2020) (Supplemental Figure 2E).

178  
179 We next performed differentially expressed genes (DEG) analysis on the transcriptomes associated with  
180 these different populations. Due to the small number of LRP cells (167 cells i.e. ~2.5% of all cells), three  
181 different statistical approaches were used to perform DEG analysis: a generalized linear model (GLM),  
182 the Mann Whitney Wilcoxon (MMW) test, and a recently published packaged called Vision (DeTomaso  
183 et al., 2019). We used previously identified LRP-enriched genes as a guide to inform our use of the results  
184 from each method. *LBD16* and *LBD29* were called in all three methods, whereas *ARF19* and *GATA23*  
185 were only called in Vision (but narrowly missed with p-values of 0.001 and 0.0004 respectively in MW

186 method). ARF7 was not significantly different in any approach. To compile the most comprehensive list,  
187 we generated a list of DEGs that were significantly different between XPP and LRP populations in at  
188 least two approaches. We called 1427 DEGs specific to LRP and 619 DEGs specific to XPP cells. Several  
189 of these genes have been previously characterized as specific in their expression patterns, and expression  
190 maps of these genes reflect this quality (**Figure 1D, Supplemental Figure S3, Supplemental Data 1**)  
191 (Zhu et al., 2006; Tejos et al., 2018).

192

193 As expected, a Gene Ontology (GO) term enrichment analysis of the DEGs with higher expression in  
194 LRP cells showed a strong enrichment for terms associated with lateral root formation, lateral root  
195 morphogenesis, lateral root development, and auxin response. Terms associated with the regulation of  
196 translation initiation and RNA processing (**Supplemental Data 1**) were also enriched, indicating that the  
197 transition from the XPP to the LRP requires a burst of *de novo* protein production. Increased protein  
198 production is associated with a stem cell state (Himanen et al., 2004), which correlates with the transition  
199 from XPP to early LRP cells which are competent to form all the types of cells of a developing root.  
200 Other GO terms associated with the DEGs more highly expressed in LRP were lateral root  
201 morphogenesis, cell cycle, and chromatin organization (**Figure 1E**).

202

### 203 **Single-cell analysis recapitulates and extends findings from previous transcriptome studies**

204 Lateral root development has been extensively characterized with transcriptomic analysis, generated using  
205 a variety of lateral root induction models and experimental designs. We compared our LRP versus XPP  
206 DEGs with published datasets to assess differences in single-cell versus whole tissue (or population)  
207 methods. We found that roughly half of the genes (including genes regulated by auxin and genes  
208 belonging to cell cycle processes) identified by a microarray analysis of induced lateral root development  
209 (Vanneste et al., 2005) were included in our set of genes upregulated in LRP cells; only five of these  
210 genes were in our set of XPP-upregulated genes (**Supplemental Figure S4A**). This result suggests our  
211 approach faithfully captured genes involved in lateral root initiation and distinguished LRP and XPP  
212 cells. A detailed time course analysis of lateral root development using a similar bend assay as what we  
213 applied (Voß et al., 2015) allowed us to compare to bulk RNA sequencing data taken at similar timepoints  
214 as used in our study. Two thirds of the genes previously found upregulated at nine hours post-bending  
215 were contained within our set of genes upregulated in LRP, none of these genes were XPP-upregulated  
216 (**Supplemental Figure S4B**). Roughly half the genes found upregulated at twenty-one hours were within  
217 our LRP-upregulated gene list; only nine were in our XPP-upregulated gene list (**Supplemental Figure**  
218 **S4B**). At both time points, approximately one thousand genes were uniquely found in LRP DEGs from

219 our study. The genes that were identified in the previous study but not in ours may be attributed to  
220 technical differences or likely are expressed in cell layers outside the pericycle.

221  
222 We also compared our data to transcriptome assays that did not directly examine lateral root initiation  
223 through root bending, but queried related processes in the root. We compared our data to a time course  
224 analysis of primary root transcriptomes after auxin treatment (Lewis et al., 2013), as auxin treatment  
225 strongly promotes lateral root initiation. We found that thirty-seven LRP-upregulated genes were strongly  
226 induced in this dataset in response to auxin, whereas only two XPP-upregulated genes were auxin-  
227 induced (**Supplemental Figure S4C**). In contrast, twenty-six XPP-upregulated genes were repressed by  
228 auxin treatment, whereas only five LRP-upregulated genes were in this repressed dataset (**Supplemental**  
229 **Figure S4C**). Another recent analysis identified genes specifically induced by ARF19-mediated auxin  
230 response (Powers et al., 2019). We found that 243 of our LRP-upregulated genes overlapped with the set  
231 of ARF19-specific auxin-induced genes, while only 19 XPP-upregulated genes did so (**Supplemental**  
232 **Figure S4D**). We conclude that our data reflect the auxin-inducibility of lateral root initiation, and  
233 specifically that this auxin inducibility was at least in part mediated by ARF19. ARF19 is unique among  
234 the ARFs in being both auxin-responsive in its own expression pattern (Wilmoth et al., 2005) and a very  
235 strong activator of transcription itself (Lanctot et al., 2020)

236  
237 Finally, we examined how our dataset compared to genes expressed in the basal meristem during lateral  
238 root specification. During specification, cells become competent to form lateral roots if they transit  
239 through the basal meristem during an auxin response maximum. Many genes exhibit similar oscillatory  
240 behavior to auxin response in the basal meristem (Moreno-Risueno et al., 2010). We found that fifty-eight  
241 genes that oscillated in phase with auxin response in the basal meristem were in our set of LRP-  
242 upregulated genes, whereas only one XPP-upregulated gene oscillated in phase with auxin  
243 (**Supplemental Figure S4E**). However, 213 XPP-upregulated genes oscillated antiphase to auxin in the  
244 basal meristem, while only twenty LRP-upregulated genes show antiphase oscillation. How specification  
245 and initiation are connected temporally and spatially and how competent cells “remember” their future  
246 cell fate is still unknown. Our results suggested that oscillatory behavior of some genes may predict their  
247 importance during initiation later in development, and in particular that genes with antiphase oscillation  
248 patterns may actively repress lateral root fate. We also compared our dataset to a study that determined  
249 genes whose expression was impacted by repressing auxin response specifically in early-stage LRP  
250 (Ramakrishna et al., 2019), and found our LRP-upregulated genes overlapped with nearly two hundred of  
251 these genes (**Supplemental Figure S4F**), again emphasizing the importance of auxin response for  
252 establishing lateral root fate.

253

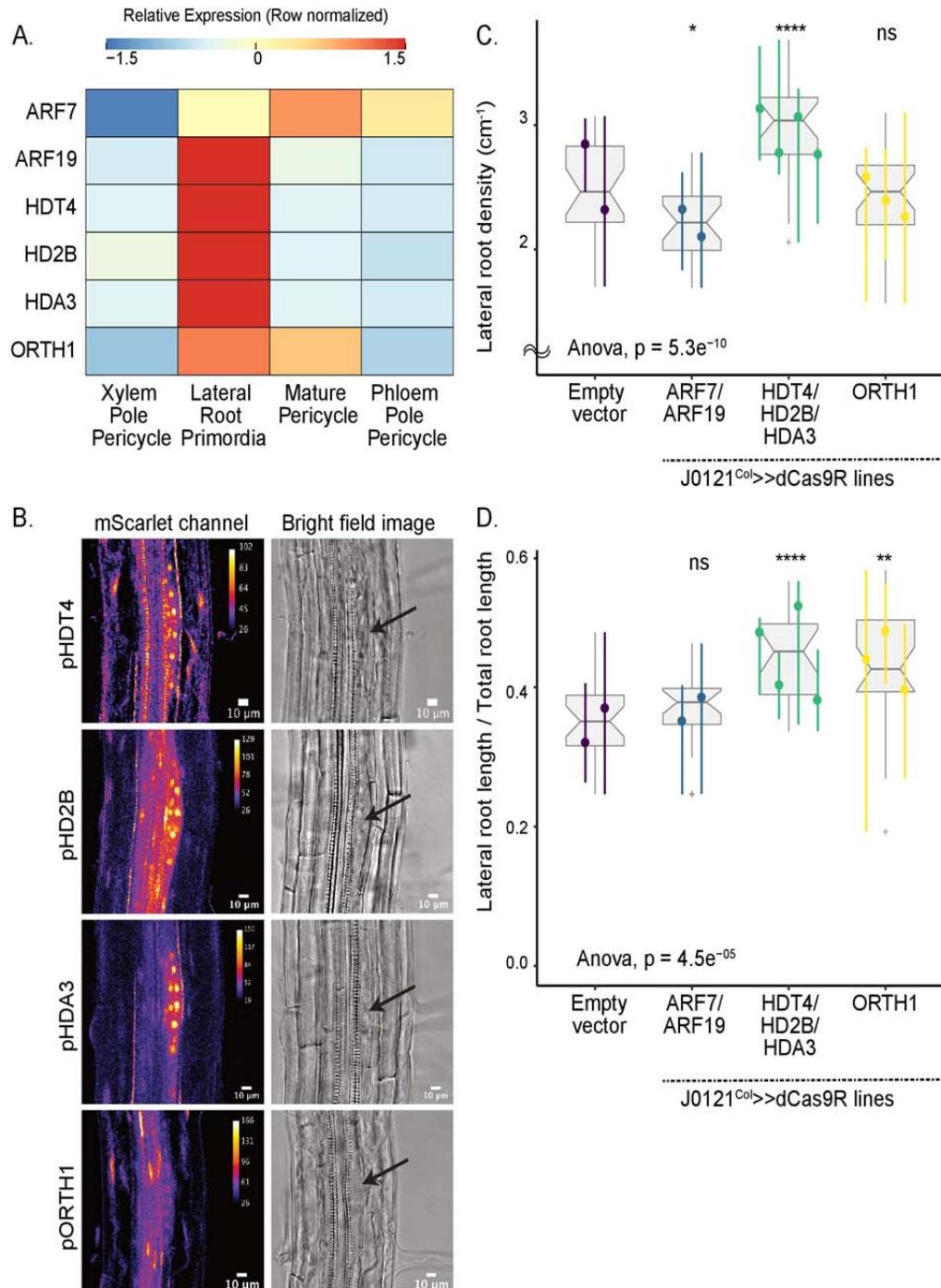
254 **Genes upregulated in LRP cells are indicative of cells undergoing fate transitions**

255 We selected timepoints that should best capture the transition of undifferentiated XPP cells into LRPs. As  
256 one of the earliest morphological steps in this process is an asymmetric cell division, followed by many  
257 subsequent cell divisions, it is not surprising that a number of LRP-enriched DEGs are involved in cell  
258 cycle control. It is already known, for example, that expression of cyclin *CYCB1;1* marks lateral root  
259 primordia (Beeckman et al., 2001) Interestingly, one of the distinguishing features of XPP cells when  
260 compared to PPP cells is that some XPP cells are arrested in G2, whereas all PPP cells are arrested in G1  
261 (Beeckman et al., 2001). This G2 arrest may prime these XPP cells to undergo rapid reintroduction into  
262 the cell cycle. We also found enrichment of chromatin remodeling factors in the set of LRP-upregulated  
263 genes. Considering lateral root initiation is the first step of organogenesis, it makes sense that broad  
264 transcriptional changes, mediated by changes in the chromatin landscape, may be required, but this has  
265 not previously been reported (**Supplemental Data 1**). Finally, we found that genes that promote cell  
266 division, differentiation, and “stemness”, mostly transcription factors, were also enriched in the set of  
267 genes upregulated in LRP cells. Most of these genes have been characterized as regulating development  
268 in other meristems, such as the primary root meristem or the shoot apical meristem, but had not been  
269 shown to play a role in lateral root development. We chose several candidate genes from these three  
270 groups, chromatin regulators (four genes), cell cycle regulators (seven genes) and stemness regulators (six  
271 genes) to carry out two types of validation experiments: (1) characterizing their spatial expression patterns  
272 with transcriptional reporters in wild-type Col-0 and *arf7arf19* mutant seedlings, and (2) phenotypic  
273 evaluation of lateral root development in transgenic line with cell-type specific repression of candidate  
274 genes.

275

276 **A novel cell-type specific dCas9-driven repressor system can reveal drivers of lateral root fate**

277 To explore the functional role of candidate genes in lateral root development, we devised a method to  
278 repress candidate genes only in the XPP cell lineage. We leveraged the enhancer trap line J0121 which is  
279 specifically expressed in XPP cells via a UAS-GAL4 driver system (Laplaze et al., 2005). We first  
280 introgressed J0121 into the Col-0 background (referred to hereafter as J0121<sup>Col</sup>), and then introduced a  
281 UAS-dCas9-TPLN300 repressor (dCas9R) construct with three gene-specific sgRNAs directed to the  
282 promoter regions of candidate genes (J0121<sup>Col</sup>>>dCas9R, **Supplemental Figure S5, Supplemental Data**  
283 **2**). This cell-type specific repression system has several advantages over traditional knockdown and  
284 knockout studies. For instance, multiple guides can be used to simultaneously repress several members of  
285 the same gene family that may have redundant functions. Additionally, many of the candidate genes we  
286 identified as enriched in LRP cells also play roles in embryonic and primary root development, greatly



**Figure 2. Validation experiments on chromatin modifier candidate genes.** A. Heatmap (row-scaled) visualizing expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale bar represents the z-score of the normalized expression values. B. Confocal microscopy images of candidate genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image of corresponding primordia (right). C. Lateral root density of *J0121<sup>Col</sup>>>dCas9RR* transgenic lines of candidate genes. D. Proportion of total root length contributed by lateral roots of *J0121<sup>Col</sup>>>dCas9R* transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines have been analyzed; each colored line in the graph represents an individual line.

287 complicating assessment of any role in lateral roots. To test the efficacy of our assay, we tested the effects

288 of repressing expression of both *ARF7* and *ARF19*. We found that these perturbation lines have  
289 significantly reduced lateral root density compared to the empty vector control as expected although they  
290 did not fully recapitulate the full suppression of lateral roots seen in *arf7arf19* null mutants  
291 (**Supplemental Figure S6, Figure 2C**). This observation is consistent with the small reduction of lateral  
292 root number seen in a GATA23-driven CRISPR/Cas9 deletion of *ARF7* and *ARF19* (Decaestecker et al.,  
293 2019). There are at least two likely explanations for the milder phenotype of J0121<sup>Col</sup>>>dCas9R  
294 compared with the *arf7arf19* null mutant. First, repression in J0121<sup>Col</sup> expression is limited to XPPs and  
295 lateral root stages I through III, so there is likely residual ARF protein that persists from expression in  
296 pre-XPP fate cells (Dubrovsky et al., 2006). Second, our synthetic repressor may not block all  
297 transcriptional activity, leading to hypermorphic rather than amorphic phenotypes. Even given these  
298 limitations, the system proved sufficiently sensitive to enable detection of cell type-specific impacts on  
299 lateral root development.

300

### 301 **Chromatin remodeling factors influence lateral root development**

302 Three histone deacetylases (HDACs) that are all in the same plant-specific gene family, *HISTONE*  
303 *DEACETYLASE 3*, *HISTONE DEACETYLASE 2*, and *HISTONE DEACETYLASE 13* (*HDA3*, *HD2B*, and  
304 *HDT4*) (Li et al., 2017; Luo et al., 2017), were all enriched in LRP cells in our DEG analysis (**Figure**  
305 **2A**), as was the E3 ubiquitin ligase *ORTHRUS 1* (*ORTH1*) which decreases DNA methylation (Kim et al.,  
306 2014). Transcriptional reporters of these genes express strongly in early stage primordia (**Figure 2B**).  
307 Expression of *HDT4* and *HDA3* were specific to LRPs in the differentiated zone of the primary root,  
308 though both were also strongly expressed in the meristematic zone of the primary root (**Supplemental**  
309 **Figure S7**). Their expression in the meristem was strongly decreased in *arf7arf19* mutants, suggesting  
310 they may be regulated by auxin (**Supplemental Figure S7**). *HD2B* was also strongly expressed in LRP  
311 cells, as well as in the primary root meristem and other pericycle cells (**Supplemental Figure S7**).  
312 *ORTH1* was broadly expressed in the vasculature of the differentiated zone of the primary root, not only  
313 in LRP cells (**Supplemental Figure S7**), which is reflected by its enrichment in mature pericycle cells in  
314 our single-cell library (**Figure 2A**). Its expression was not impacted in *arf7arf19* mutant lines  
315 (**Supplemental Figure S7**).

316

317 Using J0121<sup>Col</sup>>>dCas9R, we targeted all three *HDAC* genes for repression using distinct guide RNAs for  
318 each gene. Simultaneous repression of all three genes caused a strong phenotype, where both the density  
319 of lateral roots (**Figure 2C**) and the proportion of total lateral root length contributed by lateral roots were  
320 significantly increased (**Figure 2D**). The phenotype suggested that the HDACs may repress lateral root  
321 initiation and later stages of development. *ORTH1* repression in XPP cells did not significantly impact

322 lateral root density (**Figure 2C**), but the proportion of total root length contributed by lateral roots  
323 significantly increased (**Figure 2D**). Thus, *ORTH1* may repress lateral root growth only post-initiation.  
324 All of these chromatin regulators were expressed strongly in LRP cells, and repressing their function  
325 stimulates lateral root growth, suggesting they may act to coordinate cells and promote orderly  
326 development.

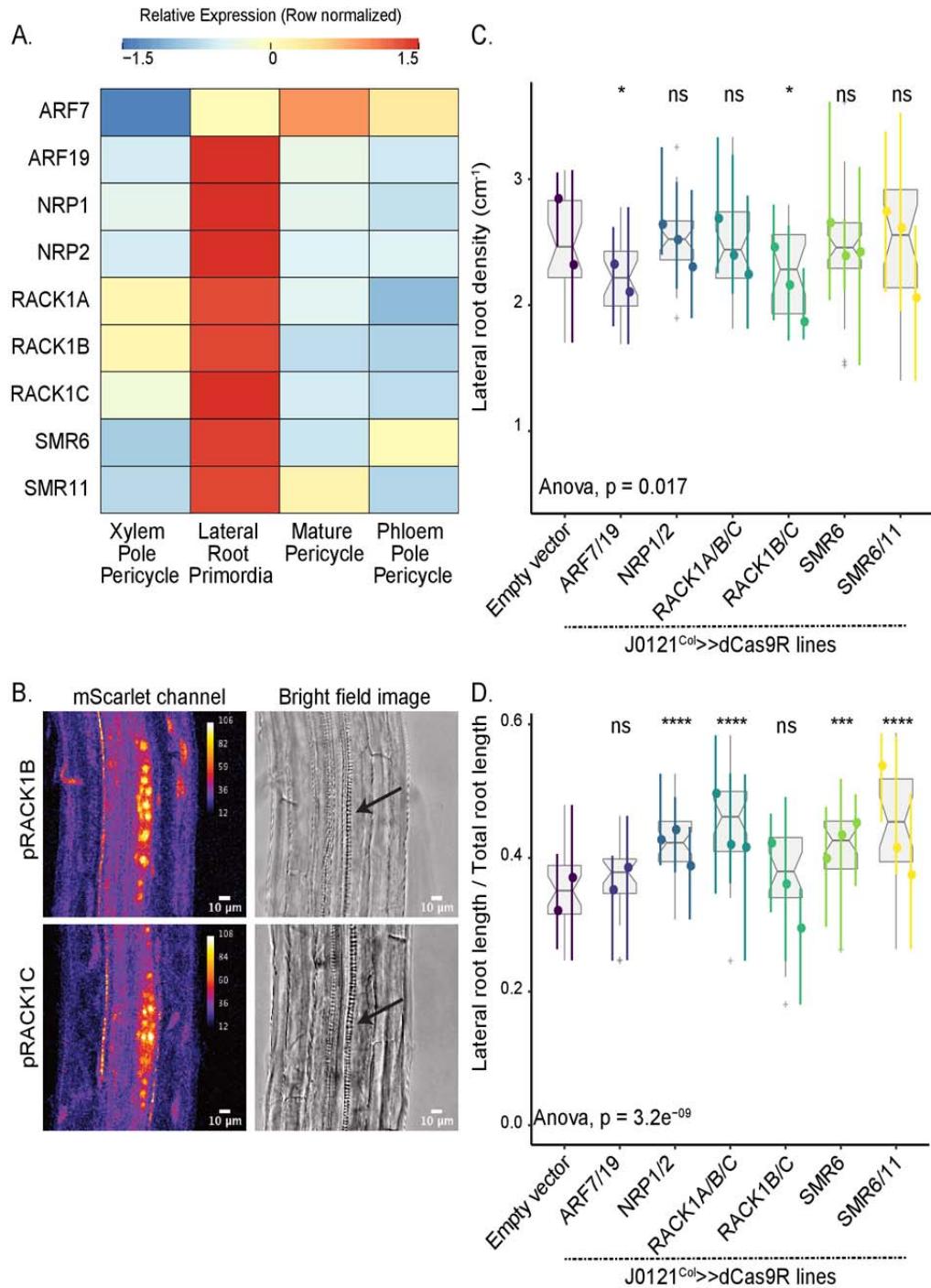
327

### 328 **Cell cycle regulators are active during lateral root development**

329 We characterized the role of five DEGs enriched in our LRP population that play a role in cell cycle  
330 regulation: *RECEPTOR FOR ACTIVATED C KINASE 1A, B and 1C* (*RACK1A*, *RACK1B* and *RACK1C*),  
331 *NAP1-RELATED PROTEIN 1* and *2* (*NRP1* and *NRP2*), and *CYCLIN-DEPENDENT PROTEIN KINASE*  
332 *INHIBITORS 6* and *11* (*SMR6* and *SMR11*) (**Figure 3A**). *RACK1B* and *RACK1C* interact with protein  
333 kinase C (Guo and Chen, 2008). *SMR6* and *SMR11* are cyclin-dependent kinase inhibitors (Yi et al.,  
334 2014), and *NRP1* is a histone chaperone required for the G2 to M transition (Zhu et al., 2006). We  
335 generated transcriptional fluorescent reporters of *RACK1B* and *RACK1C* and found their expression was  
336 indeed specific to early stage lateral root primordia as seen in the single-cell data (**Figure 3B**). Expression  
337 of both *RACK1B* and *RACK1C* was lost in the differentiated zone of the primary root in *arf7arf19*  
338 mutants, which do not form lateral roots, indicating the specificity of their expression to LRP in this zone  
339 (**Supplementary Figure S8**). Expression of these genes in the primary root meristem also was highly  
340 decreased in *arf7arf19* mutants (**Supplementary Figure S8**), suggesting ARF7 and ARF19 may be the  
341 primary ARFs regulating their expression throughout the root.

342

343 We next used J0121<sup>Col>>dCas9R</sup> to test for functional relevance of these cell cycle regulators. *NRP1* is  
344 highly related to *NRP2*, so we generated a repression line with guides targeting both genes. These lines  
345 did not show differences in lateral root density (**Figure 3C**), but did show a significantly increased  
346 proportion of summed total root length that was contributed by lateral roots reflecting longer lateral roots  
347 than in the control lines (**Figure 3D**). *RACK1B* and *RACK1C* are highly related and show redundancy  
348 with *RACK1A*, so we generated two separate sets of repression lines, one with guides targeting *RACK1B*  
349 and *RACK1C* and the other with guides targeting all three *RACK1* genes. Repression of *RACK1B* and  
350 *RACK1C* caused a significant decrease in lateral root density (**Figure 3C**). Interestingly, while repression  
351 of all three *RACK1* genes did not significantly impact lateral root density, this line did show a  
352 significantly increased proportion of total root length contributed by lateral roots (**Figure 3D**), similar to  
353 the *NRP1/NRP2* repressed line. Singular repression of *SMR6* and concurrent repression of *SMR6* and  
354 *SMR11* did not show differences in lateral root density from the control (**Figure 3C**), but again showed  
355 significantly increased proportion of total root length contributed by lateral roots (**Figure 3D**).



**Figure 3. Validation experiments on cell cycle regulator candidate genes.** A. Heatmap (row-scaled) visualizing expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale bar represents the z-score of the normalized expression values. B. Confocal microscopy images of candidate genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image of corresponding primordia (right). C. Lateral root density of *J0121<sup>Col</sup>>>dCas9R* transgenic lines of candidate genes. D. Proportion of total root length contributed by lateral roots of *J0121<sup>Col</sup>>>dCas9R* transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines have been analyzed; each colored line in the graph represents an individual line.

357 **Genes that encode pluripotency and stemness are upregulated in LRP cells**

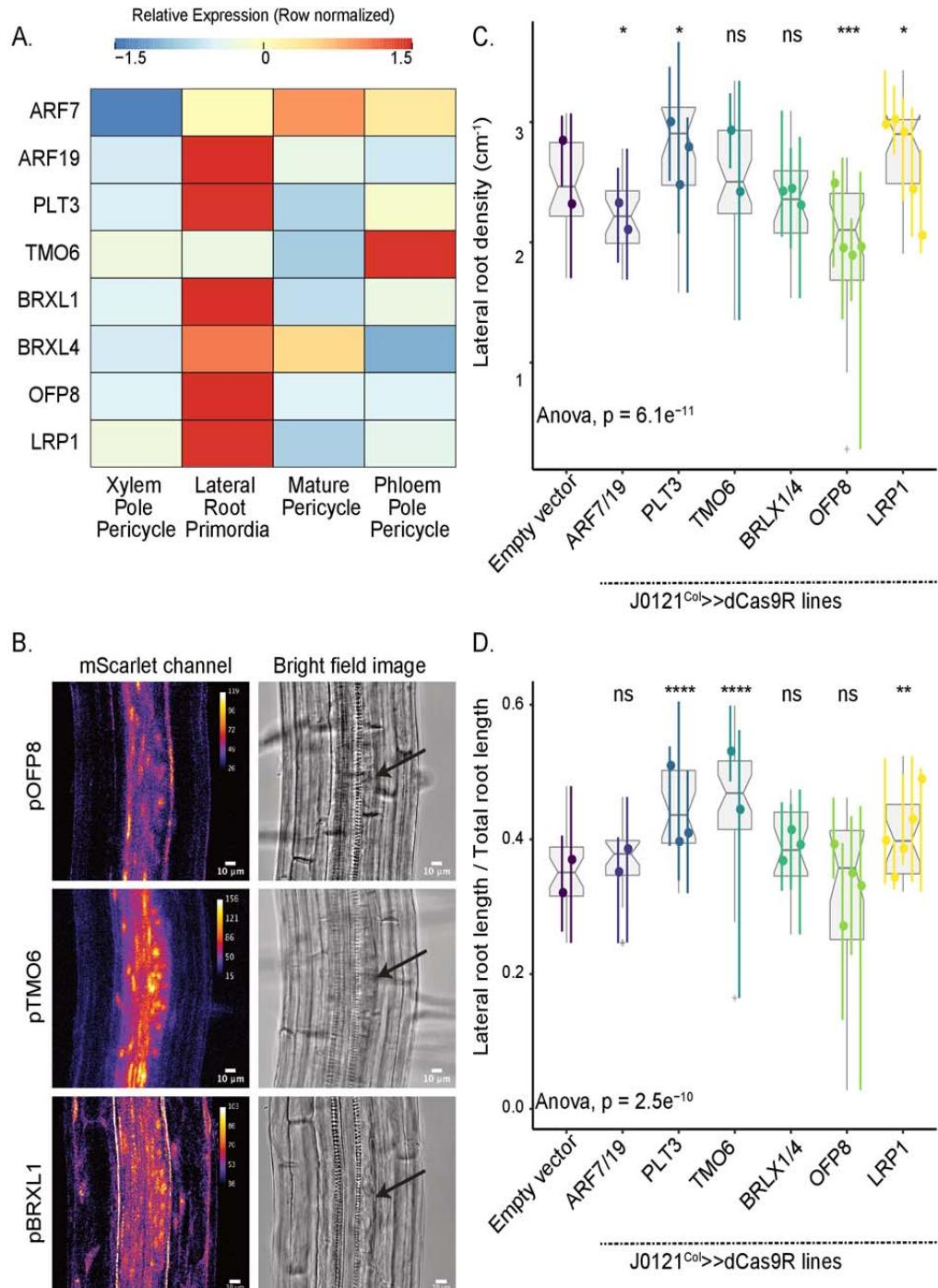
358 We chose five DEGs known to play a role in developmental transitions for further validation studies,  
359 specifically *TARGET OF MONOPTEROS 6* (*TMO6*), a Dof-type transcription factor originally isolated as  
360 a target of ARF5 in embryos (Schlereth et al., 2010), *BREVIS RADIX-LIKE 1* (*BRXL1*), a BRX-like  
361 regulator of primary root development (Briggs et al., 2006), *LATERAL ROOT PRIMORDIUM 1* (*LRP1*),  
362 which a marker and plays a role in lateral root development (Smith and Fedoroff, 1995; Singh et al.,  
363 2020), *OVATE FAMILY PROTEIN 8* (*OFP8*), a transcriptional repressor of KNOX family transcription  
364 factors (Wang et al., 2011), and *PLETHORA 3* (*PLT3*), a *PLETHORA* family gene that interprets auxin  
365 gradients in the primary root (Santuari et al., 2016). The presence of genes such as *LRP1*, *BRXL1*, and  
366 *PLT3*, known to regulate early stages of root development, confirmed that our dataset was isolating genes  
367 expected to be active early during lateral root initiation. BRXL proteins have recently been shown to play  
368 a role in promoting nuclear migration and asymmetric cell division in the development of stomata (Rowe  
369 et al., 2019; Muroyama et al., 2020), a process that is also essential during the very first stages of lateral  
370 root initiation. *OFP8* and *TMO6* were somewhat unexpected discoveries, given that ovate family proteins  
371 have primarily been characterized in fruit development (Wang et al., 2016) and *TMO* genes have  
372 primarily been characterized in embryonic development (Schlereth et al., 2010).

373

374 Expression of all of these genes showed enrichment in LRP cells, though *TMO6* expression was most  
375 enriched in PPP cells (**Figure 4A**), consistent with its known role in phloem cell division and  
376 differentiation (Miyashima et al., 2019). Reporters of *PLT3* (Galinha et al., 2007), *LRP1* (Smith and  
377 Fedoroff, 1995) and *TMO6* (Schlereth et al., 2010) have previously been published, so we only generated  
378 transcriptional reporters of *OFP8* and *BRXL1*. Both showed strong and specific expression in lateral root  
379 primordia (**Figure 4B**). Expression of the *TMO6* reporter had not been previously analyzed in XPP or  
380 LRP. We found that indeed it was strongly expressed in developing primordia, as predicted from the  
381 single-cell analysis. Nearly all of this expression was lost in *arf7arf19* mutants (**Supplemental Figure 9**),  
382 suggesting that, at least in the root, *TMO6* is primarily a target of these ARFs rather than ARF5. *TMO6*,  
383 *BRXL1* and *OFP8* were not expressed in the primary root meristem, (**Supplemental Figure 9**) making  
384 them potentially useful for targeting engineering efforts specifically to lateral roots.

385

386 Repression of these genes in XPP cells using J0121<sup>Col>>dCas9R</sup> impacted root architecture but in  
387 different ways. Repression of *PLT3* caused a significant increase in both lateral root density (**Figure 4C**)  
388 and the proportion of total root length contributed by lateral roots (**Figure 4D**), suggesting *PLT3* may  
389 repress both lateral root initiation and emergence post-initiation. Repression of *TMO6* did not impact  
390 lateral root density (**Figure 4C**), but did cause a significant increase in the proportion of total root length



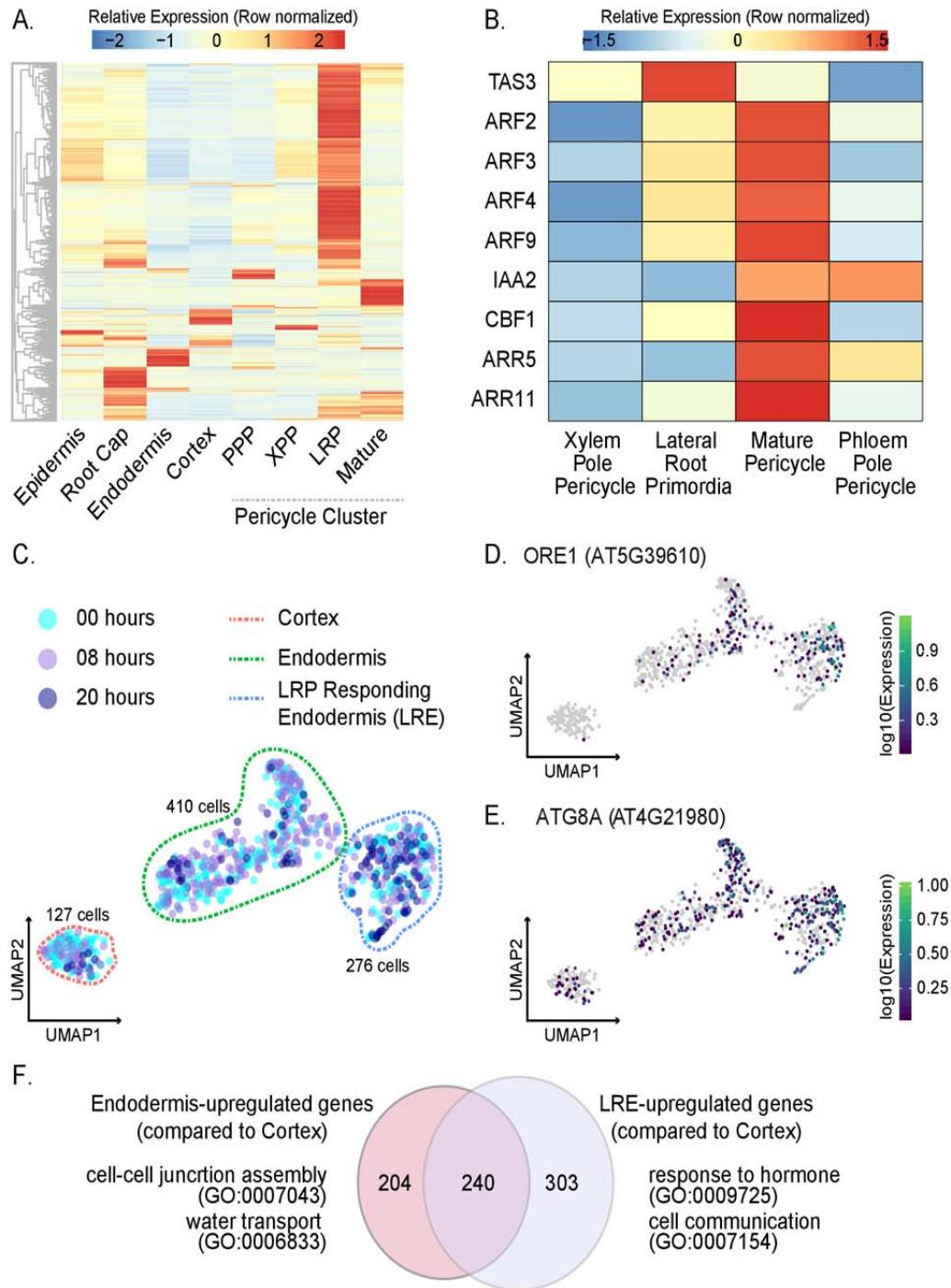
**Figure 4. Validation experiments on stemness candidate genes.** A. Heatmap (row-scaled) visualizing expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale bar represents the z-score of the normalized expression values. B. Confocal microscopy images of candidate genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image of corresponding primordia (right). C. Lateral root density of *J0121<sup>Col</sup>>>dCas9R* transgenic lines of candidate genes. D. Proportion of total root length contributed by lateral roots of *J0121<sup>Col</sup>>>dCas9R* transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines have been analyzed; each colored line in the graph represents an individual line.

391 contributed by lateral roots (Figure 4D), suggesting it may act on lateral root development post-initiation.

392 Repression of *LRP1* in XPP cells significantly increased both lateral root density (**Figure 4C**) and the  
393 proportion of total root length that are lateral roots (**Figure 4D**), a phenotype that matches previously  
394 reported overexpression lines of *LRP1*, which showed decreased lateral root density (Singh et al., 2020).  
395 Concurrent repression of *BRXL1* and its close homolog *BRXL4* did not significantly impact either lateral  
396 root density (**Figure 4C**) or the proportion of total root length that are lateral roots (**Figure 4D**), despite  
397 its strong expression in LRP cells. We did observe irregular spacing of lateral roots and shorter primary  
398 roots in *BRXL/BRXL4* repression lines, suggesting they may play a role in lateral root emergence  
399 (**Supplemental Figure 9**). Repression of *OFP8* exhibited unique behavior in our perturbation lines, as  
400 these lines showed significantly decreased lateral root density (**Figure 4C**). *OFP8* has not previously  
401 been characterized to play any role in root development, and this strong effect on lateral root initiation  
402 and its strongly specific expression in LRP is notable. *OFP8* repression did not impact the proportion of  
403 total root length contributed by lateral roots (**Figure 4D**) but these lines had shorter primary roots  
404 (**Supplemental Figure 6**).  
405

406 **Non-LRP cells populations undergo transcriptional changes and fate transitions in response to  
407 lateral root initiation**

408 Formation of a new lateral root is a self-organizing process during which a very limited number of  
409 competent XPP cells undergo repeated cell divisions to initiate lateral root organogenesis (Torres-  
410 Martínez et al., 2020). Continued development of the new root requires biophysical restructuring of the  
411 surrounding cell files. Signatures of lateral root development are seen outside the pericycle at pre-  
412 emergence stages of development, including during early initiation (Vermeer et al., 2014). Feedback on  
413 auxin signaling and changes in auxin transport patterns in the endodermis (Marhavý et al., 2013) and the  
414 vasculature (De Smet et al., 2007; Porco et al., 2016) are also essential for the first steps of lateral root  
415 initiation. Because our single-cell RNA sequencing dataset allowed us to examine the transcriptional state  
416 of these different cell layers independently, we examined which of the non-pericycle cell files contribute  
417 to transcriptional changes in response to this fate switch. For this we leveraged DEGs (945 genes)  
418 identified from a previous bulk RNA transcriptome study of bend-induced lateral root initiation (Voß et  
419 al., 2015) corresponding to the twenty hour post-bend timepoint in our study, and mapped the expression  
420 of these genes to our cell type resolved dataset (**Figure 5A**). As expected, most of these genes showed  
421 high expression in the LRP population and very low expression in the XPP population. In addition, we  
422 found strong enrichment of certain groups of genes in non-LRP populations, especially in those  
423 categorized as mature pericycle, endodermis cells and root cap cells.  
424



**Figure 5. Analysis of non-LRP cells in the single-cell library.** A. Heatmap (row-scaled) of expression patterns of previously-identified LR-specifying genes in different cell clusters. B. Expression heatmap (row-scaled) of specific auxin-inducing and auxin-repressing genes in different stele cell clusters. C. UMAP of Cortex, Endodermis, and Lateral Root Responding Endodermis (LRE) cells. D. Expression UMAP of ORE1, a NAC transcription factor that promotes autophagy of endodermal cells overlying LRP. E. Expression UMAP of ATG8A. F. Gene ontology of genes enriched in endodermis and LRE cell populations. The Venn diagram represents DEGs upregulated in endodermis in comparison to the cortex and upregulated in LRE in comparison to the cortex. A selection of GO terms for each of these sets of DEGs are shown.

425 We focused first on genes showing strong expression in the mature pericycle, as these cells could be

426 directly in contact with the initiating primordium. To assess if the genetic circuits regulating the XPP to  
427 mature pericycle transition were linked to lateral root development at the bend, we examined the genes  
428 expressed in our pseudotime analysis of the transition of XPP cells to mature pericycle cells as compared  
429 to the genes expressed in the trajectory the transition of XPP cells to LRP cells (**Supplemental Figure**  
430 **2A-D**). Genetic signatures in this mature pericycle trajectory suggested that these cells, acting as the  
431 outgroup for this fate transition, nonetheless were responding to lateral root development, though in a  
432 very distinct way from cells within the XPP to LRP trajectory. One gene that was expressed in XPP cells  
433 and LRP cells close to the branch point is *TRANS-ACTING siRNA3 (TAS3)*. *TAS3* promotes lateral root  
434 development by inhibiting the class B auxin response factors *ARF2*, *ARF3* and *ARF4* (Marin et al., 2010)  
435 Consistent with this pattern, *ARF2* transcripts were found only in the mature pericycle trajectory. While  
436 *TAS3* expression is fairly consistent across XPP, LRP, and mature pericycle cells, *ARF2*, *ARF3*, and  
437 *ARF4* show greater expression in the mature pericycle cell population as compared to the XPP population,  
438 and also show high expression in PPP cells (**Figure 5B**). As LRP never initiate from PPP, strong  
439 expression of these repressor ARFs in mature pericycle cells and PPP cells may indicate that these genes  
440 are repressing auxin response, and thus lateral root development, in these cells.

441  
442 A growing body of research has shown that LRP send signals to actively repress initiation of  
443 surrounding pericycle cells, allowing for the proper spacing of lateral roots along the axis of the primary  
444 root (Murphy et al., 2016; Toyokura et al., 2019; Trinh et al., 2019). The strong expression of genes that  
445 repress auxin response in mature pericycle cells suggests one mechanism for this inhibition. Further  
446 evidence in support of this model is the expression of another repressor ARF, *ARF9*, and the auxin  
447 repressor *IAA2* only in the mature pericycle trajectory. Uniquely expressed in this trajectory are also two  
448 cytokinin response factors, *ARABIDOPSIS THALIANA RESPONSE REGULATORS 5* and *11* (*ARR5* and  
449 *ARR11*), which are known to inhibit auxin response, repress lateral root development and coordinate  
450 uniform spacing of lateral roots (Mason et al., 2005; To et al., 2004). A target of cytokinin signaling,  
451 *CFB1*, which is specifically expressed in the flanking zone of developing LRP (Brenner et al., 2017) is  
452 also unique to this trajectory. Together, these data suggest that cells that were initially categorized as  
453 mature pericycle may be more accurately described as LRP-flanking pericycle cells, and this flanking fate  
454 is specifically induced by the initiation of a new root.

455  
456 **Lateral root development shows strong signatures in endodermal transcriptomes**  
457 We next examined the impact of lateral root initiation on the endodermal cell file which is immediately  
458 exterior to the pericycle. We first re-clustered the 813 cell partition labeled as cortex and endodermis  
459 cells. This analysis revealed three distinct cell populations. The first population was a set of 127 cells

460 which expressed cortex marker genes, the second population was a set of 410 cells which expressed  
461 endodermis marker genes, and the third population of 276 cells branching out from endodermis  
462 population (**Figure 5C, Supplemental Figure 10A, Supplemental Figure 10B**). This third population  
463 was transcriptionally most similar to the 410 cells expressing endodermis marker genes, and twenty-six  
464 percent of the cells in this third population were from the twenty hour timepoint. As a comparison, about  
465 eight percent of the 127 cell population were from the twenty hour timepoint, while about nine percent of  
466 the 410 cell population were from the twenty hour timepoint. This third population also had the highest  
467 expression of the NAC transcription factor, *ORESARA1 (ORE1)* and autophagy marker gene *ATG8a*  
468 (**Figure 5D and E**). *ORE1* is a positive regulator of programmed cell death and autophagy marker gene  
469 *ATG8a* are specifically expressed in cells overlying LRP (Escamez et al., 2020). *ORE1* expressing cells  
470 eventually die in order to make space for the lateral root to emerge (Escamez et al., 2020). As such, we  
471 categorized these three populations as cortex (127 cells), endodermis (410 cells), and lateral root-  
472 responding endodermis (LRE) (276 cells) (**Supplemental Table 1, Supplemental Figure 10A**).  
473

474 To aid in identifying differentially expressed genes between endodermis and LRE, both cell types were  
475 compared to cortex cells as an outgroup. As in the XPP-LRP comparison, only genes that were called  
476 significantly different in at least two methods were called as DEGs (**Supplemental Figure 11**). This  
477 analysis yielded over 2000 DEGs between endodermis and cortex, and over 3000 DEGs between LRE  
478 and cortex. As expected, there was large overlap between these two sets of DEGs (**Supplemental Data**  
479 **3**). A smaller set of genes were identified that were specific to the endodermis cells (204 genes), and  
480 specific to the LRE cells (303 genes), while 240 were commonly upregulated genes in endodermis and  
481 LRE (**Figure 5F**). The DEGs specific to the endodermis cells were enriched for GO terms associated with  
482 cell-cell junction assembly and water transport, while the DEGs specific to the LRE cells were enriched  
483 for GO terms associated with hormone response, auxin homeostasis, cell communication, lateral root  
484 development, and multiple stress responses (**Supplemental Data 3**).  
485

486 Pseudotime analysis was performed to identify genes driving the transition from endodermis to the lateral  
487 root endodermis. This analysis showed a branch point between the two cell types. One branch that we  
488 termed the “main branch” was composed mostly of cells from the eight hour time point, and was  
489 transcriptionally similar to the rest of the endodermis cluster. The other branch that we called the “LRE  
490 branch” led towards the LRE cluster (**Supplemental Figure 12A**). Again, two separate trajectories were  
491 analyzed, one containing only endodermis cells and another containing endodermis cells below the branch  
492 point and LRE cells. This yielded a set of 2082 genes whose expression changed over the course of the  
493 cell developmental trajectory. After removing genes upregulated in cortex cells, a small set of genes were

494 identified as specific to the main branch (154 genes) and to the LRE branch (648 genes) (**Supplemental**  
495 **Figure 12B, Supplemental Data 3**). GO analysis for the main branch specific genes showed enrichment  
496 for terms associated with responses to various stimuli. An example of a main branch specific gene is  
497 *DEEPER ROOT 1 (DRO1)* (**Supplemental Figure 12C**). Negatively regulated by auxin, *DRO1* is  
498 involved in gravity sensing in the root tip and determining lateral root branch angle (Uga et al., 2013;  
499 Guseman et al., 2017; Waite et al., 2020). *DRO1* loss of function mutants have increased horizontal  
500 lateral roots and have trouble establishing auxin gradients in response to gravistimulus. In our dataset, the  
501 majority of cells expressing *DRO1* are endodermal cells, suggesting these cells play a specific role in  
502 response to gravity. The LRE branch genes were enriched for GO terms associated with lateral root  
503 development, auxin homeostasis, auxin transport, and biosynthetic processes. Examples of LRE branch  
504 specific genes are *WRKY75* and *PINS-LIKE 5 (PILS5)* (**Supplemental Figure 12D**). *WRKY75* is induced  
505 particularly in phosphate starvation (Devaiah et al., 2007), and *PILS5* is an auxin efflux carrier regulating  
506 intracellular auxin homeostasis, both independently having a role in controlling root architecture. These  
507 along with gene ontologies pertaining to several biotic and abiotic stress responses (**Supplemental Data**  
508 **3**) indicates that LRE cell population seems to be primed to dynamically assess environment and thereby  
509 regulate lateral root emergence.

510

511

512 **Discussion**

513 Among the greatest mysteries in development is the process by which a stem cell begins proliferating and  
514 partitioning its progeny into increasingly determined cell fates. In plants, the initiation of lateral root  
515 development is among the best understood of these processes, yet many fundamental questions remain.  
516 Auxin is clearly a critical signal, but what other pathways interact with auxin response or regulate  
517 developmental steps downstream of auxin perception are still largely unknown. One key piece of missing  
518 information is a full accounting of transcriptional changes in the lateral root primordia during the critical  
519 window of initiation. In this study, we performed single-cell RNA sequencing at two timepoints on  
520 regions of roots where lateral root initiation was taking place. We identified cells of all cell types of the  
521 root outside the meristem in our population, including cells expressing lateral root markers. Through  
522 differential gene expression analysis, we identified genes upregulated in LRP cells as compared to XPP  
523 cells, many of which were indicative of cells undergoing cellular differentiation and organogenesis. We  
524 also identified a subset of pericycle and endodermal cells outside of the primordium itself that appear to  
525 be responding to the initiation of a new root.

526

527 We chose several genes for further study. Transcriptional reporters confirmed that most were expressed in  
528 early stage lateral roots, and that this expression was in many cases dependent on *ARF7* and *ARF19*.  
529 Several reporters showed additional expression in other parts of the root, including the primary root  
530 meristem. *OFP8* and *BRXL1* were expressed specifically in LRPs, making them potentially useful tools  
531 for studying and engineering root architecture. Repression of many of our candidate genes in XPP cells  
532 caused defects in lateral root development, a previously unknown role for some of them. For example,  
533 *HD2C* and *HD2B* have been linked to down-regulation of ribosomal biogenesis genes (Chen et al., 2018),  
534 a process we found to be strongly induced in lateral root primordia. This connection is consistent with our  
535 finding that repression of these genes led to higher density of lateral roots. It also highlights the fact that  
536 genes that are upregulated during primordia initiation may be important either for promoting  
537 developmental progression or for providing checks to keep cellular events coordinated or appropriately  
538 controlled. *OFP8* has been reported to act as a direct transcriptional repressor (Wang et al., 2011), and as  
539 a regulator of cell division patterns and organ shape via modification of the cytoskeleton (Snouffer et al.,  
540 2020).

541

542 Within the primordium itself, careful regulation of cell division and growth are essential for the formation  
543 of a primordium with normal morphology. The majority of our J0121<sup>Col>>dCas9R</sup> transgenic plants  
544 exhibited increased lateral root density or increased lateral root length as a proportion of total root length.  
545 These results suggest that these target genes may normally act as repressors of lateral root initiation or

546 emergence. When they are repressed, development is accelerated. One interpretation is that cell division  
547 and lateral root development are the default state of competent pericycle cells. This would be analogous  
548 to the situation in the root epidermis where becoming a root hair is the default state that must be actively  
549 repressed in non-hair cells (Berger et al., 1998). This hypothesis is supported by experiments where laser  
550 ablation of surrounding tissue causes unrestricted cell division in the pericycle cell file (Marhavý et al.,  
551 2016), and where exogenous auxin treatments trigger lateral root initiation in every pericycle cell  
552 (Himanen et al., 2002).

553  
554 Using cell-type specific repression of our candidates in XPP cells allowed us to avoid several problems  
555 with global mutant analyses. For example, *PLT3* and *BRXL1* play critical roles in the primary root  
556 meristem, making interpretation of any lateral root phenotypes difficult. A narrower scope of repression  
557 may also reduce the likelihood of compensation from paralogs. The repression lines decrease expression  
558 only in XPP cells and in LRP cells up to stage IV. This tissue-specific expression may explain the  
559 phenotypic difference between our *PLT3*-repressed line and *plt3 plt5 plt7* mutants, which show decreased  
560 lateral root emergence (Du and Scheres, 2017). It is possible that *PLT3* is playing opposing roles in  
561 different cell files of the root or at different points in lateral root development, depending on the  
562 interacting partners that are present in these cell files at these developmental stages. Alternatively, the  
563 phenotypes we observed could reflect feedback effects on other genes in the same gene regulatory  
564 network. The analysis performed here is likely to have missed phenotypes, especially those that rely on  
565 complex interactions with a soil environment or on a different set of metrics (Fitter, 1987; Lynch, 1995).  
566 For example, the irregular spacing and rate of growth of lateral roots in our *BRXL1/BRXL4*-repressed  
567 lines did not significantly impact the metrics we tested (**Supplemental Figure 9**). Introgression of J0121  
568 into different accessions would allow access to a broader array of cryptotypes (Chitwood and Topp, 2015;  
569 Ristova et al., 2018), and a more holistic view of impacts on root architecture.

570  
571 We found that our single-cell experiments captured the majority of previously reported LRP-enriched  
572 transcripts in our LRP-assigned cells, and these transcripts were not enriched in our XPP-assigned cells.  
573 We additionally found many more LRP-enriched genes in our library than in bulk transcriptomes,  
574 underlining the utility of this method in examining rare developmental events. Many of our XPP-  
575 upregulated genes oscillate antiphase to auxin response in the basal meristem. These antiphase-oscillatory  
576 genes are upregulated in cells that are *not* competent to form lateral roots. They may be actively  
577 preventing lateral root initiation in XPP cells, in alignment with the hypothesis that LRP-competency may  
578 be the default state of XPP cells. Notably, there are also 698 cells within our stele cluster that were too  
579 ambiguous in their gene expression to assign a cell label. It is possible some of these cells are precursors

580 to stele cells, such as XPP precursors that are not yet lateral root competent. How distinct lateral root  
581 competent and non-competent XPP cells are is unknown—the only known distinguishing feature between  
582 the two is a characteristic auxin response maximum or lack thereof. Additional differential gene  
583 expression between different timepoints within the XPP cluster and this non-assigned stele cell cluster  
584 may yield novel insights.

585  
586 Though lateral root development is specific to xylem pole pericycle cells, the process is not cell-  
587 autonomous. Our analysis identified a population of endodermal cells distinct from the main endodermis  
588 branch. These cells were enriched in the expression of genes falling in ontology categories for hormone  
589 and auxin response, cell-cell communication, and lateral root development, making a strong case that they  
590 are responding to developing primordia in underlying pericycle cells. They were also enriched in *ORE1*  
591 expression, a gene that has recently been shown to play a role in lateral root initiation and emergence  
592 through programmed cell death of tissue overlying LRP (Escamez et al., 2020). Consequently, this  
593 population of endodermal cells appears to be responding to lateral root initiation in neighboring pericycle  
594 cells, and forging a path for the incipient primordium.

595  
596 We were also able to identify a subset of pericycle cells that were likely directly adjacent to the  
597 primordium and responding with a distinct transcriptional program that included a combination of auxin-  
598 repressing and cytokinin-induced genes. A dynamic analysis of the gene regulatory network governing  
599 lateral root development established that early cell fate determining genes initiate multiple genetic  
600 feedback loops that divide the developing primordium into two zones, a central proliferative core and  
601 flanking cells that have inhibited expression of meristematic genes to repress cell division (Lavenus et al.,  
602 2015). Notably, PPP cells in our dataset also show strong expression of several auxin-inhibitory genes.  
603 PPP cells never initiate lateral root development, even though these cells receive the same auxin  
604 maximum signal as XPP cells during specification. It is possible these repressors of auxin signaling act to  
605 prevent spurious root development in multiple cell files.

606  
607 As human activity changes the climate and environments in which plants grow, understanding root  
608 development will help us engineer crops that are more robust to nutrient scarcities and environmental  
609 extremes. The major pathway by which eudicot plants regulate their root architecture is through  
610 modification of the position, spacing, density, and length of lateral roots. The stages of lateral root  
611 development are regulated by distinct genetic circuitry. Every stage represents an opportunity for natural  
612 and engineered modification of this developmental process. Molecular characterization of early stages of

613 lateral root at single-cell resolution gives us a more comprehensive understanding of this fate decision and  
614 the molecular pathways that tune it.

615  
616

617 **Figure Legends**

618

619 **Figure 1: Analysis of lateral root initiation using single-cell RNA-sequencing.** A. Experimental  
620 design: *Arabidopsis thaliana* seedlings were grown vertically for 4 days post-germination (dpg) then  
621 rotated (or marked, in case of control) and grown for an additional eight or twenty hours. Protoplasts were  
622 prepared from microdissected root sections for single-cell RNA sequencing. B. UMAP of all 6658 cells  
623 colored by experiment. Cell type identities were assigned to each partition based on a set of marker genes.  
624 The cell type identities are indicated on the UMAP with the number of cells corresponding to each cell  
625 type. C. UMAP of pericycle specific cells, such as lateral root primordia (LRP), mature pericycle (MP),  
626 phloem pole pericycle (PPP), and xylem pole pericycle cells (XPP) colored by experiment. D. Expression  
627 pericycle-specific UMAPs of two DEGs between LRP and XPP. E. Scatter plot of the log2 fold change of  
628 average gene expression between XPP and LRP and the Mann Whitney Wilcoxon qvalue. DEGs are  
629 colored in red. A selection of GO Terms associated with DEGs are shown above the plot.

630

631 **Figure 2. Validation experiments on chromatin modifier candidate genes.** A. Heatmap (row-scaled)  
632 visualizing expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale  
633 bar represents the z-score of the normalized expression values. B. Confocal microscopy images of  
634 candidate genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image  
635 of corresponding primordia (right). C. Lateral root density of  $J0121^{Col} \gg dCas9R$  transgenic lines of  
636 candidate genes. D. Proportion of total root length contributed by lateral roots of  $J0121^{Col} \gg dCas9R$   
637 transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison  
638 with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines  
639 have been analyzed; each colored line in the graph represents an individual line.

640

641 **Figure 3. Validation experiments on cell cycle regulator candidate genes.** A. Heatmap (row-scaled)  
642 visualizing expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale  
643 bar represents the z-score of the normalized expression values. B. Confocal microscopy images of  
644 candidate genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image  
645 of corresponding primordia (right). C. Lateral root density of  $J0121^{Col} \gg dCas9R$  transgenic lines of  
646 candidate genes. D. Proportion of total root length contributed by lateral roots of  $J0121^{Col} \gg dCas9R$   
647 transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison  
648 with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines  
649 have been analyzed; each colored line in the graph represents an individual line.

650

651 **Figure 4. Validation experiments on stemness candidate genes.** A. Heatmap (row-scaled) visualizing  
652 expression of candidate genes in the pericycle cell clusters from the single-cell library. Scale bar  
653 represents the z-score of the normalized expression values. B. Confocal microscopy images of candidate  
654 genes' transcriptional reporters in early stage lateral root primordia (left) and bright field image of  
655 corresponding primordia (right). C. Lateral root density of  $J0121^{Col} \gg dCas9R$  transgenic lines of  
656 candidate genes. D. Proportion of total root length contributed by lateral roots of  $J0121^{Col} \gg dCas9R$   
657 transgenic lines of candidate genes. For C and D, significance was determined by pairwise comparison  
658 with empty vector control and ANOVA. For each candidate gene, multiple independent transgenic lines  
659 have been analyzed; each colored line in the graph represents an individual line.

660

661 **Figure 5. Analysis of non-LRP cells in the single-cell library.** A. Heatmap (row-scaled) of expression  
662 patterns of previously-identified LR-specifying genes in different cell clusters. B. Expression heatmap  
663 (row-scaled) of specific auxin-inducing and auxin-repressing genes in different stele cell clusters. C.  
664 UMAP of Cortex, Endodermis, and Lateral Root Responding Endodermis (LRE) cells. D. Expression  
665 UMAP of ORE1, a NAC transcription factor that promotes autophagy of endodermal cells overlying  
666 LRP. E. Expression UMAP of AT8GA. F. Gene ontology of genes enriched in endodermis and LRE cell  
667 populations. The Venn diagram represents DEGs upregulated in endodermis in comparison to the cortex  
668 and upregulated in LRE in comparison to the cortex. A selection of GO terms for each of these sets of  
669 DEGs are shown.  
670

671 **Supplemental Figure 1. Marker gene expression profiles and stele cell UMAP.** A. Heatmap (column-  
672 scaled) visualizing average normalized expression of marker genes in the columella cells, epidermis cells,  
673 cortex & endodermis cells, and stele cells. Scale bar represents the z-score of the normalized expression  
674 values. B. Heatmap (column-scaled) visualizing average normalized expression of marker genes in  
675 different stele cell types. Scale bar represents the z-score of the normalized expression values. C. UMAP  
676 of stele cells colored by experiment. D. Fraction of xylem pole pericycle (XPP), lateral root primordia  
677 (LRP), mature pericycle (MP), and phloem pole pericycle (PPP) cells from each experiment.  
678

679 **Supplemental Figure 2. Xylem pole pericycle developmental trajectories.** A. UMAP of the XPP to  
680 Mature Pericycle trajectory colored by pseudotime. B. UMAP of the XPP to LRP trajectory colored by  
681 pseudotime. C. Expression UMAP (XPP and Mature Pericycle cells) of DEGs identified in both  
682 trajectories (XPP to Mature Pericycle and XPP to LRP) and in only the XPP to Mature Pericycle  
683 trajectory. D. Expression UMAP (XPP and LRP cells) of DEGs identified in both trajectories and in only  
684 the XPP to LRP trajectory. E.. Heatmap (row-scaled) visualizing average normalized expression of genes  
685 identified as differentially expressed in the XPP to LRP trajectory. Scale bar represents the z-score of the  
686 normalized expression values.  
687

688 **Supplemental Figure 3. Size-adjusted Venn diagram visualizing overlap of genes between different  
689 DEG calling methods.** For each circle of the Venn Diagram, the total number of DEGs as well as the  
690 number of DEGs up in LRP and up in XPP are shown. All genes that were called in two or more methods  
691 were used for downstream analysis.  
692

693 **Supplemental Figure 4. Comparison of XPP and LRP DEGs from the single-cell library to bulk  
694 transcriptomes.** A. Comparison to LRP-induced genes from Vanneste *et al*, 2005. B. Comparison to  
695 time-course analysis of lateral root initiation at nine and twenty-one hours post-bend from Voß *et al*,  
696 2015. C. Comparison to auxin-induced and auxin-repressed genes in the root from Lewis *et al*, 2013. D.  
697 Comparison to ARF19-specific auxin-induced genes from Powers *et al*, 2019. E. Comparison to genes  
698 oscillating in phase and antiphase to auxin in the basal meristem during lateral root specification from  
699 Moreno-Risueno *et al*, 2010. F. Comparison to auxin-induced genes during early lateral root development  
700 from Ramakrishna *et al*, 2019. Each gene set from bulk transcriptomes is compared to the XPP and LRP  
701 DEGs with a size-adjusted Venn diagram. The number of genes in each mutually-exclusive area of each  
702 Venn diagram are specified. The values in purple and turquoise denote the hypergeometric distribution  
703 for the union XPP/bulk transcriptome and LRP/bulk transcriptome respectively.  
704

705 **Supplemental Figure 5. Design of J0121<sup>Col</sup>>>dCas9R system to generate cell type-specific dCas9-  
706 repressor mediated knockdown of candidate gene expression.** A. J0121<sup>Col</sup> is an enhancer trap line  
707 where the UAS-Gal4 system drives expression of GFP in the xylem pole pericycle cell file, visualized in  
708 confocal microscopy image and B. labeled in cartoons (in green). C. Top panel indicates the enhancer  
709 trap cassette in the J0121<sup>Col</sup> and bottom panels is design of perturbation plasmids included up to three  
710 guide RNAs, tagged as location L1, L2, and L3, and a UAS promoter driving expression of dCas9-  
711 repressor cassette. Perturbation plasmids with respective cloned guide RNAs were transformed into  
712 J0121<sup>Col</sup> background to drive repression of target genes specifically in xylem pole pericycle cells.  
713

714 **Supplemental Figure 6. Example seedling traces from J0121<sup>Col</sup>>>dCas9R perturbation lines.** Roots  
715 from T2 perturbation line seedlings were quantified for various lateral root developmental phenotypes  
716 using SmartRoot.  
717

718 **Supplemental Figure 7. Transcriptional reporters of chromatin regulator candidate genes in wild  
719 type and *arf7arf19* roots.** Fluorescent microscopy images of transgenic plant lines carrying  
720 transcriptional reporters of candidate genes in early stage lateral root primordia are shown in the large  
721 left-side image of each panel. Smaller images of the same transcriptional reporters in a region of the  
722 differentiated zone of the root without any developing primordia (above) and in the root apical meristem  
723 (below) are shown on the right in each panel. The same reporters in the same regions of the root are  
724 imaged in *arf7arf19* mutant background plants on the right. The number of independent transgenic lines  
725 imaged per construct and the number of plants within each line that showed expression are reported at the  
726 bottom. The lower panel represents 1000 bp upstream of the transcription start site for each gene, with  
727 auxin response elements (TGTC/GACA) highlighted in red. Yellow bars indicate CDSs from other genes.  
728 This panel was obtained from <http://bar.utoronto.ca/cistome>.  
729

730 **Supplemental Figure 8. Transcriptional reporters of cell cycle candidate genes in wild type and  
731 *arf7arf19* roots.** Fluorescent microscopy images of transgenic plant lines carrying transcriptional  
732 reporters of candidate genes in early stage lateral root primordia are shown in the large left-side image of  
733 each panel. Smaller images of the same transcriptional reporters in a region of the differentiated zone of  
734 the root without any developing primordia (above) and in the root apical meristem (below) are shown on  
735 the right in each panel. The same reporters in the same regions of the root are imaged in *arf7arf19* mutant  
736 background plants on the right. The number of independent transgenic lines imaged per construct and the  
737 number of plants within each line that showed expression are reported at the bottom. The lower panel  
738 represents 1000 bp upstream of the transcription start site for each gene, with auxin response elements  
739 (TGTC/GACA) highlighted in red. Yellow bars indicate CDSs from other genes. This panel was obtained  
740 from <http://bar.utoronto.ca/cistome>.  
741

742 **Supplemental Figure 9. Transcriptional reporters of stemness candidate genes in wild type and  
743 *arf7arf19* roots.** Fluorescent microscopy images of transgenic plant lines carrying transcriptional  
744 reporters of candidate genes in early stage lateral root primordia are shown in the large left-side image of  
745 each panel. Smaller images of the same transcriptional reporters in a region of the differentiated zone of  
746 the root without any developing primordia (above) and in the root apical meristem (below) are shown on  
747 the right in each panel. The same reporters in the same regions of the root are imaged in *arf7arf19* mutant  
748 background plants on the right. The number of independent transgenic lines imaged per construct and the

749 number of plants within each line that showed expression are reported at the bottom. The lower panel  
750 represents 1000 bp upstream of the transcription start site for each gene, with auxin response elements  
751 (TGTC/GACA) highlighted in red. Yellow bars indicate CDSs from other genes. This panel was obtained  
752 from <http://bar.utoronto.ca/cistome>.

753

754 **Supplemental Figure 10. Marker gene expression profiles and experiment breakdown of cortex,**  
755 **endodermis, and lateral root endodermis cells.** A. Heatmap (column-scaled) visualizing average  
756 normalized expression of marker genes in the cortex, endodermis, lateral root endodermis (LRE) cells. B.  
757 Fraction of cortex, endodermis, and LRE cells from each experiment.

758

759 **Supplementary Figure 11. DEG overlaps with different methods for Endodermis/Lateral Root**  
760 **Endodermis analyses** A. Endodermis vs cortex comparison and B. LRE vs cortex comparison. For each  
761 ensemble of the Venn Diagram, the total number of DEGs, the number of DEGs up in Cortex and up in  
762 Endodermis (for A) and LRE (for B) are added to the diagram.

763

764 **Supplemental Figure 12. Pseudotime analysis of Endodermis to Lateral Root Endodermis Cells.** A.  
765 UMAP of the endodermis to LRE trajectory colored by pseudotime. B. UMAP of the expression of gene  
766 sets that differed significantly as a function of pseudotime in the main endodermis branch and the LRE  
767 Branch. C. Expression UMAP of DRO1. D. Expression UMAPs of WRKY75 and PILS5.

768

769 **Supplementary Table 1. Breakdown of cell types by experiment.**

770

771

772 **Author contributions:** A.L., H.P.G., C.Q., J.T.C and J.L.N. designed the research; H.P.G., A.L., S.G.,  
773 J.C.C., J.E.Z., W.G. and J.T.C performed research; A.L., H.P.G., K. J-B., J.T.C and J.L.N. analyzed data;  
774 and A.L., H.P.G., K. J-B. and J.L.N. wrote the manuscript.

775

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785

786

787

788 **Methods**

789

790 *Construction of plasmids*

791 Each reporter plasmid is composed of the selected promoter, the red fluorescent protein mScarlet with a  
792 nuclear localization tag (Bindels et al., 2017), and the rbcS terminator (Siligato et al., 2016). The three  
793 parts were assembled using golden gate assembly in the modified pGII-Hygr vector containing  
794 compatible Golden Gate sites (Weber et al., 2011). For each of the ten constructed reporters, the promoter  
795 sequence of the reporter corresponds to the DNA sequence in 5' of the start codon of the corresponding  
796 gene based on TAIR10 genome from <http://plants.ensembl.org/>. While aiming for a 2000 bp length, the  
797 lengths of promoters are usually smaller to avoid coding sequences of other genes. The exact sequence  
798 and length of the selected promoter for each of the 10 genes can be found in supplementary material  
799 (**Supplementary Data 2**). The promoter sequence for the TMO6 reporters corresponds to the sequence  
800 used in a previous work from (Smet et al., 2019). The promoter sequences were amplified from purified  
801 *Arabidopsis thaliana* Col-0 genomic DNA using Q5 polymerase and with primer adding the specific  
802 golden gate spacer. After gel purification, each promoter part was cloned and sequence verified in a  
803 pBLUNT entry vector. Three part Golden Gate assembly was performed using the pBLUNT promoter  
804 plasmid, mScarlet, rbcS terminator to clone reporter plasmid.

805

806 For cell type specific knockdown mediated by J0121<sup>Col>>dCas9R</sup>, Gibson cloning was used to replace the  
807 egg specific promoter and Cas9 from pHEE401E (Wang et al., 2015) with UAS promoter and dCas9-TPL  
808 fusion (Khakhar et al., 2018). The resulting plasmid is used as starting point to clone two or three guide  
809 RNA against the promoters of selected gene/genes (identified using CHOP CHOP (Montague et al.,  
810 2014) ranging from -200 to +100 region from the annotated TSS) using PCR and golden gate strategy  
811 described in (Wang et al., 2015).

812 *Plant growth conditions and sample preparations*

813 For all plant experiments, *Arabidopsis* seeds were sown on 0.5× LS 0.8% agar plates, stratified at 4°C for  
814 2 days, and grown in continuous light conditions at 22°C for respective experimental design.

815 *Microdissection of root bend and protoplast isolation*

816 For lateral root induction assays, ~150 seedling for each timepoint and treatment were rotated 90° 4 days  
817 post-germination (dpf) or in the case of the control treatment, the primary root tip was marked at this time  
818 and the plates were not turned. On the day of single-cell library preparation first the protoplasting enzyme  
819 mix was prepared adapted from (Yoo et al., 2007). Briefly, 20 mM MES (pH 5.7) containing 1.25%  
820 (wt/vol) cellulase R10 (C224 PhytoTechnology Laboratories), 0.3% (wt/vol) macerozyme R10 (M481  
821 PhytoTechnology Laboratories,), 0.4 M mannitol and 20 mM KCl was prepared and incubated in 55°C  
822 warm water bath for 10 minutes. Upon cooling to room temperature (~25 °C), 10 mM CaCl<sub>2</sub>, 1–5 and  
823 0.1% BSA was added. Root bends (or marked region in no bend control) were microdissected using a  
824 scalpel at eight hours (control and eight hour treatment groups) and 20 hours (20 hour treatment group)  
825 post-bending, approximately 1 mm from the bend or mark in both directions. Using fine forceps dissected  
826 bend tissue was transferred into 30 mm dishes containing 1 mL of protoplasting enzyme mix and gently  
827 scored using a fresh scalpel to increase exposure of interior cell files to protoplasting enzymes. The plates  
828 were then flooded with 9 mL more protoplasting enzyme mix and incubated at room temperature for one

829 hour with gentle shaking (75-80rpm). Protoplasting enzyme mix was filtered through 40  $\mu$ m cell strainer,  
830 transferred and centrifuged in 15 ml conical tubes for five min at 500g. The supernatant was carefully  
831 removed and resuspended in 50  $\mu$ L protoplasting mix without enzymes. Cell number was determined by  
832 hemocytometer and density was adjusted to ~1000cell/ $\mu$ L

833 *Construction and selection of transgenic Arabidopsis thaliana lines*

834 Floral dip (Clough and Bent, 1998) was used to introduce constructs into Col-0 and *arf7arf19* lines  
835 (Okushima et al., 2007). T1 seedlings were selected on 0.5X LS (Caisson Laboratories, Smithfield, UT) +  
836 25 $\mu$ g/ml Hygromycin B + 0.8% bacto-agar. Plates were stratified for two days, exposed to the light for six  
837 hours, and then grown in the dark for three days (Harrison et al., 2006). Hygromycin resistant seedlings  
838 were identified by their long hypocotyl, enlarged green leaves, and long root. Transformants were  
839 transferred on soil, and T2 seeds were collected.

840

841 *Lateral root bend assay and confocal microscopy*

842 For each reporter, one Col-0 T1 line representative of other characterized T1 lines was selected to  
843 perform lateral root bend assay. For each reporter, 20 T2 seeds of the corresponding T1 line were placed  
844 on 0.5 LS+0.8% bacto-agar plate following a specific pattern to avoid seedling collision during the lateral  
845 root bend assay. The plate was stratified during 120 hours, grown vertically for 96 hours at 22°C, rotated  
846 90°C while keeping vertically and grown for an additional 20 hours.

847

848 *Confocal microscopy of reporter lines at root bends*

849 Seedlings were fixed at 4 dpg + 20 hours using 4% formaldehyde using vacuum infiltration followed by  
850 cleared using ClearSee solution (Kurihara et al., 2015). Fixed and cleared seedlings were mounted on  
851 microscopic slides using 50% glycerol and parafilm edges to avoid coverslips pressing on the root.  
852 Seedlings were imaged at the bend region using a SP5 confocal microscope. Images were processed using  
853 FIJI.

854

855 *Comparison between Col-0 and *arf7arf19* lines*

856 To perform comparative imaging of Col-0 and *arf7arf19* reporter lines, seeds of selected T1 lines for both  
857 Col-0 and *arf7arf19* reporter lines were placed on the same 0.5 LS+0.8 phytoagar plate. The selected Col-  
858 0 and *arf7arf19* lines for each reporter is specified below the microscope images of supplemental figure 7  
859 to 9 as being highlighted in bold. Plates were stratified for 2 days, and grown vertically at 22°C for 10  
860 days. Then, seedlings were imaged using a Leica DMI 3000B microscope at the root tip region, at the  
861 region above the root tip corresponding to the initiation of root hair and at the lateral root initiation  
862 region. As the *arf7arf19* line does not develop lateral roots, the theoretical lateral root initiation region is  
863 identified by identifying a lateral root primordium in the Col-0 seedling and imaging at a similar region.

864

865 *Lateral root phenotypes of repression lines*

866 For cell type specific knockdown mediated by J0121<sup>Col</sup>>>dCas9R we leveraged an established GAL4-  
867 UAS system (Laplaze et al., 2005) of enhancer trap line J0121. We backcrossed the J0121 line, made in  
868 the C24 background, eight times into the Col-0 background to produce a strain we refer to as J0121<sup>Col</sup>.  
869 We confirmed that J0121<sup>Col</sup> retained strong GFP expression in xylem pole pericycle and exhibited Col-0-  
870 like root growth dynamics. Transformants were selected as described above and T2 seeds for at least 10  
871 lines were collected. T2 seeds were grown vertically for 10dpg in 100mm square plate on 0.8% bacto agar

872 and were scanned on a flatbed scanner (Epson America, Long Beach, CA) for phenotyping. Since the T2  
873 generation is a segregating population for the transformed plasmid, seedlings were genotyped for the  
874 presence of vector backbone to identify positive seedling. Roots for positive seedlings were traced using  
875 ImageJ and SmartRoot plugin (Lobet et al., 2011) and analyzed and plotted using R package archiDART  
876 (Delory et al., 2016) package plot were generated using ggplot2. Density was measured as the total  
877 number of lateral roots divided by the length of the primary root. Proportion of lateral root length was  
878 measured as the summed length of all lateral roots divided by the summed length of all lateral roots and  
879 the primary root length.

880

#### 881 *Single-cell RNA-sequencing Protocol*

882 Single-cell RNA-Seq was performed using the 10X scRNA-Seq platform, the Chromium Single Cell  
883 Gene Expression Solution (10X Genomics). Two replicates were produced for each timepoint of the  
884 experiment for a total of six samples. We also generated two replicates from a transgenic plant line that  
885 slows the rate of degradation of IAA14 (Guseman et al., 2015), dissecting root bends in this line twenty  
886 hours after bending. This line shows delayed lateral root development, and we initially thought to  
887 compare its transcriptomes to our wild type treatment groups. Unfortunately, one of the replicates of this  
888 line failed at the 10X droplet-binding stage, so we did not obtain the same number cells from this  
889 treatment group as from our other groups. Consequently, we excluded these cells from further analysis.

#### 890 *Estimating Gene Expression in Individual Cells*

891 Single-cell RNA-sequencing reads were sequenced using an Illumina NextSeq 500 and then mapped to  
892 the TAIR10 *Arabidopsis* genome using the software Cellranger (v.3.0.1). Cellranger produces a matrix of  
893 UMI counts where each row is a gene and each column represents a cell. The ARAPO RT gene annotation  
894 was used. For the analysis, reads from two 00 hour replicates, two 08 hour replicates, and two 20 hour  
895 replicates were aggregated using the aggr command in cellranger to normalize to an equivalent number of  
896 mean reads per cell across samples. This resulted in a mean of 14,516 reads per cell, a median of 1,411  
897 genes per cell, and a median of 2,873 UMIs per cell.

#### 898 *Running Monocle 3: Dimensionality Reduction, and Cell Clustering*

899 The filtered output of the Cellranger pipeline (./outs/filtered\_gene\_bc\_matrices\_mex/) was parsed into R  
900 (v. 3.5.0). Particularly the matrix.mtx file was parsed using the readMM() function from the Matrix  
901 package (<https://cran.r-project.org/web/packages/Matrix/Matrix.pdf>), and the barcodes.tsv file and the  
902 genes.tsv file were parsed using the read.table() function. Genes that were expressed in less than 10 cells  
903 were removed from the analysis. In addition, the 346 genes induced due to protoplast generation process  
904 were also removed from the analysis (Birnbaum et al., 2003). The barcodes table was updated to label  
905 cells by Sample Number and Experiment. Finally the expression matrix, the barcode table, and the gene  
906 table were converted into a CellDataSet (CDS) using the new\_cell\_data\_set() function in Monocle 3  
907 (cole-trapnell-lab/monocle3, 2020) (v. 0.1.2; <https://cole-trapnell-lab.github.io/monocle3/>)

908

909 All Monocle 3 analysis was performed on a High Performance Computing cluster using 128 GB of RAM  
910 spread across eight cores. We visualized cell clusters and trajectories using the standard Monocle  
911 workflow. Monocle internally handles all normalization needed for dimensionality reduction,  
912 visualization, and differential expression. The CDS was normalized and pre-processed using the  
913 preprocess\_cds() function with the following parameters:

914

```
915 num_dim=100,  
916 method="PCA",  
917 norm_method="log",  
918 scaling=T,  
919 residual_model_formula_str="~ Sample_Number"  
920  
921 Preprocessing involves reducing the dimensionality of the data (the number of genes) using principal  
922 component analysis (PCA). Here, we retain the first 100 PCs for further dimensionality reduction, in  
923 addition we reduce batch effect across samples. Then, the PCA matrix was used to initialize a nonlinear  
924 manifold learning algorithm implemented in Monocle 3 called Uniform Manifold Approximation and  
925 Projection (UMAP) (McInnes et al., 2018). This allows us to visualize the data into two or three  
926 dimensions. Specifically, we projected the data onto two dimensions using the reduce_dimension()  
927 function using the following parameters:  
928  
929 reduction_method="UMAP",  
930 preprocess_method="PCA",  
931 umap.metric="cosine",  
932 umap.min_dist=0.1,  
933 umap.n_neighbors=15L,  
934 umap.nn_method="annoy",  
935 umap.fast_sgd=F  
936  
937 Next, cells were clustered into partitions using PhenoGraph (Levine et al., 2015) and into clusters using  
938 the Leidan community detection algorithm (Traag et al., 2019) with the cluster_cells() function using the  
939 following parameters:  
940  
941 reduction="UMAP",  
942 k=20,  
943 louvain_iter=1,  
944 partition_qval=0.05,  
945 weight=T,  
946 resolution=c(10^seq(-6,0)).  
947  
948 This resulted in 5 cell partitions, and 43 cell clusters. Next, for each cell partition, a cell trajectory was  
949 drawn atop the projection using Monocle's reversed graph embedding algorithm, which is derived from  
950 SimplePPT (Mao et al., 2017) using the learn_graph() function using the following parameters:  
951  
952 use_partition=T,  
953 close_loop=F,  
954 learn_graph_control=list(prune_graph=T)  
955  
956 To further analyze the partition we annotated as stele, those cells were reclustered together and were  
957 reanalyzed using Monocle 3 as previously described except during preprocessing, instead of performing  
958 PCA on all the genes, PCA was performed on just a set of stele cell marker genes reported earlier (Mao et
```

959 al., 2017). Specifically the “use\_genes” option was used in the preprocess\_cds() function, and a list of  
960 gene names was provided. Lastly, cells were clustered using the cluster\_cells() function, and the  
961 “resolution” parameter was set to 0.001. This produced 3 partitions, and 7 clusters.  
962

963 To further analyze the clusters we annotated as Phloem Pole Pericycle, Mature Pericycle, Xylem Pole  
964 Pericycle, and Lateral Root Primordia, those cells were reclustered together and were reanalyzed using  
965 Monocle 3 as previously described except during preprocessing, instead of performing PCA on all the  
966 genes, PCA was performed on just a set of stele cell marker genes reported in (Brady et al., 2007). Again,  
967 Specifically the “use\_genes” option was used in the preprocess\_cds() function, and a list of gene names  
968 was provided. Next, the data was reduced onto two dimensions using the reduce\_dimension function but  
969 the “umap.min\_dist” parameter was set to 0.01. Afterwards cell clusters were called as before using the  
970 cluster\_cells() function except the “resolution” parameter was set to 0.0005. Next a cell trajectory was  
971 created using the learn\_graph() function. Finally cell clusters were recalled using the cluster\_cells()  
972 function except the “resolution” parameter was set to 0.001. This produced 1 partition and 4 clusters.  
973 To further analyze the partition we annotated as Cortex and Endodermis, those cells were reclustered  
974 together and were reanalyzed using Monocle 3 as previously described except during preprocessing,  
975 instead of performing PCA on all the genes, PCA was performed on just a set of cortex and endodermis  
976 cell marker genes reported in (Brady et al., 2007). This produced 2 partitions and 15 clusters.  
977

#### 978 *Estimating Doublets*

979 Single Cell Remover of Doublets (Scrublet) was used to predict doublets in our scRNA-Seq data  
980 (Wolock et al., 2019). Using python 3.5, Scrublet was run using default settings as described by the  
981 example tutorial that is available as a Python notebook  
[982 \(https://github.com/AllonKleinLab/scrublet/blob/master/examples/scrublet\\_basics.ipynb\)](https://github.com/AllonKleinLab/scrublet/blob/master/examples/scrublet_basics.ipynb). The only  
983 significant change was that expected double rate was set to 0.1; in the tutorial it is 0.06.  
984

#### 985 *Assigning Cell Types*

986 A set of known marker genes derived from earlier studies using green fluorescent protein (GFP) marker  
987 lines of the *Arabidopsis* root were used to identify cell types (Brady et al., 2007; Cartwright et al., 2009).  
988 The average gene expression of each marker set was used to assign cell types to cells, with cells being  
989 assigned the cell type it had the highest average expression  
990

#### 991 *Calling Differentially Expressed Genes: Xylem Pole Pericycle vs. Lateral Root Primordia*

992 Differentially expressed genes between the cluster of cells labeled Xylem Pole Pericycle and the cluster  
993 of cells labeled Lateral Root Primordia were called using three different approaches.  
994 The first approach involved running a generalized linear model to predict the average log express of each  
995 gene as a function of the cell type label. This was done using a subsetted CDS containing only the Xylem  
996 Pole Pericycle cells and the Lateral Root Primordia cells, and the Monocle 3 function fit\_models() with  
997 the following parameters:  
998

```
999 model_formula_str = " ~cell_type",  
1000 expression_family="negbinomial",  
1001 clean_model=T
```

1002 where “cell\_type” is a column in the dataframe returned by the colData() function that describes the cell  
1003 type label associated with a cell/barcode. Using an FDR cutoff of 0.1, 1204 genes were called as  
1004 differentially expressed between Xylem Pole Pericycle and Lateral Root Primordia. Of these, 424 were  
1005 more highly expressed in Xylem Pole Pericycle, and 780 were more highly expressed in Lateral Root  
1006 Primordia.

1007

1008 The second approach involved using the Mann-Whitney-Wilcoxon test to determine if the rank-sum of  
1009 the normalized expression values for each gene differed between the Xylem Pole Pericycle population  
1010 and the Lateral Root Primordia population. Mann-Whitney-Wicoxon test p-values were adjusted for  
1011 multiple test comparisons using the Benjamini-Hochberg procedure via the R function p.adjust() from the  
1012 stats package. Normalized expression values were calculated by taking the UMI matrix, obtained using  
1013 Monocle 3’s counts() function and dividing by the size factors of each cell using Monocle 3’s  
1014 size\_factors() function. Using an adjusted p-value cutoff of 0.0001, 2088 genes were called as  
1015 differentially expressed with 604 genes more highly expressed in Xylem Pole Pericycle and 1484 genes  
1016 more highly expressed in Lateral Root Primordia.

1017

1018 The last approach involved using the analysis tool Vision. The normalised expression matrix for only  
1019 XPP and LRP cell clusters was exported from Monocle. The gene signature of pericycle cell population  
1020 from (Parizot et al., 2012) were used for running Vision() and analyse() function. LRP cell population  
1021 was selected in browser view mode to identify DEG against XPP population. Vision identified 4900  
1022 DEGs using an FDR of less than 0.05.

1023

#### 1024 *Pseudotime Analysis: Xylem Pole Pericycle Cells Development*

1025 Pseudotime analysis was performed on two subsetted CDSs, one containing only Xylem Pole Pericycle  
1026 cells and Mature Pericycle cells, and the other containing only Xylem Pole Pericycle cells and Lateral  
1027 Root Primordia cells. Cells in both CDSs were assigned a pseudotime on the cell trajectory using  
1028 Monocle 3’s order\_cells() function with the Xylem Pole Pericycle serving as the root of the trajectory.  
1029 Genes whose expression changed as a function of pseudotime were identified using a generalized linear  
1030 model. This was done on both CDSs using the fit\_models() function and the following parameters:

1031

```
1032 model_formula_str = " ~pseudotime",  
1033 expression_family="negbinomial",  
1034 clean_model=T
```

1035 Using an FDR cutoff of 0.1, 1394 genes were identified as changing as a function of pseudotime in the  
1036 CDS containing only Xylem Pole Pericycle cells and Mature Pericycle cells, and 1014 genes were  
1037 identified as changing as a function of pseudotime in the CDS containing only Xylem Pole Pericycle cells  
1038 and Lateral Root Primordia cells with an overlap of 510 genes.

1039

#### 1040 *Calling Differential Expressed Genes: Endodermis vs. Lateral Root Primodia Responding Endodermis*

1041 As previously described, a generalized linear model was used to identify differentially expressed genes  
1042 between the cluster of cells labeled Endodermis, and the cluster of cells labeled Lateral Root Primodia  
1043 Responding Endodermis. Using an FDR cutoff of 0.1, 1251 genes were identified as differentially  
1044 expressed, with 748 genes being more expressed in Endodermis and 503 genes being more expressed in

1045 Lateral Root Endodermis. To identify additional DEGs, the MMW test was performed comparing  
1046 Endodermis to Cortex and Lateral Root Endodermis to Cortex.

1047

1048 *Pseudotime Analysis: Endodermis Cells*

1049 Pseudotime analysis was performed on two subsetted CDSs, one with only Endodermis cells, and the  
1050 other containing only Endodermis below the branch point, and Lateral Root Primodia Responding  
1051 Endodermis. As previously described, cells were assigned a pseudotime along the cell trajectory with the  
1052 Endodermis cells below the branch point serving as the root. As previously described, a generalized linear  
1053 model was used to identify differentially expressed genes as a function of pseudotime. Using an FDR  
1054 cutoff of 0.1, 2063 genes were identified in the CDS with only Endodermis, and 2079 genes were  
1055 identified in the CDS with only Endodermis and LRP Responding Endodermis with an overlap of 2060  
1056 genes.

1057

1058 *GO Term Enrichment Analysis*

1059 GO term enrichment analysis was performed using PANTHER (<http://pantherdb.org/>) (Mi et al., 2019).  
1060 For GO term enrichments for XPP, LRP, Endodermis, and LRE, genes that were significant in at least 2  
1061 methods were used for analysis. All genes in the *Arabidopsis* genome were used as a background.  
1062 Fisher's Exact test was used and the False Discovery Rate was calculated for multiple test correction. The  
1063 complete annotation data set for biological process, molecular function, and cellular component GO terms  
1064 were used for analysis.

1065

1066 *Accession Numbers*

1067 The GEO accession number for the scRNA-seq data reported in this paper is GSE158761  
1068 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158761>).

1069

1070 **Supplementary Data files**

1071 Supplemental Data 1. Xylem Pole Pericycle, Lateral Root Primordia, and Mature Pericycle DEG analysis  
1072 sheet and GO Terms

1073

1074 Supplemental Data 2. Cloning Primers for generation of plasmids used to generate reporter and  
1075 J0121<sup>Col</sup>>>dCas9R transgenic lines

1076

1077 Supplemental Data 3 . Cortex, Endodermis, and Lateral Root Endodermis DEG analysis sheet and GO  
1078 Terms

1079

1080

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