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# A root phloem pole cell atlas reveals common transcriptional states in protophloem adjacent cells

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# 36 Abstract

37 Single cell sequencing has recently allowed the generation of exhaustive root cell atlases. 38 However, some cell types are elusive and remain underrepresented. Here, we use a second-39 generation single cell approach, where we zoom in on the root transcriptome sorting with 40 specific markers to profile the phloem poles at an unprecedented resolution. Our data highlight the similarities among the developmental trajectories and gene regulatory networks communal 41 to protophloem sieve element (PSE) adjacent lineages in relation to PSE enucleation, a key 42 43 event in phloem biology. 44 As a signature for early PSE-adjacent lineages, we have identified a set of DNA-binding with one finger (DOF) transcription factors, the PINEAPPLEs (PAPL), that act downstream of 45

46 *PHLOEM EARLY DOF (PEAR)* genes, and are important to guarantee a proper root nutrition
47 in the transition to autotrophy.

48 Our data provide a holistic view of the phloem poles that act as a functional unit in root49 development.

50 Main text

# 51 INTRODUCTION

In plants, organs originate from meristems postembrionically and are patterned by mobile 52 53 signals and the positional information generated in the individual immobile cell types. 54 Determining cell type-specific transcriptional programs is key to understanding the positional 55 cues guiding plant development<sup>1</sup>. However, despite the importance of phloem in vascular 56 plants and radial growth pre-patterning<sup>2</sup>, phloem gene expression is not yet well characterized. 57 During root development, the term phloem is oftentimes used as a synonym of the protophloem sieve element (PSE), the cell type that undergoes a unique differentiation 58 59 process to specialize in the transport of sap from source photosynthetic organs to distant sink tissues. This simplification is probably the result of the extensive knowledge we have about PSE specification<sup>2,3</sup> and differentiation <sup>4–11</sup>. However, in the Arabidopsis primary root, the phloem pole is composed of six cells belonging to four distinct cell types: the central PSE is flanked by two phloem pole pericycle (PPP) cells to the outside and one metaphloem sieve element (MSE) cell to the inside, and both SE cells are in direct contact with the two lateral companion cells (CC)<sup>12</sup> (Fig 1a).

66 In the Arabidopsis root, both conductive elements (MSE and PSE) derive from the same stem 67 cell<sup>13</sup> but MSE differentiates later, when PSE cells are no longer functional. Despite having a similar function to PSE, MSE ontogeny is less well characterized<sup>14</sup> and few factors have been 68 directly related to MSE development. An exception are the partially redundant homologs 69 70 OCTOPUS (OPS, At3g09070) and OCTOPUS-LIKE 2 (OPL2, At2g38070) identified as 71 important for MSE entry into differentiation<sup>15</sup>. Despite some commonalities between PSE and 72 MSE, a recent study highlighted MSE differentiation is independent of adjacent or preceding 73 PSE<sup>14</sup>, underlining the peculiarities of this cell type. The conducting cell types and CC originate 74 from different progenitors in the Arabidopsis root<sup>13</sup>. CC are believed to be essential to support 75 enucleated PSE function<sup>16</sup> and their intimate relationship has been evidenced by a common 76 molecular switch controlling SE/CC fate in vitro and in hypocotyls<sup>17</sup>, while in the primary root 77 undifferentiated CC and MSE can transdifferentiate to PSE cells if these are misspecified<sup>18</sup>. 78 The CC function in leaves consists of loading nutrients into the SE but their role in the root 79 remains elusive<sup>19</sup>. Traditionally, it was thought they were involved in phloem unloading<sup>20</sup>, that 80 is, the exit of the nutrients from the sieve element pipe so that they reach meristematic cells 81 for food. However, it was recently demonstrated this process happens through funnel 82 plasmodesmata connecting PSE to PPP12.

Despite being considered a non-vascular tissue, PPP and the associated vasculature share a
high overlap in gene expression<sup>21</sup> and are different in size and ultrastructure to the xylem pole
pericycle (XPP) population<sup>22</sup>, exhibiting specific gene expression<sup>23</sup> from early stages, mirroring
the diarch pattern in the Arabidopsis vasculature<sup>24</sup>.

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87 In the last 15 years, transcriptomics has been the stepping stone to learn about plant 88 organogenesis. However, even if markers for mature CC and PPP were used for transcriptomics<sup>1,25,26</sup>, the lack of specific markers for early phloem, combined with the 89 90 difficulties to access phloem cells, deeply embedded in the root cylinder, have hampered the 91 study of these populations, oftentimes masked under the concept "stele", that groups pericycle 92 and vasculature <sup>27–29</sup>. The more recent root single-cell atlases confer a detailed root panoramic 93 but even here phloem cells remain underrepresented compared to more accessible root layers<sup>30-32</sup>. 94

Combining fluorescent activated cell sorting (FACS) and SMART-seq single cell technologies 95 96 allowed the profiling of 758 PSE cells at an unprecedented resolution, identifying the 97 bifurcation of MSE and procambium lineages<sup>33</sup>. In this study, we have generated a phloem 98 pole cell atlas of 10204 cells by sorting phloem marker lines combined with single cell sequencing. This allowed us to gain resolution not just in the PSE lineage but in all the 99 surrounding cells (CC, PPP, MSE) in the phloem poles, all of which are underrepresented in 100 101 general root cell atlases. We investigated not only the specificities of each cell type but also 102 the transcriptional commonalities between them. We additionally identified a second set of 103 DOF transcription factors (TF) expressed in the PSE adjacent cells, downstream of PEAR TF, 104 that are important in the transition to autotrophy in young seedlings.

# 105 RESULTS

In order to profile phloem cells, we took advantage of new and existing fluorescent markers expressed in SEs, CC and PPP from early meristematic cells until differentiation (Fig S1a). This allowed us to enrich our data with cells of interest, by using FACS and preparing singlecell sequencing libraries using the 10x Chromium droplet-based protocol. This resulted in a total of 10,204 high-quality cells, defined as those having at least 2000 detected genes and no more than 10% of reads assigned to mitochondrial genes (the resultant sample of cells had a median of 17,455 reads/cell and a median of 4,564 genes/cell). The raw count data was 113 normalised using variance stabilising transformation<sup>34</sup> and integrated across batches using 114 the mutual nearest neighbours algorithm<sup>35</sup>, although our main conclusions are robust to normalisation and batch effects. These cells were grouped into 15 clusters using the Louvain 115 116 algorithm on a shared-nearest-neighbour cell graph and visualised using uniform manifold approximation and projection (UMAP)<sup>36</sup> (Fig. 1b). Using signature marker 117 118 genes<sup>1,2,5,12,17,19,37</sup>(Fig 1c), we identified all the cell types included in the phloem pole. We 119 manually annotated groups of clusters as: PSE conducting cells (clusters 12, 2, 6), CC 120 (clusters 5, 3) and a third to PPP (clusters 7, 4, 14, 11), all emerging from a central group of 121 less mature cells (clusters 8, 9, 10, 13). Clusters 10 and 1 express MSE genes (Fig 2b). In 122 turn, clusters 13 and 12 contain G2/M cell cycle markers, indicating cells undergoing division. 123 While it is usually difficult to infer the identity of cycling cells, in the case of cluster 12 most of 124 the cells express early PSE markers as well as cell division markers, pointing towards PSE-125 dividing cells. For example, PEAR1 or CVP2 are detected in all of the cells of this cluster and 126 cell-cycle genes such as KNOLLE, AUR1 or CYCB1 are also detected in over 57% of those 127 cells. Finally, cluster 15 corresponds to the outer layers of the root, as an apparent 128 contamination during cell sorting.

129 Separated from the rest, clusters 7, 4 and 14 were contributed to mainly by pS17::GFP and 130 pAPL::3xYFP markers (Fig 2a), and expressed genes characteristic of PPP such as S17 (At2g22850) and GLUCAN SYNTHASE-LIKE 4 (CALS8, At3g14570) (Fig 1c). In turn, cluster 131 11, mainly contributed to by pS17::GFP and the pMAKR5::MAKR5-3xYFP sortings, 132 133 represents mature pericycle cells, since in addition to PPP markers it also expresses markers for XPP (At1g02460, At4g30450<sup>38</sup>, At2g36120, Fig 2c) and PPP (Fig 1c). This is likely because 134 135 MEMBRANE-ASSOCIATED KINASE REGULATOR 5 (MAKR5, At5g52870) is expressed in the 136 whole pericycle layer high up in the root and pericycle cells come together with PPP cells for 137 similarity.

Considering genes that were statistically more highly expressed in PPP-specific clusters, we built reporter lines for two genes, which were confirmed to have PPP-specific expression. One of these, *At3g27030*, was expressed in PPP and late PSE, while the other, *METHYL*  141 ESTERASE 7 (MES7, At2g23560), was expressed early in PPP and soon afterwards
142 becomes more broadly expressed in the vasculature and endodermis (Fig 1d).

In turn, the known CC genes are expressed in cluster 5 (SISTER OF APL, (SAPL, 143 At3g1273012)), with cluster 3 expressing mature CC genes (ATPase3 (AHA3, At5g573502), 144 SODIUM POTASSIUM ROOT DEFECTIVE 1 (NAKR1)<sup>39</sup>, SUCROSE PROTON 145 146 SYMPORTER 2 (SUC2, At1g2271040). AHA3 in particular was statistically more highly 147 expressed in this cluster and allowed the discovery of new CC genes by correlation, which were validated building reporter lines (Fig 1d). One of these was At2g32210, which is 148 149 expressed first in PSE and then switches to a strong CC-MSE expression, with a weak expression in the epidermis. In turn, METACASPASE 3 (MC3, At5g64240), was expressed in 150 151 late PSE and started being expressed in CC after enucleation, first in a patchy way and then 152 getting continuous and mostly CC-exclusive. Cloning reporter lines for other genes expressed in these clusters, we found a gene expressed in PSE and CC (PHOSPHATIDYLINOSITOL-153 154 SPECIWC PHOSPHOLIPASE C5 (PLC5, At5g58690)), previously described to be expressed in vascular tissues<sup>37</sup> and At2g38640<sup>41</sup> mostly specific of mature CC (Fig S1b). Therefore, we 155 156 have been able to validate our cell annotation (shown on the UMAP in Fig 1b) in vivo by using 157 new genes highly expressed in these clusters.

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## 159 Spatiotemporal patterns of differentiation in the atlas

160 From our initial cell annotation, it seemed clear that our data also captured the temporal aspect 161 of cell differentiation in the phloem. For example, marker genes usually expressed in more differentiated cells, showed higher expression at the terminal clusters of our UMAP projection 162 163 (3, 6, 11, 14, Fig S1c), while those closer to the cycling cells are less mature. To validate this 164 hypothesis, we compared our data with a microarray dataset<sup>1</sup> of manually microdissected root 165 longitudinal sections (3 to 5 cells thick), assigning each of our cells to the longitudinal section 166 with which they had the highest Spearman correlation (Fig S2a). Using this strategy, we 167 observed that the cells towards the centre of our UMAP matched with the meristematic sections of Brady *et al.*, with a temporal progression towards the terminal clusters of our
UMAP, until the more mature cells cap each trajectory. This analysis validates our hypothesis
of a temporal trajectory that is well captured by our UMAP projection and cell clustering.

171 To further infer developmental trajectories and order our cells along a continuous pseudotime,

172 we used Slingshot<sup>43</sup> (Fig 3a). Setting a unique origin for all in cluster 13 (cycling cells), we

173 obtained 5 different trajectories (Fig 3a), reflecting the known developmental trajectories in the

174 root. Furthermore, these trajectories agreed with RNA velocity analysis using scVelo<sup>44</sup>, with

175 velocity vectors aligning towards the end of these trajectories (Fig S2c).

Trajectories 1-3 account for PPP, CC and PSE respectively. Trajectory 5 is for outer layersand we will not focus on it.

While PSE trajectory is independent from all others, PPP and CC have cluster 5 in common. While other clusters were unequivocally assigned to a single trajectory (see for instance cluster 3, with *slingshot* assigning a probability close to 1 of belonging to the CC trajectory, or cluster 4, with a probability close to 1 of belonging to the PPP trajectory, Fig S2e) or shared by all trajectories (like early phloem cells in cluster 8, Fig S2e), cluster 5 was not a clear cut, with a probability of around 0.75% of belonging to trajectory 2 (CC) and around 0.25 of belonging to trajectory 1 (PPP), Fig S2e.

185 Regarding gene expression, cluster 5 does not express any canonical CC or PPP marker 186 strongly. However, these markers (SUC2, NAKR1, AHA3 for CC or S17 for PPP) are only highly expressed in more mature cells. Cluster 5 has 64% cells expressing the CC marker 187 188 SAPL (409/638 cells) and 20% expressing the PPP marker S17 (127/638 cells), with 12% of 189 the cells in this cluster expressing both genes simultaneously (76/638 cells). This indicates 190 more cells in cluster 5 express CC markers than PPP markers. This matches our observations 191 in the root, when SAPL starts to be expressed earlier in development than S17 (Fig S2g). The fact that a small percentage of cells express both markers at the same time despite being 192 193 specific for different cell types would indicate transcriptional reporters are not always 194 highlighting weak gene expression, so it is possible that our transcriptional data paint broader 195 expression domains than the ones visible with the specific marker lines (see for example the broader SAPL expression domain compared to the cells sorted using pSAPL::VENUSerreporter line, Fig 2a,b).

We tried to distinguish incipient PPP from early CC in cluster 5 but there is no known PPPspecific marker expressed earlier in development than *S17*. However, these intermediate PPP cells should have been collected in the sorting experiments "*pMAKR5:MAKR5-3xYFP whole root*" and "*pAPL:3xYFP*" (Fig 2a), and should be present in the UMAP. These cells would sit in between the early PPP cells, sorted using "*pMAKR5:MAKR5-3xYFP root tip*" enriched in root tips, and those expressing *S17*, sorted using *pAPL::3xYFP* and *pS17::GFP* markers.

Therefore, cluster 5 gathers CC and PPP cells that exist in the same transcriptional state but
 are fated to differentiate into different cell types.

The developmental trajectories obtained reinforce clusters 8, 9 and 10 as early CC, PPP and SE cells. Given these populations are contributed mainly by cells sorted using *MAKR5* and *PEAR1del* (Fig 2a), we can conclude that these clusters correspond to the early phloem cells, containing three different identities (MSE, PPP and CC), still undifferentiated.

There is no gene statistically enriched in cluster 9 and those few in cluster 8 (Table S1) are broadly expressed in whole root single cell data. Except for PSE, when we detect cycling cells expressing PSE markers in cluster 12, it is hard to distinguish an early identity in the other trajectories. However, when early phloem cells are compared to the early cells in general root cell atlases, phloem early cells cluster together more than expected by chance compared to

215 other early cells, suggesting early phloem cells have a specific signature (Fig 4f,g).

216 An important event in phloem development is the enucleation of PSE, since at that moment 217 this cell type loses the nucleus and stops directing phloem progression, becoming dependent 218 on neighbouring cells for survival and probably triggering changes in their transcriptomes. In 219 order to map the enucleation point in the UMAP and know which cells are neighbouring PSE 220 before and after enucleation, we needed to coordinate trajectories, since each trajectory has 221 a different pseudotime. To coordinate them we used our knowledge of ALTERED PHLOEM 222 DEVELOPMENT (APL) expression, which is expressed at different times in all three 223 trajectories, combined with the enucleation markers NAC DOMAIN CONTAINING PROTEIN 224 86 (NAC086, At5q17260) and NAC45/86-DEPENDENT EXONUCLEASE-DOMAIN PROTEIN 225 4 (NEN4, At4g39810) (Fig 3b, Fig S2f). APL is first expressed in PSE and at the time of enucleation is transcriptionally activated in CC and MSE<sup>4</sup>. In reporter lines like pAPL::3xYFP 226 227 we perceive a strong signal in PPP as well (Fig S1a), a circumstance that we took advantage 228 of for sorting, but the phloem pole cell atlas transcriptomics data do not reflect a strong APL 229 expression in mature PPP (Fig 3b", Fig S2d). In another reporter line, pAPL::YFPer we also 230 observe a signal in PPP that gets weaker going shootward (Fig S2d). Based on reporters and transcriptomics data, the signal in PPP is probably not the product of gene expression in this 231 232 cell type but likely caused by direct unloading from PSE that lags and gets diluted in 233 successive cell divisions.

While *APL* expression increases in PSE trajectory until enucleation, it starts being detected in PPP and CC trajectories in the common cluster 5 (Fig 3b), indicating this is the transition zone when *APL* starts building up in the neighbouring cell types before enucleation. Therefore, the PSE trajectory is contemporary to the early phloem cells, cluster 5 coincides with PSE enucleation preparation and clusters for mature PPP and CC contain cells that are neighbouring an enucleated PSE (Fig 3b').

## 240 First stages of MSE development identified

MSE is difficult to identify since there are no specific markers available for this cell type. However, by reducing the diversity of cells in our sample using cell sorting, we were able to gain some insights about this elusive population of cells. Slingshot identified a trajectory (trajectory 4, Fig 3a) that is mainly formed by cluster 10 (Fig S2b), which is mostly contributed by *MAKR5* sortings, pointing this could be early MSE cells (Fig 2a). In cluster 10, we find cells expressing MSE markers like *sAPL*, *APL* (Fig 2b), and other genes expressed in MSE and other cell types but excluded from PSE (*At5g47920, PAPL1*, Fig 2b, Fig 7).

In addition, we know procambial markers like *PIN-FORMED 4* (*PIN4, At2g01420*) become
 excluded from MSE cells early in development<sup>42</sup> and we find this marker absent from cluster

10 (Fig 2b). Out of 31 genes identified as highly expressed in cluster 10 compared to others
(FDR < 1%), 29 were S-phase genes (histones), indicating these are still early cells and</li>
therefore harder to characterise further.

253 We also know that MSE cells should not display PSE markers in early stages, since these are 254 no longer expressed in MSE after lineage bifurcation, but will express these signature genes 255 later in development. For this reason, we interpret that cluster 1 is a more developed MSE, 256 since we find early SE genes like PEAR1 and S32 (At2g18380) expressed in this cluster, which 257 is mainly contributed by the APL sorting. Cluster 1 belongs to CC trajectory, possibly because 258 CC and MSE at this stage share some transcriptional expression, as evidenced by reporters like At2g32210 (Fig 2b), SAPL and the cases shown in Fig 6b, highlighting how phloem pole 259 260 cell fates are intertwined along development.

While we have end points for our PSE, CC and PPP trajectories, we don't expect to have an endpoint for MSE, since this cell type differentiates further away from the meristem<sup>14</sup>. Out of the 7 genes identified to be expressed in both sieve elements<sup>14</sup>, we detected all the genes in PSE clusters but only *DESIGUAL2* (*DEAL2, At4g21310*) in cluster 1 and CC, confirming we have not sampled mature MSE cells. However, we are convinced we have identified the first stages of MSE development in clusters 10 and 1.

#### <sup>267</sup> The atlas represents a continuum of phloem development

268 In order to explore the depth of our data, we integrated our phloem atlas with existing root 269 single cell datasets<sup>30,32,45</sup> (Fig 4a). After filtering, this process rendered a UMAP with 113,340 270 reclustered cells, of which 9% belonged to our project, 7.69% to Wendrich et al., 4.84% to 271 Denyer et al. and 78.4% to Shahan et al. We used markers to identify cell types (Fig 4b, d) 272 and projected the clusters of the phloem pole cell atlas in the integrated dataset to confirm our 273 trajectories (Fig 4d). The relative position of the original clusters is similar in the integrated 274 data as it was in our analysis (Fig 1b). The exception are the cells which we named as "outer 275 layers", which are dispersed in different parts of the integrated UMAP.

276 When projecting the cells of each project in the UMAP, a continuity can be observed in the 277 cells contributed by our atlas covering the gaps in the other data (Fig 4e). Indeed, most of PSE 278 cells (cluster 28), a majority of the intermediate PPP and CC cells (cluster 27) and the early cells in cluster 26 (PPP) were provided by our dataset (Fig S3), demonstrating the difficulty to 279 280 sample phloem without using an active strategy to enrich this population. Most of the cells 281 classified as "Phloem" by Shahan et al. coincide with our clusters 4 and 14, which we 282 annotated more specifically as PPP cells (Fig 4c). There are also a few cells near our cluster 283 3, which we annotated as CC cells. We also noticed cluster 3 (companion cells) is split in this 284 integrated dataset (Fig 4d, orange arrowheads), between mature CC (orange dots on the right 285 of the plot, that is, cells expressing mature CC markers, like SUC2 and NAKR1, see Fig S3a) 286 and the orange cells in the less mature CC expressing SAPL (See Fig S3a). Perhaps the more 287 mature CC have higher overall similarity to more mature cells of other phloem cells (such as 288 PPP for example), more represented in the integrated dataset than in the original atlas.

While other atlases, in particular Shahan et al., excelled in harvesting mature cells (including mature MSE cells, Fig S3a,b), the continuity observed in the UMAP allowed us to track phloem developmental trajectories more accurately (Fig 3a) and enrich populations that were underrepresented in other general root atlases (Fig 4e, Fig S3a,b).

We also wanted to compare root phloem with a recently published single cell dataset on leaf, containing 478 vascular cells<sup>46</sup>. In Arabidopsis leaves (Fig 5a), veins are often formed by multiple sieve elements usually surrounded by at least two CC and one phloem parenchyma cell. In turn, phloem parenchyma cells, which are more irregular and have a much less dense cytoplasm compared to CC, are often in contact with one or more CC, sharing comparatively many more connections than other interfaces<sup>47</sup>.

When the root and leaf data were integrated and clusters were annotated using marker genes (Fig 5d), we noticed PPP and phloem parenchyma cells blended in two clusters (Fig 5b,c). Cluster 9 of the integrated data was formed by CC cells, which are present in both leaf veins and roots. However, cluster 6 of the integrated data contained a mixture of cells annotated as

303 mature root pericycle cells and phloem parenchyma cells from leaves, showing expression of 304 signature PPP (Fig 5e), phloem parenchyma genes (Fig 5f) and XPP (Fig 5g) in both datasets 305 (see methods on how we assessed the degree of mixing of the cells from the two datasets in 306 cluster 6). Phloem parenchyma leaf genes are expressed in clusters 11 and 14 in the phloem 307 pole cell atlas, corresponding to pericycle and PPP respectively (Fig 5f). Pericycle tissue is 308 present in roots and stems, but not in leaves, and phloem parenchyma cells are found in the 309 aerial tissue and root secondary phloem but they are not found in the primary root. Despite 310 being different cell types with different origins, the transcriptional overlap between phloem 311 parenchyma and mature pericycle is another indication of the importance of positional information for cell function in plants, reinforcing the role of PSE as phloem organiser. These 312 313 data also suggest parenchymatous cells share similarities across different organs and 314 underscore their relevance for phloem.

#### 315 Phloem pole cells share transcriptional programmes

316 In order to identify groups of genes showing distinct expression patterns in the phloem poles, 317 we built a gene co-expression network from our scRNA-seq data using the algorithm 318 implemented in *bigScale2*<sup>48</sup>, which uses a gene-gene correlation metric specifically tailored 319 for sparse single-cell data. This resulted in a gene-gene network containing 5,238 vertices 320 (genes) and 370,794 edges (connecting two genes if their correlation was above 0.9). The 321 biological validity of this network was confirmed by the fact that out of 59,545 edges containing 322 genes both present in our network and in known TF-target lists (Arabidopsis Gene Regulatory Information Server, AGRIS<sup>49</sup>), 51,658 (~86%) were preserved as linked pairs in our network. 323 324 To identify groups of genes with correlated expression profiles, we used the Louvain algorithm 325 and obtained a total of 16 gene modules (Fig S4, Table S2), and summarised their expression 326 as the first principal component of a PCA, which we refer to as an eigengene<sup>50</sup>. Among them, 327 most of the modules were broad in all the trajectories with different temporal patterns. Module 328 6 seems to represent genes with high expression in PSE (Fig S4). In contrast, module 1, which

329 contains 1,367 genes (Table S3), displays an increasing expression in both PPP and CC 330 trajectories and a lower-than-average expression in PSE (Fig 6a). Reporter lines for genes in this module followed these predictions: in addition to genes with broader expression (like 331 332 MES7, Fig1d), we identified genes showing a "ring" pattern, expressed specifically in all the 333 cells around PSE (Fig 6b, Fig S5). While At3g11930, At2g02230 (PHLOEM PROTEIN 2-B1, 334 PP2-B1), At5g47920, At3g16330<sup>2</sup> and its sister gene At1g52140, and At4g27435 do not show 335 a strong expression in PSE, At5g59090 (SUBTILASE 4.12, SBT4.12), At2g20562 (TAXIMIN 336 2, TAX2) and At1g26450 are expressed in late PSE in addition to being expressed in a ring 337 pattern. Some of the genes found are expressed in some of the cells around PSE (incomplete 338 ring) and other cell types (Fig S5). For instance, At4g27435 is expressed in CC and 339 occasionally in PPP and protoxylem plus lateral root cap. At3g21770 (PER30) and At3g11930 340 are found in the ring around PSE but extend to procambium higher up (Fig S5, Fig 6b).

Out of the nine genes with a ring expression pattern as observed with reporter lines (see above), seven were found in module 1, with *TAX2* (*At2g20562*) not included in our network and *At4g27435* found in module 4, which includes genes expressed in all trajectories. Despite module 1 being the largest on our network, this result is more than would be expected by chance (hypergeometric test, p-value = 0.0005).

346 Because of the large size of module 1, we tried to refine our analysis by sub-clustering the 347 genes within this module, to identify a more specific group of candidate "ring genes" as defined 348 by the reporter analysis above. This resulted in 15 sub-modules, with five of them containing 349 over 100 genes (Fig S6). Six of the seven "ring genes" from module 1 fell within the same sub-350 module 1 (the exception was At3g16330), which again is more than would be expected by 351 chance (hypergeometric test, p-value = 0.0009). While we do not expect that all of the 326 352 genes in this sub-module have a ring expression pattern, this analysis highlights that this 353 pattern is widespread for a variety of phloem genes, which group together by similarity in 354 expression pattern. On the other hand, a gene such as MES7, which we saw was not entirely 355 ring-specific, fell in a different sub-module. Therefore, our network analysis suggests that there is a complex ring-specific pattern of expression shared across several genes in the phloempole.

The complex patterns in the cells around PSE point out that PSE-adjacent cells share some common developmental programs that are maintained even when cells differentiate into their specific identities, suggesting the transcriptional signature of phloem cells is influenced by multiple positional cues.

This set of genes could be important to understand how PSE relates to its neighbouring cell types before and after enucleation. Indeed, as observed in the UMAPs, the ring pattern is frequent right after PSE enucleation, suggesting a shift in the phloem pole governance after PSE enucleation.

## 366 PINEAPPLE ring genes are expressed in early phloem

Among the genes in module 1, sub-module 1, that also extend their expression into the less 367 mature clusters, we found a DOF transcription factor, DOF1.5 (COGWHEEL1, COG1). This 368 369 gene and the sister gene DOF2.3 (CYCLING DOF 4, CDF4), are expressed in early phloem 370 cells (Fig 7a). CDF4 encodes a differentiation factor in columella cells, repressed by WOX5<sup>51</sup>. 371 The role of COG1 in roots is unknown but this transcription factor is a negative regulator of 372 phytochrome signaling<sup>52</sup> and promotes brassinosteroid biosynthesis by upregulating PIF4 and 373 PIF5, leading to hypocotyl elongation<sup>53</sup>. Both genes have been involved in regulating tolerance 374 to seed deterioration<sup>54,55</sup> as well as flowering time<sup>56</sup>.

Transcriptional fusions of both genes confirmed the expression of both TF in PPP, CC and MSE from 40 µm from the QC, remarkably earlier than the other ring genes described above. While both genes form a ring around PSE reminiscent of a pineapple slice (the expression is weaker or absent in PSE, Fig S7i), *DOF1.5* (from now on *PINEAPPLE1*, *PAPL1*) is also expressed in the epidermis (Fig 7b) and *DOF2.3* (*PAPL2*) is found in columella cells with a broader domain towards the QC (Fig S7f, 17f'). The ring pattern observed with the GFP fusion construct extends one layer towards procambium when fused to 3xYFP expression (Fig S7a), indicating a weaker expression in this layer. Translational fusions show these transcription factors are nuclear localised and not mobile (Fig 7d, Fig S7a-c, S7e), since transcriptional and translational patterns are coincident. This indicates that PAPL transcription factors act cellautonomously. Together with the translational domain of *MAKR5*, the expression domain of *PAPL* genes indicate complex expression patterns in the phloem are relevant from an early stage.

388 PAPL genes, as other genes in module 1, were predicted to be downstream of PEAR in 389 microarray data<sup>2</sup>. PEAR transcription factors move to PSE-adjacent cells to control periclinal 390 cell divisions and other transcriptional programs non-cell autonomously. This is evidenced by 391 markers like SAPL and At3g16330 becoming ectopically expressed after broad PEAR 392 overexpression or SAPL being expressed in PSE upon PSE plasmodesmata closure<sup>2</sup>.

To validate PAPL genes are downstream of PEAR, PAPL reporter lines were expressed in 393 pear1pear2 double mutant, which resulted in a delay in PAPL expression, from 40 to 120 µm 394 395 from the QC (Fig 7c, S7g). Since PEAR genes are highly redundant, we also introduced 396 PAPL1 constructs in the pear sextuple mutant, pear sext<sup>42</sup>, where we observed a loss in its 397 usual meristematic expression (Fig S7d). In parallel, closing PSE plasmodesmata connections 398 to the neighbouring cell types using *icals3m* tool, the ring expression of PAPL1 is altered (Fig. 399 7e) and overexpressing PEAR1 leads to ectopic expression of PAPL2 (Fig 7f). These results 400 validate that PAPL genes are downstream of PEAR and indicate that PEARs are needed and 401 sufficient to express PAPL genes in the early phloem.

In addition to the *PAPL* genes, we validated that some of the genes in module 1 act downstream of PEAR TF. Indeed, PEARs are sufficient to induce *SBT4.12*, *At3g11930*, *MES7* and *PER30*, since these genes become ectopically expressed upon induction of *PEAR2* expressed under a ubiquitous promoter (*pRPS5A*) (Fig S7j). In *pear sext.*, the expression pattern of *PER30* and *MES7* was modified, while *SBT4.12* expression was decreased and *At3g11930* spread towards the meristem (Fig S7j).

#### <sup>408</sup> PAPL proteins link *PEAR* genes to root physiology

409 Next, we decided to check if PAPL genes were downstream of PEAR genes to control periclinal cell divisions. Since PAPL expression is delayed in pear1pear2 double mutant and 410 411 absent from early phloem, we chose this mutant as a background to express PAPL1 under 412 the WOODEN LEG (WOL) promoter. When inducing PAPL1 expression (20h treatment or germinated directly in beta estradiol and grown for 5 days), we did not observe a phenotype 413 similar to PEAR1 overexpression with increased periclinal cell divisions in the root<sup>2</sup> (Fig S8a-414 415 h). A similar result was observed when PAPL1 was overexpressed in the stele in wild type background (Fig S8i-p). These observations indicate PAPL genes do not control periclinal cell 416 417 divisions downstream of PEARs.

To gain insight into the function of *PAPL* genes, and after checking *papl* single mutants didn't show any obvious root phenotype, we generated double mutants (*papl1-1 papl2* and *papl1-2 papl2*). Bulk RNA sequencing identified *CYCLING DOF 2*, *CDF2*, as upregulated in *papl1-1papl2* (Table S4). This gene encodes another DOF transcription factor expressed in the cortex, pericycle and procambium, partially overlapping with *PAPL* expression (Fig S7h). Presuming this gene was upregulated to compensate for the lack of *PAPL* genes, we generated a triple mutant using a *cdf2* T-DNA allele<sup>56</sup> (*papl1-1papl2cdf2-1, 3papl*).

425 The triple mutant root was shorter than wild type in several conditions (Fig S9a) but the effect 426 was more pronounced growing the seedlings in media without sucrose (Fig 7g, Fig S9a,b). A 427 triple mutant with a new allele for CDF2 generated using CRISPR/Cas9 technology showed 428 similar results (3papl-2, Fig S9h). While wild type plants grown in media without sucrose often 429 showed a bimodal distribution in terms of root growth (Fig S9a,b), the proportion of roots 430 arresting growth in 3papl was higher (Fig 7g, Fig S9f). Even if there is high variation between seed batches, the average root length of the mutant is lower than that of the wild-type (Fig 431 432 S9h). Contrary to other phloem development mutants apl and pear sext., adding 1% sucrose 433 to the media mostly suppressed the mutant phenotype of 3papl (Fig 7h, Fig S9e). In this

434 scheme, compared to other mutants, root length in pear1pear2 mutant was not so affected 435 by the absence of sucrose in the media. When grown with sucrose, it was rescued to wt levels. 436 Since the mutants could be rescued by transferring them to sucrose, we aimed to identify the 437 time point at which sucrose is needed for 3papl. For this experiment, we transferred plants 438 from sucrose supplemented to sucrose-depleted media and vice versa. The more time the 439 mutant seedlings spent without sucrose, the more difficult it was for them to recover root 440 growth (Fig 7h, Fig S9c). Those recovering managed to grow well (Fig S9f). Spending at least 3 days in sucrose was necessary for the mutant seedlings to grow normally while spending 441 442 only two days in sucrose was not enough for root growth recovery (Fig 7h, Fig S9c). This 443 phenomenon was not observed in wild type roots grown and transferred in parallel (Fig S9g). 444 In the confocal, the root meristem of seedlings that got arrested, looked shorter and stunted 445 (Fig S9d). PAPL genes were expressed at this stage in both sucrose and non-sucrose 446 conditions showing similar patterns as observed in more mature seedlings (Fig S10a). Other 447 phloem marker genes, like MAKR5, APL and ring gene SBT4.12 were expressed similar to 448 wild type in *3papl* mutant background, suggesting there are no defects in phloem development 449 in 3papl (Fig S10b-d). On the contrary, MAKR5 expression is delayed in pear1pear2 mutant 450 background (Fig S10b) and APL expression is highly reduced in pear sext<sup>33</sup>, suggesting PAPL 451 genes do not fulfil the same roles as PEAR genes. These markers and SUC2 are expressed similarly when the plants are grown in media containing or depleted of sucrose (Fig S10e-h). 452 453 To better understand the 3papl phenotype, we carried out metabolic profiling of leaves and 454 roots of seedlings grown in a sucrose-depleted media across six developmental stages (2-7 455 days post-sowing, dps) (Table S5). We identified 7 and 5 metabolites in leaves and roots, 456 respectively, with significant differences between WT and mutant in at least one of the time 457 points (<5% false-discovery rate from a linear mixed model fit to the whole data, see methods; 458 Fig 8a). One of those metabolites was sucrose, with a significant difference only in the roots, 459 where it started at lower levels in the mutant (days 2 and 3) and then continued to increase to 460 reach levels comparable to the WT at the end of the experiment at day 7 (Fig 8b). A similar 461 pattern, with more significant points, was observed in fructose, which is a component of 17

462 sucrose, and to a lesser extent in glucose, the other monosaccharide forming sucrose (Fig 463 8b). It has been described that by the time the radicle emerges, all the sugars stored in the Arabidopsis seed have been consumed. Within 48 hours after germination (approximately at 464 day 3 after sowing), lipid and protein reserves are exhausted and seedlings need to switch to 465 autotrophic growth 57,58. The data suggest PAPL genes could be important after the seedling 466 467 has transitioned to autotrophic growth, facilitating sugar transport to sink tissues like roots. 468 The continued increase in sucrose in the mutants could be due to the, on average, smaller 469 size of 3papl seedlings and stunted growth, which could therefore lead to reduced sucrose 470 consumption and therefore its observed continued accumulation.

# 471 DISCUSSION

Our manuscript demonstrates the power of tissue-specific transcriptomes combining FACS
and single cell sequencing to study elusive cell populations underrepresented in organ general
cell atlases. The use of droplet-based technologies also allowed us to gather more cells and
a higher resolution than plate-associated methods.

The phloem pole cell atlas is allowing a holistic understanding of phloem. While there are specific genes for PPP and CC, these cell types share the first stages of their developmental trajectory. Trajectory analysis also revealed the connection between CC and early MSE, providing new insights on early stages of MSE development. The commonalities among the different cell types were validated by gene regulatory network analysis and reporter lines confirmed the relevance of the ring expression pattern in all the cells around PSE.

PSE differentiation involves enucleation and becoming dependent on adjacent cells for survival. Using *APL* expression as a standard, we mapped the enucleation point in the atlas. While PSE organizes the phloem pole in the meristem neighboured by unspecialized cells, PSE enucleation marks the onset of cell differentiation for adjacent cells and switches on similar gene regulatory networks in PSE-surrounding lineages, as evidenced by the ring pattern shown by many genes right after PSE enucleation. The coordinated expression in the cells of the phloem pole highlights the importance of positional information and cell to cell communication to preserve phloem function when PSE delegates control in the adjacent cells. They also underpin the relevance of PPP cells, which we believe should be considered a built-in part of phloem.

A phloem plasticity zone was recently described in the root meristem, when CC and MSE cells
could act as a reservoir for PSE identity<sup>18</sup>. This further supports the coordination between the
pole identities to ensure correct phloem morphogenesis. It would be interesting to investigate
if PPP can also transdifferentiate to PSE if required.

In turn, the similarities between root pericycle cells and phloem parenchyma cells in leaves
suggests parenchymatic cells share characteristics despite being present in different organs
with variable anatomic configurations and reinforces PSE as the phloem pole organiser.

The modular analysis of the atlas identified the DOF *PAPL* genes, characterised by early expression in the ring domain and the inability to introduce new periclinal cell divisions when overexpressed in procambium.

502 Contrary to other phloem mutants, like apl, the presence of sucrose in the media almost 503 completely suppresses the root growth phenotype of 3papl. Regarding PEARs, the root length 504 in the pear1pear2 mutant was not so affected by the absence of sucrose in the media and it 505 was rescued to wt levels when grown with sucrose. The fact that the subtle root length 506 phenotype is rescued by sucrose leaves the possibility open that different doses of the phloem-related DOF genes are responsible for the phenotype as opposed to the type of DOF 507 508 genes. However, we don't favour this scenario because of the functional differences of the 509 DOF genes based on the overexpression phenotypes.

510 Since phloem is in charge of nutrient transport and a smaller amount of sucrose and its 511 component fructose is detected in both mutant leaves and roots at 3 dps when root anatomy 512 is comparable between wt and mutant, we interpret *PAPL* genes regulate nutrient allocation 513 between the leaf source organs and the root sink in young seedlings, when embryo reserves 514 are scarce. *PAPL* genes could either regulate phloem loading, long distance transport or 515 phloem function and more studies are required to determine their precise role.

# 516 MATERIALS AND METHODS

#### 517 Plant growth conditions

All *Arabidopsis thaliana* lines used in this study were in Col-0 background except *pear1* mutant allele, which is in Ler background, conferring *pear1pear2* mutant a mixed Ler appearance. Plants were grown in ½ MS Basal salts media (0.5 MS Salts, 1% Difco agar, with or without 1% sucrose) at 23°C and long day conditions, except for sorting experiments, when they were grown using 1x MS Basal salts at 23°C with 30% humidity and 188 µM of light, long day conditions, to be able to compare with other transcriptomic data.

- papl1-1 (cog1-6, from gene At1g29160, PAPL1, DOF1.5, COG1) has a single nucleotide 524 525 deletion (G) at position +85, which generates a premature stop codon. This mutant was identified as a cog1-D suppressor<sup>53</sup>. papl2 (At2g34140, PAPL2, DOF2.3, CDF4) has a 4 bp 526 527 deletion (CAAG) at position +99 creating a premature stop codon. The cdf2 T-DNA allele (GK782H09) is a knockdown allele<sup>56</sup>. Triple mutant was obtained by crossing the double 528 529 mutant papl1-1papl2 to cdf1r23556, selecting for mutant 3papl and homozygous wild type 530 alleles for all other genes. The second triple mutant (3papl-2) was obtained by generating a new cdf2 allele by using CRISPR/Cas9 technology directly on the double mutant papl1-1papl2. 531 532 The process rendered a 5 bp deletion (CCCGG) at position +953 (cdf2-2), which generated a 533 premature stop codon shortly afterwards.
- 534

535 5  $\mu$ M Beta estradiol or 10  $\mu$ M DEX were used in the inducible constructs for the indicated 536 times. Plants induced with DEX were treated for 24 hours.

## 537 Sorting and single cell sequencing

538 Seedlings from the different marker lines were grown vertically over mesh (Normesh, 100 μm)
539 for five days in the conditions specified above. Approximately one third of the root including

540 the root tip was chopped with razor blades and the tissue transferred to a 70 µm strainer 541 submerged in 7 ml of the protoplasting solution for an hour with gentle shaking at room temperature<sup>59</sup>. In the case of the sample "MAKR5 enriched in root tips", we submerged the 542 543 root tips of intact roots in eppendorfs containing the protoplasting solution for 15 minutes, 544 which is enough time for the meristems to be enzymatically cut from the rest of the root. Then 545 the separated root tips were transferred to 70 µm strainers, incubated with 7ml of protoplasting 546 solution in 4 cm radius petri dishes at room temperature for 45 minutes and from then onwards 547 were treated as the other samples. Washed protoplasts suspended in solution A were taken 548 at room temperature to the sorting facilities and the process from chopping to sorting took 549 approximately 2-2.5h. For the gating, a wild type Col0 sample was run first to establish the 550 fluorescent negative gate. Then this sample was subsequently stained with DAPI and DRAQ5 551 to gate for intact cells that contained DNA, respectively. The corresponding sample containing 552 fluorescent protoplasts was then stained subsequently with DAPI and DRAQ5 and underwent 553 FACS. Gating helped enrich intact (DAPI negative), YFP/GFP positive, DNA containing cells 554 (DRAQ5 positive) that were sorted with a 130 µm nozzle using a High speed Influx Cell Sorter 555 (BD Biosciences). Sorted protoplasts were harvested in W5 solution (154 mM NaCl, 125 mM 556 CaCl 2, 5 mM KCl, 5 mM MES (2-(N morpholino)ethanesulfonic acid) in BSA coated 1.5 ml 557 Eppendorf tubes. Cells were centrifuged for 12 minutes at 200g to eliminate the excess of supernatant. Immediately, Single-cell RNA-seq libraries were prepared in the Cancer 558 Research UK Cambridge Institute Genomics Core Facility using the following: Chromium 559 560 Single Cell 3' Library & Gel Bead Kit v3, Chromium Chip B Kit and Chromium Single Cell 3' Reagent Kits v3 User Guide (Manual Part CG000183 Rev C; 10X Genomics). Cell 561 562 suspensions were loaded on the Chromium instrument with the expectation of collecting gel-563 beads emulsions containing single cells. RNA from the barcoded cells for each sample was subsequently reverse-transcribed in a C1000 Touch Thermal cycler (Bio-Rad) and all 564 565 subsequent steps to generate single-cell libraries were performed according to the 566 manufacturer's protocol with no modifications. cDNA quality and quantity was measured with Agilent TapeStation 4200 (High Sensitivity 5000 ScreenTape) after which 25% of material was
used for gene expression library preparation.

Library quality was confirmed with Agilent TapeStation 4200 (High Sensitivity D1000 ScreenTape to evaluate library sizes) and Qubit 4.0 Fluorometer (ThermoFisher Qubit<sup>™</sup> dsDNA HS Assay Kit to evaluate dsDNA quantity). Each sample was normalized and pooled in equal molar concentration. To confirm concentration pool was qPCRed using KAPA Library Quantification Kit on QuantStudio 6 Flex before sequencing. All samples were sequenced using Illumina NovaSeq6000 sequencer with following parameters: 28 bp, read 1; 8 bp, i7 index; and 91 bp, read 2.

## 576 Analysis of single-cell RNA-seq

Here we give a briefer description and overview of our analysis steps, but the full details of our
analysis pipeline (e.g. specific package functions and options used) can be seen in our code
repository at <a href="https://github.com/tavareshugo/publication\_Otero2021">https://github.com/tavareshugo/publication\_Otero2021</a> PhloemPoleAtlas.

580 To obtain unique molecular identifier (UMI) counts for each gene, the raw sequencing reads 581 were aligned to the reference Arabidopsis TAIR10 genome using the Araport11 gene 582 annotation (both downloaded from Ensembl release 45) using 10x Genomics Cell Ranger 583 v3.1.0<sup>60</sup>. The data were processed and quality-filtered using several Bioconductor packages<sup>61</sup>. Empty droplets were inferred and removed using dropletutils v1.8.0<sup>62</sup>, and data normalisation 584 585 was done using both the pooling method implemented in scran v1.16.0<sup>63</sup> and the variance-586 stabilising transformation from sctransform v0.2<sup>34</sup>. To adjust for potential batch effects, data 587 from the different samples (i.e. sorted with different GFP fusion markers and/or from different 588 public datasets) were integrated using the Mutual Nearest Neighbours (MNN) algorithm implemented in batchelor v1.4.035. After initial data exploration and quality checks, we retained 589 590 cells with at least 2000 detected genes and genes detected in at least 100 cells (a gene was 591 considered to be detected if it had at least 1 UMI count). Downstream analysis was done on 592 these filtered data, batch-normalised using MNN and using variance-stabilised transformed

values. However, our conclusions were qualitatively robust to the specific choice of normalisation methods. For data visualisation purposes, we have projected the data to two dimensions using uniform manifold approximation and projection (UMAP), using a neighbourhood size of 30 cells (sizes of 7, 15 and 100 were also explored and give comparable results). We have also visualised the UMAP in three dimensions, which did not provide further insights into the data compared to the two-dimensional projection.

599 Cell clustering was performed by first defining a "shared nearest neighbours" graph and then 600 identifying modules in the graph using the Louvain algorithm (using scran v1.16.0<sup>63</sup> and igraph 601 v1.2.6<sup>64</sup>. To annotate our cells we used a set of genes with known expression patterns (from 602 promoter fusion microscopy experiments) and calculated, for each cluster, the percentage of 603 cells where each marker gene was detected as well as the (z-score scaled) average 604 expression of the gene in that cluster.

605 To identify cluster-specific genes, we used pairwise Wilcoxon rank sum tests between a given 606 target cluster and all others using the findMarkers() function in the R/Bioconductor package 607 scran v1.16.062. We specifically tested for genes upregulated in the target cluster, to identify 608 highly-expressed genes specific to each cluster (rather than also including genes that are 609 specifically absent from the cluster). The results of the pairwise tests for a given target cluster 610 were then consolidated to obtain a summary p-value (and corrected false-discovery rate) for 611 how enriched each gene is in a given cluster. We summarised the pairwise p-values for a null hypothesis that the gene is not differentially expressed in at least 8 out of the 15 clusters, 612 613 allowing us to flexibly identify genes that were highly expressed in across multiple cell types 614 (e.g. mature ring cells such as PPP and CC) but not others. We also did a more stringent 615 summary of p-values (null of no differential expression in 12/15 clusters) to obtain genes more 616 specific to particular clusters of interest (namely cluster 10, which was a candidate for early 617 MSE cells).

The same pipeline was applied to the public datasets, also integrated using MNN. The quality of this data integration was confirmed by checking that the majority of our annotated cells were clustering together with the same cell types in other datasets. We produced two sets of data 621 integration, one with root data and another with leaf data. Details of the public datasets used622 are given in (Table S6).

To explore how well cells from leaf and root datasets mixed in clusters where they co-occurred 623 624 (namely cluster 6, which contained both leaf phloem parenchyma and root phloem pole 625 pericycle cells) we used the same shared-nearest-neighbours cell graph used for clustering 626 and calculated the proportion of edges between root-leaf cells (the vertices of the graph). This 627 value was then compared with a null expectation, obtained by randomly shuffling the cell tissue 628 labels 1000 times and calculating this proportion each time. The 95% inter-percentile range of 629 this null distribution was then used to compare with the observed value. The graph had 19.2% 630 leaf-root edges in this cluster, which is only slightly lower than expected by chance (median 631 23.5%, 95% CI 22.8%-24.2%, obtained from 1000 random shuffles of the cell labels). This 632 result suggests that the cells from the two datasets are well mixed. This is in contrast with 633 cluster 17, for example, which consists of poorly clustered cells that occur separated in the UMAP. In this case, there were 26.7% leaf-root edges, almost half of the null expectation for 634 635 that cluster, which was 44.9%.

636 To further temporally annotate our phloem pole atlas dataset we used several approaches. 637 Early dividing cells were identified by checking the expression of all annotated cyclins and 638 other cell cycle markers such as AUR1 (AT4G32830) and KNOLLE (AT1G08560). We also 639 cross-referenced our data with a published dataset that profiled the transcriptome of 640 longitudinal root sections using microarray technology<sup>1</sup>. Based on 9,674 common genes 641 between the two datasets, we assigned each of our cells to the longitudinal section of Brady 642 et al. that had the highest Spearman correlation with it. We also used the RNA velocity method 643 implemented in scVelo v0.2.2 to infer developmental dynamics in our data<sup>44</sup>. Finally, cells were 644 assigned to lineages and ordered by pseudotime using slingshot v1.6.043. We first reduced the dimensionality of the (batch-normalised) counts to 10 components using diffusion maps, 645 646 which is a dimensionality reduction method suited to capture developmental transitions in the 647 data<sup>65,66</sup>. In this latter case we used a semi-supervised approach, where the starting point for 648 the inferred trajectories was set to the cluster highly expressing cell-cycle markers and 24 649 identified as the earliest cluster when cross-referencing with the Brady et al. dataset. In this 650 manner we obtained biologically meaningful trajectories (without setting this constraint several 651 more trajectories were obtained but with an ordering of cells which was the reverse of what 652 was expected from our other analyses). We obtained smooth gene expression patterns for 653 each trajectory using generalised additive models, as implemented in tradeSeq v1.2.0, which 654 were then used to explore gene expression patterns along the slingshot trajectories.

655 To cluster genes based on their similarity of expression across the cells, we built a co-

- 656 expression network using a modified version of bigSCale<sup>48</sup>, adapted to work on any species
- 657 (rather than the original version suited only for mouse and human). The modified package is
- 658 available from

#### 659 https://github.com/tavareshugo/bigSCale2/tree/support-any-species.

660 Summarily, bigScale builds a gene correlation matrix not from the original count data (which 661 in scRNA-seq is too noisy and sparse), but from a z-score statistic calculated between pairs 662 of cell clusters. These clusters are iteratively generated to ensure the z-scores capture as 663 much diversity in gene expression patterns across the cells as possible. In this way, 664 correlations between genes are more robust to the noisy and sparse nature of single-cell 665 RNA-seq data. This correlation matrix was then thresholded at 0.9 to obtain a gene-by-gene 666 adjacency matrix, resulting in a network with 5,238 vertices (genes) and 370,794 edges. We identified gene modules using the Louvain algorithm, resulting in 16 modules. From each 667 668 module, we calculated an eigengene following the procedure in WGCNA vX<sup>50</sup>, which 669 essentially summarises the expression of all genes of a module as the first principal 670 component score from a principal components analysis (PCA) done on those genes. The 671 largest of these modules - module 1 containing 1,367 genes - contained several genes of 672 interest for our analysis, and was therefore re-clustered with Louvain to generate 15 sub-673 modules. This was further justified by the fact that the variance explained by this module's 674 eigengene was relatively low (21.44%), suggesting some heterogeneity in expression 675 patterns within the module. To further interpret these results, the eigengenes from these

676 sub-modules were joined with the pseudotime trajectories from slingshot, although we note

677 that no information about trajectories was used to build the network itself. Therefore, the fact

678 that the different approaches (gene network and pseudotime analysis) reveal groups of

679 genes with similar patterns of expression is a strengthening point in our analysis.

## 680 Generation of reporter lines and confocal images

Promoter::VENUSer fusions were generated for the genes At3g27030, At2g23560 (MES7),
At2g32210, At5g64240 (MC3), At5g58690 (PLC5), At2g38640, At3g11930, At2g02230 (PP2B1), At5g47920, At4g27435, At5g59090 (SBT4.12), At3g21770 (PER30), At1g26450,
At1g29160 (PAPL1, DOF1.5, COG1), At2g34140 (PAPL2, DOF2.3, CDF4), At5g39660
(DOF5.2, CDF2).

- Translational fusions were also generated for *At2g20562* (*TAX2*), *PAPL1* and *PAPL2*. 3xYFP
  constructs were also generated for transcriptional fusions of *PAPL1* and translational fusions
  of *PAPL1* and *MAKR5*.
- Promoter fragments between 622-4879 bp were amplified by PCR and cloned using MultiSite-Gateway (Table S7). Transcriptional fusions to *VENUS* with an ER tag or translational fusions to YFP were generated in vectors with either resistance to Basta or Hygromycin or a Fast Green/Fast Red selection system. All the constructs were transformed in Col0 background and at least 2 independent lines were analysed for each.
- Roots from 5-7-day-old seedlings were either imaged in the confocal directly after mounting them in 50 µg/ml propidium iodide or fixed for 45 minutes in 4% paraformaldehyde in PBS and cleared using ClearSee solution (10% (w/v) Xylitol, 15% (w/v) sodium deoxycolate, 25% (w/v) urea, water to the final volume)<sup>67</sup>. Cleared roots were then stained with SCRI Renaissance 2200 and observed under the confocal. Images were acquired at 512x512 resolution using the confocal Leica SP8. Images were analysed in ImageJ v2.1.0/1.53c.

#### 700 Bulk RNA-seq transcriptomics

Wild type and *papl1-1papl2* seedlings were grown on mesh in ½ MS media with sucrose in the above-mentioned conditions for 5 days. Root meristems from wild type and mutant were manually and individually dissected in parallel under a stereomicroscope using 18G needles. Meristems were preserved in RNAlater RNA stabilisation reagent (Qiagen) until 120 meristems per replicate were gathered. 3 replicates for each mutant and wild type were used for RNA extraction.

707 RNA was extracted using the RNeasy Plant Mini kit from Qiagen and RNA integrity and concentration were checked using TapeStation and Qubit 2.0 fluorometer (Life Technologies) 708 709 respectively. After quality control in Novogene company, the best 3 replicates for mutant and 710 wild type were used for library construction and sequencing following the Novogene pipeline. 711 Briefly, mRNA was enriched by using oligo dT beads and fragmented randomly. cDNA 712 synthesis was performed using random hexamers and reverse transcriptase. After first-strand 713 synthesis, the second strand is synthesized by nick-translation. Library is ready after a round 714 of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment. Library concentration was quantified using a Qubit 2.0 fluorometer (Life 715 716 Technologies), Insert size was checked on Agilent 2100 and quantified more accurately by 717 guantitative PCR. Libraries were fed into the HiSeg XTEN platform for sequencing. Original 718 raw data were transformed to Sequence Reads by base calling and raw data recorded in 719 FastQ files. Low quality reads or reads containing adaptors were filtered out. TopHat2<sup>68</sup> 720 v2.0.12 was used to map the reads to the reference genome (TAIR10). HTSeq<sup>69</sup> v0.6.1 721 software was used to analyze the gene expression level using the union mode. Fragments 722 Per Kilobase of transcript sequence per Millions base pair sequenced (FPKM) value of 0.1 or 1 was set as the threshold to determine whether a gene is expressed or not. To compare 723 724 gene expression levels under different conditions, FPKM distribution diagram and violin plot were used. For biological replicates, the final FPKM would be the mean value. The differential 725 726 gene expression analysis consisted of read-count normalization, model-dependent mean value estimation and FDR value estimation based on multiple hypotheses testing. DESeq<sup>70</sup>
 v1.10.1 software was used for these steps. The results of this analysis are given in Table S4.

## 729 Root length measurements and statistical analysis

To quantify root growth, an EPSON Perfection V700 Photo scanner was used to obtain images
of the seedlings in plates. A ruler was also scanned to calibrate the images. Roots were
measured manually one by one using ImageJ v2.1.0/1.53c.

733 Because of the nature of the data, which often had a bimodal distribution, we opted for using a non-parametric bootstrap approach for our statistical analysis. This was done by resampling 734 735 the data 500 times and estimating the difference between groups of interest (either WT vs 736 mutants or between sucrose treatments, as detailed in the respective figure legends). We thus 737 obtained distributions of root length differences, which we used to obtain confidence intervals 738 (based on a 95% inter-percentile range) and a bootstrap p-value calculated as the number of 739 samples with absolute difference less than a "null" distribution centred on zero. Our p-values 740 therefore have a lower bound of 1/501 ~ 0.002, which we deemed to be of sufficient statistical 741 resolution for our analyses (we added an offset of 1 to both the numerator and denominator 742 to avoid p-value = 0, which would mis-represent the precision of our analysis). Whenever 743 relevant, the bootstrap analysis took into account experimental and seed stock batches by summarising the results at those levels first, before then comparing the groups of interest; this 744 745 ensured that the uncertainty in our estimates captures those different levels of potential 746 variation. The results of these analyses are provided in Table S8.

## 747 Experimental Design

Experiments were repeated independently for the following number of times. In Fig 1d, *MES7*reporter was imaged 3 times and reporters for *MC3*, *At3g27030* and *At2g32210* (also in Fig
2b) were imaged twice.

For Fig 6b, reporters for *At3g11930*, *At5g47920* and *At1g26450* were imaged twice, *PP2-B1*, *SBT4.12* and *TAX2* reporters were imaged 3 times. For figure 7, *pPAPL1::GFP* was imaged 6 times, *pPAPL1::GFP* in *pear1pear2* was imaged 3 times, *pPAPL1::PAPL1-YFP* (also in Fig S7b) was imaged twice, *pPAPL1:GFP* in *pPEAR1::icas/3m* was imaged twice and *pPAPL2:YFPer* in *pRPS5A::PEAR1-GR* was imaged once.

- For Fig S1a, *pMAKR5::MAKR5-3xYFP* was imaged 7 times, *pS17:GFPer* was imaged twice, *pAPL::3xYFP* was imaged 3 times and *pSAPL::YFPer* (also in Fig 2b) was imaged four times.
- For Fig S1b, the reporter for *PLC5* and *At2g38640* were imaged 3 times.
- For Fig S2d, *pAPL::YFPer* was imaged once. For Fig S5, reporters for *At3g16330* and *PER30*were imaged 3 times, while the reporter for *At4g27435* was imaged 4 times.
- 761 For figure S7, pPAPL::3xYFP was imaged 6 times, pPAPL::PAPL1-3xYFP was imaged 3 762 times and this construct in pear sext. background was imaged 3 times. pPAPL2:PAPL2-YFPer 763 was imaged 3 times, pPAPL2::VENUSer was imaged 3 times and this construct in pear1pear2 764 mutant background was imaged twice. pCDF2:VENUSer was imaged 3 times. For constructs 765 in Fig S7j, reporters in the overexpressor background, induced and control, were imaged once 766 while reporters were imaged twice in pear sext. background. For Fig S8, each line was 767 observed independently twice. For Fig S9d, roots were imaged for this figure once but these 768 two backgrounds were imaged many times with reporter lines in them.
- For Fig S10a, reporters with and without sucrose were imaged twice. For Fig 10b, *pMAKR5::MAKR5-3xYFP* in *3papl* mutant was imaged 3 times and it was also imaged 3 times in *pear1pear2* mutant background. Reporter in Fig S10c was imaged 3 times while the reporter in Fig S10d was imaged once. Reporters in Fig S10e-g were imaged twice and reporters for Fig S10h were imaged once (Col0) or three times (mutant background).
- For the experiment shown in Fig 7g and S9e, the total number of seedlings measured for each
  genotype was: 488 *3papl*; 273 PAPL1-32; 37 PAPL1-51; 33 PAPL1-71; 343 PAPL2-11; 79
  PAPL2-23; 37 PAPL2-31; 314 PAPL2-73; 382 wt. Seedlings were split across 5 experimental
  batches and came from different seed stocks (N = 24 46 with a median of 36 seedlings per

experimental batch and seed stock combination). Both seed stock and experimental batchwere taken into account in the statistical analysis.

780

## 781 Metabolic profiling

782 Arabidopsis plants were grown across six developmental stages (from day to day 7) on mesh 783 in solid media containing sucrose or devoid of sucrose. Each day of the time course, leaves 784 and roots were harvested separately and snapped frozen in liquid nitrogen. 50 mg of leaves and 20 mg of roots were ground using a Tissue Lyser. Extraction was performed according to 785 786 Lisec et al. (2006)<sup>71</sup>, with modifications. In detail, 750 µl/300 µl of extraction buffer (100% 787 methanol plus the internal standard adonitol, Sigma) were added to root and leaf samples 788 respectively. Samples were vortexed and transferred to a shaker at 70 °C for 15 minutes. 375 789 µl/200 µl of chloroform and 750 µl/350 µl of water were added to the tubes for leaves and roots respectively, and samples were centrifuged for 10 minutes at maximum speed. 400 µl (roots) 790 791 and 200 µl (leaves) of supernatant were dried for each sample using the speedvac. Samples 792 were kept at -80°C until processing.

The dried samples were derivatized for 2 hours at 37 °C in 50 µl of 20 mg ml– 1 methoxyamine hydrochloride (Sigma-Aldrich, cat. no. 593-56-6) in pyridine followed by a 30 min treatment at 37 °C with 100 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA reagent; Macherey-Nagel, cat. no. 24589-78-4). For each sample, 1 µl was injected in splitless mode to a chromatograph coupled to a time-of-flight mass spectrometer system (Leco Pegasus HT TOF-MS; Leco Corp., St Joseph, MI, USA), using an autosampler Gerstel Multi-Purpose system (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany). Chromatograms and mass spectra

800	evaluation, as well primary metabolites identification based on the expected retention time and
801	mass fragmentation were performed using the software Xcalibur software (Thermo Fisher
802	Scientific). The software ChromaTof (Leco) was used to confirm the peaks and retention times for
803	expected metabolite fragments.
804	To estimate differences between WT and 3papl metabolite levels, we fit a joint hierarchical
805	model to the peak areas of all metabolites, including terms for genotype, stage (dps), tissue
806	and their interactions. The advantage of using this model is that we could include a random
807	effect term to account for multiple measurements per sample (each sample contributed 21
808	data points, one for each metabolite). Due to the skewed distribution of peak areas, the data
809	were modelled on a log-scale, which produced well-behaved normally distributed residuals.
810	The model was fit with the Ime4 <sup>72</sup> v1.1-27.1 R package and we obtained estimates of the
811	difference between the two genotypes for each metabolite and tissue using the emmeans
812	v1.6.2-1 R package. The p-values from the <i>emmeans</i> contrasts were corrected for multiple
813	testing using the false discovery rate method.
814	Additional information on metabolomics analysis and metabolites annotation are reported in
815	table S9 (sheets checklist and overview) according to the guidelines provided in Alseekh et
816	al <sup>73</sup> .
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819	Data availability

- Sequencing data from 10x Chromium single-cell RNA-seq is available from NCBI's
  Gene Expression Omnibus through GEO accession number GSE181999<sup>74</sup>:
  <a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181999">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181999</a>.
- Sequencing data from bulk RNA-seq is available from NCBI's GEO accession number
  GSE182672<sup>75</sup>:

#### 825 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182672.

All other data (phenotypic scoring, microscopy imaging, plasmid maps) are available from the Cambridge Apollo Repository (<u>https://doi.org/10.17863/CAM.74836</u>). A persistent DOI will be available upon acceptance<sup>76</sup>.

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## availability

830 Analysis code, with instructions on how to run it, is available from:
831 https://github.com/tavareshugo/publication Otero2022 PhloemPoleAtlas.

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# **Author Contributions**

852	S.O. performed the experiments, I.S. identified PAPL1 and At3g16330 expression patterns
853	which appeared as PEAR targets in microarray data, P.R. provided pear1pear2 double
854	mutant, pPEAR(del)::3xYFP and advised on experimental design, Y.L. and H.T. analysed
855	gene regulatory networks, P.R., M.B., L.K., B.B. and J-o.H. participated in sample collection
856	for sorting and metabolomic profiling, M.B. imaged <i>pSUC2:GFP</i> , J-o.H. provided
857	<i>pSAPL::YFPer</i> line, V.D. and A.R.F. carried out the metabolic profiling and data analysis, F.P.
858	and T. L. provided the papl2 and papl1-2 alleles, H.T. designed and performed the single cell
859	data and statistical analysis, S.O., H.T. and Y.H. conceptualised and designed the study. S.O.
860	wrote the manuscript with input from Y.H., H.T., P.R. and L.K. All authors read, edited and
861	discussed the manuscript.

# 862 Competing Interests statement

863 The authors declare no competing interests.

# <sup>864</sup> Figure legends

#### 865 Figure 1. A root phloem pole cell atlas containing PSE, MSE, CC and PPP cells.

866 a) Root schematic highlighting the cells in the phloem pole coloured by identity (adapted from<sup>42</sup>) with a 867 close-up of the phloem pole (a'). One of the radial cuts shows the middle part of the phloem pole and 868 the other shows the side view of the phloem pole b) UMAP plot showing the classification of 10,204 869 cells clustered by cell identity and developmental stage (colours indicate clusters, labelled with a 870 number). The sample of cells has a median of 4,564 detected genes (10%-90% percentiles: 2,600-871 6,780) and a median of 17,445 total UMIs per cell (10%-90% percentiles: 5941-52689). c) Cluster 872 annotation based on markers with known tissue- or cell-specific expression. The size of the points 873 represents the percentage of cells in a cluster where the gene was detected (i.e. at least 1 UMI). The 874 colour shows the scaled average expression of the gene (z-score, i.e. number of standard deviations 875 above/below the gene's mean across all cells). d) Newly identified genes significantly enriched in PPP

876 (*At2g23560*, *At3g27030*) and CC (*At2g32210*, *At5g64240*). UMAPs show the particular cluster877 weighted normalised expression of each gene in the phloem pole cell atlas. UMAP and microscopy
878 pictures are representative images of the transcriptional reporter lines, where the gene promoter is
879 fused to *VENUSer*. Scale bar in the longitudinal sections is 25 µm while it is 10 µm in the cross sections.
880 White arrowheads point to PSE cells as a reference point. The numbers in each panel indicate samples
881 with similar results, of the total independent biological samples observed.

#### 883 Fig 2. MSE cells identification and identity of cluster 11.

884 a) Cells were plotted in the UMAP separated by sorting experiment, as indicated in each panel, to show which sorting experiment provided every cell. Colour indicates point density (lighter colour indicates 885 886 higher density of points), with grey areas meaning an absence of cells. Numbers in each panel indicate 887 the number of filtered cells contributed by that sorting experiment. We sorted MAKR5 twice, one 888 enriching in root tips (MAKR5) and another sorting the usual one third of the root (MAKR5 differentiated). b) UMAPs showing the cluster-weighted normalised expression of marker genes used to identify MSE 889 890 identity. PEAR1, S32 and DEAL2 are expressed in sieve elements. APL is genuinely expressed in PSE, CC and MSE. At5g47920, SBT4.12 and PAPL1 are expressed in a ring expression pattern, including 891 892 MSE and other cell types. PIN4 is used as a negative control, since it is excluded from sieve elements 893 early in development<sup>3</sup>. SAPL and At2g32210 are expressed in CC and MSE. Black arrowheads point 894 to clusters 1 and 10. In the confocal cross sections, the scale is 10 µm. White arrowheads point to PSE 895 as a reference point and yellow arrowheads point to MSE. The numbers over each picture indicate 896 samples with similar results, of the total independent biological samples observed c) UMAPs for xylem 897 pole pericycle markers, which are found in cluster 11 together with other PPP markers, indicating this 898 is a late pericycle cluster. 899

#### 900 Fig 3. Developmental trajectories and mapping of the PSE enucleation point.

901 a) Developmental trajectories inferred using Slingshot coloured according to pseudotime, with more 902 mature cells in yellow. The origin for all trajectories was set in the clusters containing cycling cells. b) 903 APL expression is plotted along the PPP, CC and PSE trajectories, with the cells coloured by cluster 904 number in the UMAP. The black line is a smoothed trend estimated from a non-parametric generalised 905 additive model. b') APL is used as a standard to coordinate the three trajectories. Cluster 5 groups the 906 cells with an increasing expression of APL in PPP and CC trajectory, mapping the enucleation point in 907 the adjacent cells. The position of each cell type is indicated in the UMAP in relation to PSE enucleation 908 b") APL expression plotted in a UMAP of the phloem pole cell atlas

#### 910 Fig 4. Phloem cell types in the integrated UMAP.

911 a) A new UMAP containing 113340 cells was generated by integrating cells from Denyer et al. 2019<sup>30</sup>, Wendrich et al. 2020<sup>32</sup> and Shahan et al. 2020<sup>45</sup>. Colours are used to differentiate cell clusters. b) 912 913 914 Different markers were plotted in the UMAP to identify the phloem pole cell types: SAPL (CC and MSE), S17 (PPP), PEAR1 (PSE, MSE), SUC2 (mature CC), CALS8 (PPP and CC), KNOLLE (cycling cells). 915 c) Integrated UMAP showing cells coloured according to the annotation from Shahan et al. d) Integrated 916 UMAP coloured by the original clusters from the Phloem Pole Atlas. Orange arrowheads point to the 917 two parts of cluster 3, split in the integrated dataset e) Integrated UMAP with the cells contributed by 918 each individual project plotted on top (number indicated below, percentage of the total in brackets), 919 using a coloured scale to indicate cell density. Green arrowheads point to the clusters mostly 920 contributed by our dataset. f) Cluster 3 of the integrated dataset containing root early cells and dividing 921 cells. Cells in cluster 3 (early cells) are indicated in the first panel while the other panels in the row show the expression of G2/M genes in the integrated dataset, marking dividing cells g) Contribution of each 922 923 single cell project to cluster 3. Observe the grouping of early phloem cells (black arrowhead) compared 924 to the higher dispersion of early cells in other datasets.

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#### Fig 5. Similarities in the gene expression between leaf phloem parenchyma and root pericycle.

a) Schematic of the leaf minor vein showing phloem anatomy. Notice the different composition in terms
of cell identities, cell number and organization compared to the root. Adapted from b) UMAP integrating
the phloem pole cell atlas with the leaf single cell dataset. Cells were combined and reclustered,
coloured by source (leaf in green, root in black). Notice the separation of the leaf specific clusters

933 (bundle sheath and mesophyll cells) and overlap in clusters 6 (PPP / phloem parenchyma) and 9 (CC). 934 c) Percentage of cells contributed by each dataset in each cluster. Y axis shows the cluster number 935 with the number of cells in it between brackets. Root cells are coloured in black, leaf cells are in green. 936 d) Cluster annotation of the root-leaf UMAP based on markers with known tissue-specific expression. 937 The size of the points represents the percentage of cells in a cluster where the gene was detected (i.e. 938 at least 1 UMI). The colour shows the scaled average expression of the gene (z-score, i.e. number of 939 standard deviations above/below the gene's mean across all cells) e) Violin plots showing the 940 expression of PPP markers in leaf (green) and root (black) cells for phloem pole pericycle markers (e), 941 942 phloem parenchyma markers (f) and XPP markers (g) The confocal picture in f shows the expression of pSWEET11::SWEET11-2A-GFP in PPP in roots. In f, the gene expression of the respective genes 943 is shown in the phloem pole cell atlas. The numbers under the black/green violin plots indicate the 944 number of cells in cluster 6 of the leaf/root UMAP expressing each gene, with the percentage between brackets. In the confocal picture, the scale is 10 um and the white arrowhead points to PSE as a 945 946 reference point. 947

#### 949 Fig 6. Identification of a gene expression pattern common to non-PSE cells frequent after PSE 950 enucleation.

951 a) The module 1 eigengene profile with its expression along PPP, CC and PSE trajectories. b) New 952 genes with an expression pattern validating the gene profiles grouped in module 1. All the genes 953 presented in this panel are expressed forming a ring around PSE at the time of PSE enucleation. 954 SBT4.12 and TAX2 are also expressed in late PSE, with TAX2 also showing expression in the 955 epidermis. UMAPs show the particular cluster-weighted normalised expression of each gene in the 956 phloem pole cell atlas and microscopy pictures are representative images of the transcriptional reporter 957 lines where the gene promoter is fused to VENUSer. Scale bar in the longitudinal sections is 25 µm 958 while it is 10 µm in the cross sections. White arrowheads point to PSE cells as a reference point. "X" 959 marks xylem cells. Each gene has also been plotted in PPP (green), CC (orange) and PSE (purple) 960 Slingshot trajectories, showing average expression values in the Y-axis and pseudotime in the X-axis. 961 c) Expression profile of sub-module 1 eigengene, the sub-module of module 1 which is enriched for 962 genes with ring-specific expression. This sub-module contains all the genes in the panel except for 963 TAX2, which was not present in our network.

964 The numbers in each panel indicate samples with similar results, of the total independent biological 965 samples observed.

#### 967 Fig 7. PAPL genes are PEAR targets that influence root nutritional status.

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a) UMAPs showing the expression of PAPL1, PAPL2 and CDF2 in the phloem pole cell atlas. Note 968 969 these genes are also expressed in the early phloem cell clusters. b) pPAPL1::GFP/GUS expression 970 domain, showing expression in the cells around PSE from 40µm from the QC and in the epidermis c) 971 Phloem expression of pPAPL1::GFP/GUS is delayed until 120µm in pear1pear2 mutant background d) 972 pPAPL1::PAPL1-YFP (Col0) translational domain coincides with the transcriptional domain. e) The ring 973 pattern of pPAPL1::GFP/GUS gets distorted upon PSE plasmodesmata closure using the cals3m tool 974 (pPEAR1::XVE>>cals3m). f) PAPL2 (pPAPL2::VENUSer) becomes ectopically expressed upon PEAR1 975 overexpression in the meristem (pRPS5A::PEAR1-GR). g) Average root length of 6 days post-sowing 976 (dps) seedlings in 3papl, WT and complementation lines in 3papl background with genomic constructs 977 for PAPL1 (3 lines) or PAPL2 (4 lines) in sucrose-depleted media. The median and 95% confidence 978 interval are shown (methods). Number of seedlings measured: 488 3papl; 273 PAPL1-32; 37 PAPL1-979 51; 33 PAPL1-71; 343 PAPL2-11; 79 PAPL2-23; 37 PAPL2-31; 314 PAPL2-73; 382 WT. The same 980 data is also shown in Fig S9h, separately for each experimental batch and seed stock (see 981 "Experimental Design" section in the methods). Statistical analysis comparing each mutant genotype to 982 the WT is in Table S8. h) Transfer experiment between sucrose and sucrose-depleted plates of 3papl 983 seedlings. Days spent with and without sucrose are represented by grey and purple bars, respectively, 984 and roots were measured at 8 dps. Number of seedlings: 131 3papl control; 24 3papl seedlings on 985 average per transfer experiment. Statistical analysis comparing each pair of conditions is in Table S8.

In confocal pictures b, e and f, primed letters show the cross sections of each respective letter. Scale
bars: 25 µm in longitudinal sections; 10 µm in cross sections. White arrowheads point to PSE cells and
"X" marks xylem cells. The number in each confocal picture indicates samples with similar results of the
total independent biological samples analysed.

#### 991 Fig 8. Difference in WT and *3papl* metabolite levels in leaves and roots.

992 a) Overview of the different metabolites with significant differences between WT and mutant in at least 993 one of the time points. The point and error bars show, respectively, the mean and 2 times the standard 994 error (i.e. an approximate 95% confidence interval) of the log-fold-change between WT and mutant 995 metabolite levels, estimated from our linear model (see methods). The asterisk highlights points that 996 were statistically significant after adjusting for multiple testing across all the tests (false discovery rate 997 of 5%). b) Average metabolite levels for sucrose in mutant and wild-type. The bars denote the 95% 998 confidence interval estimated from our linear model (see methods). The points show the raw data for 999 individual samples. N = 6 - 8 for each timepoint/tissue/genotype combination (3 of them had 6 replicates, 1000 8 had 7 replicates and 13 had 8 replicates). 1001

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