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Metaphloem development in the Arabidopsis root tip

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Summary statement: Metaphloem sieve element differentiation in Arabidopsis roots follows a robust developmental trajectory.

35 **ABSTRACT**

36 The phloem transport network is a major evolutionary innovation that enabled plants to dominate
37 terrestrial ecosystems. In the growth apices, the meristems, apical stem cells continuously
38 produce early, so-called protophloem. This is easily observed in Arabidopsis root meristems,
39 where the differentiation of individual protophloem sieve element precursors into interconnected,
40 conducting sieve tubes is laid out in a spatio-temporal gradient. The mature protophloem
41 eventually collapses as the neighboring metaphloem takes over its function further distal from the
42 stem cell niche. Compared to protophloem, metaphloem ontogenesis is poorly characterized,
43 primarily because its visualization is challenging. Here we describe an improved protocol to
44 investigate metaphloem development in Arabidopsis root tips in combination with a set of new
45 molecular markers. We found that mature metaphloem sieve elements are only observed in the
46 late post-meristematic root although their specification is initiated as soon as protophloem sieve
47 elements enucleate. Moreover, unlike protophloem sieve elements, metaphloem sieve elements
48 only differentiate once they have fully elongated. Finally, our results suggest that metaphloem
49 differentiation is not directly controlled by protophloem-derived cues but rather follows a distinct,
50 robust developmental trajectory.

51

52 INTRODUCTION

53 The evolution of vascular tissues enabled plants to conquer land because it allowed the
54 separation of the sites of photosynthesis from the sites of nutrient and water acquisition (Lucas et
55 al., 2013). In extant angiosperms, the xylem vessels form hollow tubes to transport water and
56 inorganic ions from the root system to the shoot system. This transport is mainly driven by the
57 water potential differential between the soil and the atmosphere, and therefore by purely physical
58 forces (Endo et al., 2019; Pratt and Jacobsen, 2017). Closely associated with the xylem is the
59 phloem, which is composed of inter-connected sieve elements that form the conducting sieve
60 tubes and their neighboring companion cells. Unlike xylem vessels, sieve elements are not dead,
61 but during their differentiation process, they drastically alter their cellular makeup to optimize the
62 transport flow. Most noticeable, they lose their nucleus and vacuole. Thus, sieve elements depend
63 on the neighboring companion cells for the maintenance of their transport functions. Phloem sieve
64 tubes mediate the long distance bulk transport of phloem sap, a viscous mix of sugars,
65 metabolites as well as systemic signaling molecules, from source to sink organs, for example
66 from mature photosynthesizing leaves to roots (Lopez-Salmeron et al., 2019). This transport is
67 driven by a differential in osmotic pressure, which builds up through the controlled loading of
68 osmotic sugars in the source tissue phloem and their unloading in the sink tissue phloem
69 (Knoblauch et al., 2016; Zhang and Turgeon, 2018). The growth apices of plants, the meristems,
70 are terminal sinks, whose activity is sustained by phloem sap delivered through the early, so-
71 called protophloem. In root meristems, protophloem is produced by apical stem cells that reside
72 adjacent to the quiescent center (QC) and matures while neighboring tissues still divide or
73 undergo expansion growth (Esau, 1977; Lopez-Salmeron et al., 2019). Eventually, its sieve
74 elements become non-functional and are completely obliterated as protophloem is replaced by
75 emerging metaphloem. Although the metaphloem sieve elements share a common precursor with
76 protophloem sieve elements (Bonke et al., 2003; Rodriguez-Villalon et al., 2014), the metaphloem
77 only matures after the expansion growth of the surrounding tissues is completed (Esau, 1977).
78 Metaphloem is then retained as the main conducting phloem, although it can later be replaced by
79 secondary phloem in species that undergo secondary growth.

80 Non-invasive investigation of phloem development is challenging, on the one hand
81 because sieve elements are thin and highly anisotropic cells, and on the other hand because the
82 phloem is buried deep inside plant organs. Routine observation of protophloem by confocal
83 microscopy is however possible in the root tip of *Arabidopsis thaliana* (Arabidopsis), where its
84 development is laid out in a spatio-temporal gradient of ~20 cells from stem cell daughter to
85 mature sieve element (Furuta et al., 2014; Rodriguez-Villalon et al., 2014). Arabidopsis root tips

86 produce two protophloem strands, which are arranged opposite each other inside the stele,
87 flanking an axis of xylem cells (Fig. 1A). The last two decades have seen tremendous advances
88 in our understanding of protophloem ontogeny. Through its dissection by genetic approaches,
89 numerous protophloem-specific mutants and molecular markers have become available. These
90 studies underline the essential character of root protophloem, whose absence or disturbed
91 development has grave, systemic consequences on root meristem growth and maintenance
92 (Anne and Hardtke, 2017; Bonke et al., 2003; Rodriguez-Villalon et al., 2014). Whether the
93 defects in the protophloem of pertinent mutants also extend to metaphloem remains largely
94 unknown, mainly because of the difficulty in visualizing metaphloem development and a paucity
95 of specific molecular markers for non-invasive investigation. Here we set out to mend this gap by
96 developing a toolbox for the analysis of metaphloem development.

97

98 **RESULTS AND DISCUSSION**

99 **An optimized protocol for metaphloem visualization by confocal microscopy**

100 Imaging of the Arabidopsis root tip by confocal microscopy techniques is routine but can be tricky
101 depending on the tissue targeted for investigation. In particular, this applies to the cells inside the
102 stele, which are small in diameter compared to the surrounding ground tissue or epidermis (Fig.
103 1A). For instance, whereas the diameter of cortex cells reaches ~25 to ~50 micrometers,
104 protophloem cells are a mere ~5 micrometers across and therefore roughly 20 times smaller in
105 their horizontal cross section profile (Fig. 1A). Together, the vascular tissues inside the stele
106 occupy merely ~10% of the area in a root meristem cross section, although they represent ~40%
107 of cell files. Despite progress in staining and fixation techniques, visualization of these cells can
108 sometimes be challenging. For instance, while developing protophloem sieve elements (PPSEs)
109 can be readily identified because of their early differentiation and associated cell wall build up
110 (Truernit, 2014), mature, enucleated PPSEs are difficult to observe. Initially, PPSEs elongate from
111 the ~20 micrometer typical of dividing cells to an intermediate stage of ~50 micrometer during
112 which the principal differentiation steps occur. Once they are enucleated, they still elongate rapidly
113 to about twice their length as they become the conductive unloading terminus of the PPSE cell
114 file (Ross-Elliott et al., 2017). It is likely their high anisotropy in combination with a still elongating,
115 soft cell wall that is responsible for the compression of maturing PPSEs by neighboring tissues
116 once they lose their elevated turgor during fixation. Elongating cells possess relatively soft primary
117 cell walls to facilitate directional expansion. Stabilizing secondary cell walls are only deposited
118 during the final stages of differentiation, when the cells have reached their final size and adapt to
119 their future roles. The phenomenon of cell shrinkage or collapse upon fixation is generally
120 observed once all tissues have started to elongate further distal, in the generic cell elongation
121 zone of the root meristem (Fig. S1A). The developing metaphloem sieve elements (MPSEs) are
122 thus particularly affected, rendering their observation difficult with existing standard protocols, like
123 chloral hydrate clearing (McBryde, 1936) or mPS-PI staining (Truernit et al., 2008). By contrast,
124 the recently developed ClearSee (Kurihara et al., 2015) and TDE (2'2-thiodiethanol) clearing
125 (Musielak et al., 2016) protocols not only preserved the structure of this delicate area (Fig. S1B)
126 but also the fluorescence of reporter proteins.

127 Starting from these recent advances, we sought to develop a protocol that would leave
128 the elongation area intact and permit routine observation of MPSEs. Through a test series with
129 various combinations and concentrations of described detergents, clearing agents and fixation
130 steps (Kurihara et al., 2015; Musielak et al., 2016; Ursache et al., 2018), we established an
131 optimized procedure that maintained the integrity of the root elongation zone and allowed us to

132 observe the progressive development of MPSE cell files (the “TetSee” protocol, see Materials and
133 Methods) (Fig. S1C). Starting from the second formative division in the phloem lineage, the
134 division that gives rise to the PPSE and MPSE cell files (Bonke et al., 2003; Rodriguez-Villalon et
135 al., 2014), we could follow MPSE files across overlapping 3D renderings of serial confocal
136 microscopy images (Fig. 1B). The morphologically visible onset of MPSE differentiation, as judged
137 by intensified calcofluor white cell wall staining, was on average observed as far as ~1,400
138 micrometers from the QC. This was substantially later than the onset of morphological
139 differentiation of PPSEs (~120 micrometer from the QC), trichoblasts (~620 micrometer from the
140 QC) or protoxylem (~680 micrometer from the QC) (Fig. 1C). Thus, MPSEs only differentiated
141 visibly once all other tissues had already matured, with the exception of the metaxylem, which
142 differentiated around the same time or slightly later.

143 **Metaphloem sieve elements differentiate after they have reached their final cell size**

144 Interestingly, whereas cell elongation and differentiation are tightly linked in PPSEs (Furuta et al.,
145 2014; Rodriguez-Villalon et al., 2014), MPSEs elongated to roughly their final size before any cell
146 wall build up became apparent (Fig. 1B). Observation of other cellular rearrangements indicative
147 of MPSE differentiation, notably enucleation, proved to be difficult because of the high anisotropy
148 of MPSEs and against the background from neighboring tissues, for example when nucleic acid
149 dyes such as DAPI were used. However, our morphology-based observations were corroborated
150 by analyses of a generic molecular marker of cellular differentiation in Arabidopsis, the MINIYO
151 (IYO) protein (Sanmartin et al., 2011). Constitutively expressed IYO-GFP fusion protein is barely
152 visible in the cytosol but accumulates in the nucleus once cells differentiate. In the root tip, IYO-
153 GFP was therefore clearly visible in the quickly differentiating distal root tissues, the columella
154 and lateral root cap (Fig. 2A). Among the proximal root tissues, protophloem is the first to
155 differentiate and consistently, nuclear IYO-GFP accumulation became first apparent in
156 differentiating PPSEs (Fig. 2A) (Sanmartin et al., 2011). Interestingly, they were followed by their
157 companion cells with some distance, suggesting that PPSE companion cells only differentiate
158 once PPSEs are fully elongated and functional (Fig. 2A). In the stele, developing protoxylem
159 displayed nuclear IYO-GFP next (Fig. 2B), followed with some delay by MPSE cell files (Fig. 2C).
160 In fact, nuclear IYO-GFP accumulation was only observed in developing MPSEs after developing
161 protoxylem vessels had already completed their secondary wall build up and after they had
162 themselves fully elongated (Fig. 2D). In summary, both our morphological and molecular analyses
163 suggest that unlike in PPSEs, cell elongation and terminal differentiation do not coincide in
164 MPSEs.

165 **A set of new molecular markers for the investigation of metaphloem development**

166 Although nuclear IYO accumulation is a very useful generic indicator of the onset of cellular
167 differentiation (Sanmartin et al., 2011), it is not a marker for cell specification. We therefore sought
168 to identify tissue-specific molecular markers that would allow us to trace the incipient beginnings
169 of MPSE development. To this end, we mined the literature for genes that are specifically
170 expressed in mature phloem in other contexts (Anstead et al., 2012; Bonke et al., 2003; Cayla et
171 al., 2015; Khan et al., 2007; Sankar et al., 2014) and chose seven genes for further investigation.
172 Moreover, we intersected existing phloem-related gene expression data sets (Brady et al., 2007;
173 Clark et al., 2019; Kondo et al., 2016; Zhao et al., 2005) to identify a set of 14 additional
174 metaphloem marker candidates. For some of them, existing reporter plasmids could be obtained,
175 but for the majority we cloned promoter constructs that drive the expression of a nuclear localized
176 fluorescent reporter (NLS-CITRINE). After their transformation into Col-0 wildtype plants, eight
177 out of the 21 reporters showed activity in developing root phloem: the described *SISTER OF*
178 *ALTERED PHLOEM DEVELOPMENT* (*SAPL*) (Ross-Elliott et al., 2017), *EARLY NODULIN-LIKE*
179 *9* (*ENODL9*) (Khan et al., 2007), *SIEVE ELEMENT OCCLUSION-RELATED 2* (*SEOR2*) (Anstead
180 et al., 2012) and *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2* (*SND2*) (Kim et
181 al., 2020) reporters (Fig. 3A-D); and the new reporters *DESIGUAL 2* (*DEAL2*) (Wilson-Sanchez
182 et al., 2018), *SIEVE ELEMENT MARKER 1* (*SEMA1*; AT2G35585), *SEMA2* (AT1G61760) and
183 *SEMA3* (AT3G26350) (Fig. 4A-D).

184 However, none of the reporters was exclusively active in the (incipient) metaphloem,
185 rather, all markers were also expressed in the late developing protophloem. Among them, the
186 *SAPL* expression was particular, because although it was expressed in late differentiating PPSEs
187 similar to the other markers, thereafter it was highly specific for companion cells, both in the proto-
188 and metaphloem, and not detected in developing MPSEs (Fig. 3A). Notably, *SAPL* was
189 continuously expressed from the early coincidence with PPSE differentiation onward beyond
190 differentiated MPSEs and was not observed in any other cell file. This suggests that the four
191 companion cell files subsequently serve both PPSE and MPSE maintenance. The other markers
192 were expressed in PPSEs as well as MPSEs, with varying levels of specificity. All of them were
193 expressed in developing PPSEs, after the onset of cell wall build up and coincident with the partial
194 elongation that occurs before enucleation. *ENODL9*, *SEOR2* and *SEMA1* were most specific for
195 developing sieve elements (Figs. 3C,D and 4B). However, whereas *SEOR2* and *SEMA1*
196 expression gradually ceased upon PPSE differentiation and only became active again later,
197 *ENODL9* expression switched to the incipient MPSE file earlier and stayed on until MPSE
198 differentiation terminated (Fig. 3D). Moreover, *SEOR2* expression reappeared earlier than

199 *SEMA1* expression (Figs. 3C and 4B). The other reporters also displayed some marked
200 expression outside of PPSEs/MPSEs. The *SND2* reporter was strongly expressed in late
201 developing MPSEs, however it was also observed in developing metaxylem (Fig. 3B). *DEAL2*,
202 *SEMA2* and *SEMA3* all switched expression to the cell files surrounding PPSEs after enucleation
203 (Fig. 4A,C,D). In the metaphloem, *DEAL2* was expressed in MPSEs but also in the directly
204 neighboring cell files, likely the companion cells (Fig. 4A). A similar pattern was observed for
205 *SEMA3* (Fig. 4D), whereas *SEMA2* appeared to be specific for MPSEs (Fig. 4C). In summary, we
206 were able to identify a set of reporters for metaphloem development that mark different stages as
207 well as cell types (Fig. 4E). Their investigation confirmed that unlike what has been reported for
208 PPSEs, cell elongation and differentiation are uncoupled in MPSEs, and also show that both sieve
209 element types are associated with the same companion cell files.

210 **Metaphloem development is not affected by CLE45 treatment**

211 The continuous expression of *ENODL9* in the MPSE cell files as soon as PPSEs enucleate also
212 suggested that MPSE specification starts as soon as PPSE development is finished. This could
213 mean that premature MPSE differentiation is prevented by lateral inhibition through cues derived
214 from developing PPSEs. One such candidate signal are secreted CLAVATA3/EMBRYO
215 SURROUNDING REGION-RELATED (CLE) signaling peptides, because low concentrations of
216 certain synthetic CLE peptides suppress PPSE development when applied to roots (Depuydt et
217 al., 2013; Hazak et al., 2017; Ito et al., 2006; Kinoshita et al., 2007; Rodriguez-Villalon et al.,
218 2014), as does dosage increase of CLE45 (Czyzewicz et al., 2015b; Rodriguez-Villalon et al.,
219 2014). Interestingly, CLE45 as well as CLE26 and CLE25 are specifically expressed in developing
220 PPSEs (Czyzewicz et al., 2015a; Ren et al., 2019; Rodriguez-Villalon et al., 2014; Rodriguez-
221 Villalon et al., 2015). CLE peptide signaling is however apparently not strictly required for
222 protophloem development (Anne et al., 2018; Fukuda and Hardtke, 2020), rather it appears to act
223 as a safeguard mechanism that maintains plasticity of phloem pole cells during their meristematic
224 stage (Gujas et al., 2020).

225 Upon CLE45 treatment, the expression of both markers tested, *SEMA3* and *SEOR2*,
226 disappeared from the protophloem, consistent with their prohibitive effect on PPSE formation (Fig.
227 5A-D). However, both markers persisted in developing MPSEs (Fig. 5B,D), in line with the
228 observation that their differentiation appeared unaffected. Notably, this observation also
229 confirmed once more that MPSE specification is position- rather than lineage-dependent, because
230 the PPSE and MPSE cell files arise from the same stem cell daughter through a periclinal division
231 that is suppressed by CLE45 application (Rodriguez-Villalon et al., 2014; Rodriguez-Villalon et
232 al., 2015). Moreover, the absence of pertinent phenotypes in *cle25* mutants (Ren et al., 2019) as

233 well as in receptor mutants that are fully insensitive against all three CLE peptides (Anne et al.,
234 2018) corroborates the conclusion that PPSE-derived CLE peptides do not impinge on MPSE
235 development under normal circumstances. In summary, CLE45 peptide treatment efficiently
236 suppressed PPSE formation, but did not interfere with MPSE development.

237 **MPSE development follows a robust developmental trajectory**

238 In the protophloem, CLE45 signaling is quantitatively antagonized by the vascular plant -specific
239 *OCTOPUS* (*OPS*) gene. *OPS* is thus a positive regulator of PPSE differentiation that is expressed
240 from early on in the protophloem and insulates developing PPSEs against the effects of autocrine
241 CLE45 signalling (Breda et al., 2017; Breda et al., 2019). In *ops* loss-of-function mutants,
242 developing PPSEs frequently fail to differentiate (including failure to build up cell wall and thereby
243 appearing as so-called gap cells), which causes discontinuities in the protophloem strands and
244 disturbs the transport of phloem sap into the meristem (Anne and Hardtke, 2017; Rodriguez-
245 Villalon et al., 2014; Truernit et al., 2012). *OPS* is also weakly expressed in the incipient MPSE
246 cell file, against a background of low, ubiquitous expression of its homolog *OPS-LIKE 2* (*OPL2*)
247 that increases in developing metaphloem (Ruiz Sola et al., 2017). Whereas *opl2* single mutants
248 did not display apparent phenotypes, except (in our hands) a more variable root growth vigor (Fig.
249 S2A), the *ops opl2* double mutant is the only described genotype with MPSE defects so far (Fig.
250 S2B) (Ruiz Sola et al., 2017), apart from mutants that lack protophloem and metaphloem
251 altogether. Compared to *ops* single mutants, root growth vigor was further diminished in *ops opl2*
252 double mutants (Fig. S2A,C) and they also displayed aggravated PPSE differentiation defects
253 (Fig. S2B,D) (Ruiz Sola et al., 2017). The latter were more severe than evident from simple gap
254 cell presence-absence counts, because *ops opl2* double mutants often had only one
255 distinguishable PPSE strand. To better understand how MPSE and PPSE differentiation is
256 affected in *ops* single and *ops opl2* double mutants, we introduced some of our reporter genes
257 into these backgrounds.

258 In *ops* single mutants, the *SEMA3* reporter was expressed at the later stages of PPSE
259 differentiation as in wildtype, but absent in developing PPSEs that failed to differentiate (Fig. S3A),
260 underlining their different cellular identity. In developing MPSEs, *SEMA3* expression appeared to
261 be unaffected (Fig. S3B,C). By contrast, *SAPL* reporter activity was still observed in gap cells
262 (Fig. S3D), which corroborates earlier observations and is in line with the recent proposal that
263 they adopt companion cell identity (Gujas et al., 2020) as well as the strong continuous companion
264 cell-specific *SAPL* expression after PPSE differentiation. Again, *SAPL* expression appeared
265 unaffected in the developing metaphloem region of *ops* mutants (Fig. S3E). Together, these
266 findings reiterate that the defects in *ops* mutants are protophloem-specific (Ruiz Sola et al., 2017;

267 Truernit et al., 2012). Interestingly, *SAPL* expression could still be detected in protophloem gap
268 cells later on (Fig. S3F), indicating that PPSE cells that fail to differentiate properly in the
269 protophloem differentiation window fail to catch up.

270 In *ops ops* double mutants, the *SEOR2*, *DEAL2*, *SEMA2* and *SEMA3* markers displayed
271 normal expression, except their apparent absence in gap cells (Fig. 6A-D). Despite the described
272 MPSE differentiation defects (Ruiz Sola et al., 2017), which we could also observe in optical cross
273 sections (Fig. S2B), our markers were however essentially continuously expressed in developing
274 MPSEs of *ops ops* mutants (Fig. 6A-D). Thus, we could not detect corresponding “metaphloem
275 gap cells”, possibly because differentiating MPSEs are quite long (200-300 micrometer) and
276 because surveying extended stretches of MPSEs was difficult. Nevertheless, although *ops ops*
277 double mutants typically displayed marker expression in both PPSE and MPSE strands (Fig. 7A),
278 this pattern also often deviated from wildtype. Upon closer inspection, this could be attributed to
279 the reappearance of reporter expression in undifferentiated protophloem cell files long after the
280 zone of normal PPSE differentiation, clearly visible from elongated PPSEs that expressed the
281 respective marker (Fig. 7B). Thus, the observation that one of the two protophloem poles in *ops*
282 *ops* mutants was frequently absent (Fig. S2B) (Ruiz Sola et al., 2017) could also reflect a strongly
283 delayed differentiation of one PPSE strand. *In extremis*, the delay was such that it overlapped
284 with reporter expression in the neighboring MPSE cell files (Fig. 7C). We had not observed such
285 atypical differentiation in *ops* single mutants. This not only indicates that PPSE differentiation can
286 be substantially delayed in *ops ops* mutants, but also that the onset of MPSE differentiation is
287 largely independent of such delays. Corroborating the independent trajectory of MPSE
288 differentiation, in *ops ops* roots where only one PPSE cell file showed differentiating cells (any
289 gap cells notwithstanding) and marker expression was also absent from the failed PPSE cell file
290 later on, marker expression in both MPSE cell files appeared to be normal (Fig. 7D) and could
291 typically be observed shortly after protoxylem cells with secondary cell walls were visible, as in
292 wildtype. Together with the observed activity of our markers in CLE45-treated roots, our analyses
293 therefore suggest that MPSE development follows a robust trajectory that is largely independent
294 from PPSE development.

295 Finally, it is noteworthy that OPS action is exquisitely dosage-sensitive (Breda et al., 2017;
296 Breda et al., 2019) and promotes PPSE differentiation by quantitatively antagonizing CLE45
297 signaling via the receptor kinase BARELY ANY MERISTEM 3 (Breda et al., 2017; Breda et al.,
298 2019; Fukuda and Hardtke, 2020). Moreover, an excess of ectopic OPS activity leads to
299 premature differentiation across root tissues (Breda et al., 2019). Thus, our results are consistent
300 with the notion that the differential expression levels of OPS family proteins such as *OPS* and

301 *OPL2* along the gradient of developing PPSE versus MPSE cell files contributes to the correct
302 spatio-temporal separation of their differentiation.

303 **A toolbox for the investigation of metaphloem development**

304 In summary, our study extends our toolbox for the investigation of sieve element development in
305 the *Arabidopsis* root, with a special focus on the so far poorly described differentiation of the
306 metaphloem, and provides first forays into its genetic control. Our results highlight commonalities
307 between PPSE and MPSE development, but also suggest that metaphloem development follows
308 a robust trajectory that is not directly influenced by adjacent or preceding PPSE development
309 under normal circumstances. Combined with state-of-the-art technical advances, such as single
310 cell RNA sequencing or tissue-specific gene knock-out (Smetana et al., 2019; Wendrich et al.,
311 2020), our observations should enable more targeted future approaches to dissect metaphloem
312 development and discover its unique features.

313

314 MATERIALS AND METHODS

315 Plant culture, transformation and common molecular biology followed previously described
316 standard procedures (Cattaneo et al., 2019; Graeff et al., 2020; Kang et al., 2017).

317 Plant materials and growth conditions

318 Seeds were surface sterilized using 3% sodium hypochlorite, sown onto half strength Murashige
319 & Skoog agar medium (0.9% agarose) supplemented with 0.3% sucrose and stratified for 3 days
320 at 4°C. Plants were grown under continuous white light (intensity ~120 µE) at 22°C. All mutants
321 and marker lines were in the *Arabidopsis thaliana* Columbia-0 (Col-0) wild type background. The
322 *ops* and *ops opl2* mutant lines were described previously (Ruiz Sola et al., 2017; Truernit et al.,
323 2012). CLE45 peptide treatments were performed as described (Anne et al., 2018).

324 Database mining and selection of sieve element marker candidates

325 For the selection of the sieve element (SE) marker candidates, expression of 576 genes enriched
326 in cells expressing the S32 phloem marker (AT2G18380) was analysed along the root, in cells
327 expressing *SUC2* (AT1G22710) (Brady et al., 2007), in cells expressing *CVP2* (AT1G05470)
328 (Clark et al., 2019), in a general root and seedling gene expression dataset (Gan et al., 2011),
329 and in the “VISUAL” phloem and xylem datasets (Kondo et al., 2016). Twenty candidate genes
330 that i) showed expression in the phloem poles, ii) showed increased expression further away from
331 the meristem, iii) showed relatively higher expression in the root, and iv) appeared in the VISUAL
332 phloem datasets were tested as SE markers.

333 Transgene constructs

334 For the construction of SE markers, respective promoter fragments of 1,500 bp to 2,500 bp were
335 amplified from genomic Col-0 DNA using suitable oligonucleotides with overhangs (attB1/2
336 extensions for *ENODL9*, *SEOR2*, *SND2*, and attB4/1r extensions for *DEAL2* and *SEMA1-3*) for
337 subsequent *Gateway*TM cloning (see Table S1). The manufacturer’s solutions (*ThermoFisher*
338 *Scientific* article numbers 11791020 and 11789020) and protocols were used for all cloning
339 reactions. Amplified fragments were cloned into suitable entry vectors and the *ENODL9*, *SEOR2*
340 and *SND2* promoters were transferred into the pMDC205 destination vector in front of a GFP
341 reporter with an ER retention signal (Curtis and Grossniklaus, 2003). The *DEAL2* and *SEMA1-3*
342 promoters were recombined together with NLS-CITRINE in a multisite gateway reaction into the
343 pK7m24 vector backbone. Flowering Col-0 plants were transformed using the floral dip method
344 and transformants were selected either on ½ MS media containing 25 mg/ml hygromycin B or 25
345 mg/ml kanamycin following a fast selection procedure (Harrison et al., 2006).

346 **Tissue fixation and clearing (TetSee protocol)**

347 For microscopy, 7-day-old seedlings were fixed in a solution of 4% PFA in PBS buffer and
348 transferred into a vacuum of 25 to 30 mmHg/Torr for 15 to 30 min. Subsequently, seedlings were
349 washed three times in PBS for 5 min. For clearing of the samples, different protocols were used
350 and assessed for the quality of the tissue preservation along the root. While standard protocols
351 like chloral hydrate clearing (McBryde, 1936) or mPS-PI staining (Truernit et al., 2008) caused
352 the shrinking of the cells in the early elongation zone, the recently developed ClearSee (Kurihara
353 et al., 2015) and 2'2-thiodiethanol-clearing (Musielak et al., 2016) protocols preserved the
354 structure of this delicate area as well as the fluorescence of reporter proteins. The two protocols
355 were further optimized and combined into the “TetSee” (2'2-Thiodiethanol-ClearSee) protocol.
356 Briefly, the washed seedlings were transferred into TetSee X solution (15% Na-deoxycholate,
357 25% urea, 10 % glycerol, 5% 2'2-thiodiethanol [Merck, Product No. 166782], 1% Triton X-100)
358 and kept for 3 days at 4°C with daily changes of the TetSee X solution. For microscopy, the
359 TetSee X solution was removed and replaced by TetSee solution (the TetSee X solution without
360 Triton X-100) containing 0.25 mg/ml calcofluor white (CCFW; Sigma, Product No. F3543).
361 Seedlings were incubated in the CCFW staining solution for 6 h or overnight, washed once in
362 TetSee solution and then transferred onto microscopy slides with TetSee solution as mounting
363 medium.

364 **Microscopy**

365 For morphological assessment of phloem differentiation, roots were prepared as described above
366 and the CCFW-stained cell walls were imaged using a Zeiss LSM880 confocal microscope with
367 a 40x objective. A 405 nm laser was used for CCFW excitation, and the cell wall signal was
368 recorded in a range from 450 to 480 nm. For imaging of the SE marker lines, GFP or CITRINE
369 were sequentially excited with 488 nm and their emission recorded from 500 to 560 nm. Tile scans
370 and Z scans were combined in order to obtain continuous images of the vasculature from the root
371 meristem to the differentiated metaphloem. Additionally, a Nikon Spinning disc CSU-W1 confocal
372 microscope with a 40x objective was used to record images of the CLE45-treated and the *ops*
373 *opl2* SE marker lines. Analysis of the images and generation of 3D renderings from the Z stacks
374 were performed using the *GNU icy* software.

375

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379 **COMPETING INTERESTS**

380 The authors declare no competing interests.

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385

386 **FIGURE LEGENDS**

387 **Fig. 1. Development of metaphloem sieve elements (MPSEs) in the Arabidopsis root tip.**

388 (A) Schematic overview of tissue arrangement and development in an Arabidopsis root meristem,
389 based on confocal microscopy images of a longitudinal half section and a horizontal cross section.
390 (B) Confocal microscopy, optical sections illustrating MPSE development in a 7-day-old
391 Arabidopsis Col-0 wildtype root tip stained with calcofluor white (CCFW; blue fluorescence) and
392 propidium iodide (PI; reddish fluorescence) using the optimized “TetSee” protocol. Left overview
393 panels indicate the approximate positions of the magnified images in the right panels. The bottom
394 right panels are labeled counterparts of the raw images in the corresponding top right panels. The
395 common stem cell precursors for the protophloem sieve element (PPSE) and MPSE cell files are
396 labeled in red in the left-most assembly. Note the formative division giving rise to the developing
397 PPSE strand (labeled green) and incipient MPSE strand (labeled yellow). Size bars are 10
398 micrometer. (C) Distance of the first visibly differentiated cell from the quiescent center, for
399 different root tissues. Box plots display 2nd and 3rd quartiles and the median, bars indicate
400 maximum and minimum.

401 **Fig. 2. Differentiation timing in the Arabidopsis root tip.** (A-D) 3D renderings of confocal

402 image stacks, focused on the vasculature. Consecutive sections of a 7-day-old CCFW-stained
403 root expressing the IYO-GFP fusion protein (green fluorescence) under control of the constitutive
404 35S promoter are shown. Left panels: CCFW-GFP fluorescence composite images; right panels:
405 GFP fluorescence only. Nuclear IYO-GFP accumulation indicates cellular differentiation.
406 Differentiating PPSEs are pointed out by a red arrowhead, their companion cells by white
407 arrowheads (A). Protoxylem cells (blue arrowheads) start to differentiate before MPSEs (orange
408 arrowheads) (B), who enter differentiation once secondary wall build up in protoxylem cells
409 becomes apparent (C,D).

410 **Fig. 3. Reporter genes for phloem specification and differentiation I.** (A-D) 3D renderings of

411 confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that express
412 the indicated reporter genes (green fluorescence). Left overview panels (generic wildtype root)
413 indicate the approximate positions of the magnified images in the right panels. Bottom panels:
414 CCFW-GFP fluorescence composite images; top panels: GFP fluorescence only. Note that for
415 better visibility of details, images are not always to the same scale.

416 **Fig. 4. Reporter genes for phloem specification and differentiation II.** (A-D) 3D renderings of

417 confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that express
418 the indicated reporter genes (green fluorescence). Left overview panels (generic wildtype root)

419 indicate the approximate positions of the magnified images in the right panels. Bottom panels:
420 CCFW-GFP fluorescence composite images; top panels: GFP fluorescence only. (E) Schematic
421 summary of the tissue-specific expression patterns for the reporter genes shown in figures 3 and
422 4.

423 **Fig. 5. MPSE reporter genes do not respond to CLE45-treatment.** (A-D) 3D renderings of
424 confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that express
425 the indicated reporter genes (green fluorescence). Left overview panels (generic wildtype root,
426 treated with mock or CLE45) indicate the approximate positions of the magnified images in the
427 right panels. Bottom panels: CCFW-GFP fluorescence composite images; top panels: GFP
428 fluorescence only. Roots were either grown on mock (A,C) or 15 nM CLE45 peptide (B,D).

429 **Fig. 6. Phloem reporter gene expression in *ops opl2* double mutants I.** (A-D) 3D renderings
430 of confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that
431 express the indicated reporter genes (green fluorescence) in *ops opl2* double mutant background.
432 Left overview panels (generic *ops opl2* root) indicate the approximate positions of the magnified
433 images in the right panels. Center panels: CCFW-GFP fluorescence composite images; right
434 panels: GFP fluorescence only. Note that for better viewing of details, images are not always to
435 the same scale.

436 **Fig. 7. Phloem reporter gene expression in *ops opl2* double mutants II.** (A-D) 3D renderings
437 of confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that
438 express the indicated reporter genes (green fluorescence) in *ops opl2* double mutant background.
439 Left panels: CCFW-GFP fluorescence composite images; right panels: GFP fluorescence only.
440 PPSE or MPSE cell files expressing molecular markers are pointed out by red or orange
441 arrowheads, respectively.

442

443 **SUPPLEMENTARY FIGURE LEGENDS**

444 **Fig. S1. Imaging of elongating root cells using different fixation protocols.** (A-C) Confocal
445 microscopy, optical sections of 7-day-old CCFW-stained root meristems (white fluorescence)
446 fixed with different protocols as indicated. Red arrowheads point out elongating PPSEs.

447 **Fig. S2. Root phenotypes of *ops op12* double mutants.** (A) Primary root length of 12-day-old
448 seedlings of indicated genotypes. Box plots display 2nd and 3rd quartiles and the median, bars
449 indicate maximum and minimum. (B) Confocal microscopy, longitudinal and horizontal optical
450 cross sections of 7-day-old CCFW-stained root meristems (black fluorescence). Full red
451 arrowheads point out normally differentiating PPSEs, open red arrowheads point out cells in the
452 PPSE file that fail to differentiate ("gap cells). (C) Images of 12-day-old seedlings of the indicated
453 genotypes. (D) Frequency of PPSE cell files with gap cells in root meristems of the indicated
454 genotypes (n=20-30).

455 **Fig. S3. Phloem reporter gene expression in *ops* single mutants.** (A-F) 3D renderings of
456 confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that express
457 the indicated reporter genes (green fluorescence) in *ops* single mutant background. Left overview
458 panels (generic *ops* root) indicate the approximate positions of the magnified images in the right
459 panels. Center panels: CCFW-GFP fluorescence composite images; top panels: GFP
460 fluorescence only. Note that for better viewing of details, images are not always to the same scale.
461 Open arrowheads in (A), (D) and (F) point out cells in the PPSE file that fail to differentiate ("gap
462 cells).

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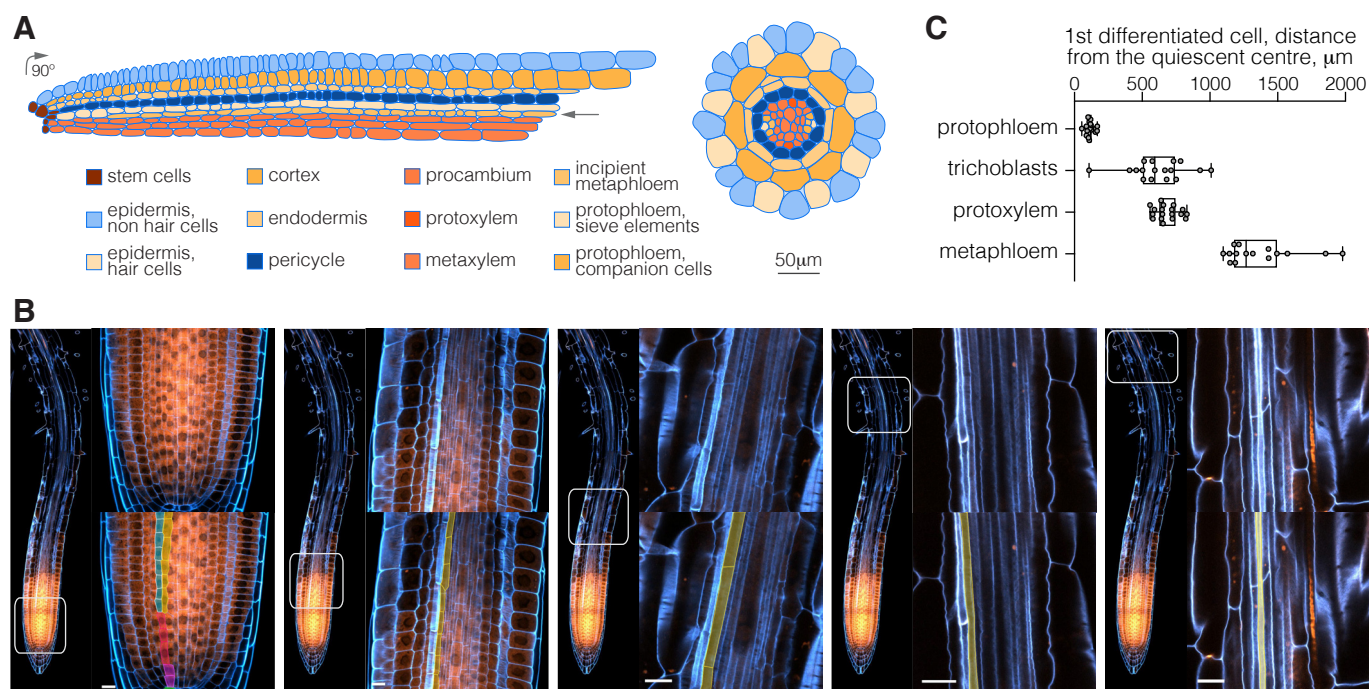


Fig. 1. Development of metaphloem sieve elements (MPSEs) in the Arabidopsis root tip. (A) Schematic overview of tissue arrangement and development in an Arabidopsis root meristem, based on confocal microscopy images of a longitudinal half section and a horizontal cross section. (B) Confocal microscopy, optical sections illustrating MPSE development in a 7-day-old Arabidopsis Col-0 wildtype root tip stained with calcofluor white (CCFW; blue fluorescence) and propidium iodide (PI; reddish fluorescence) using the optimized "TetSee" protocol. Left overview panels indicate the approximate positions of the magnified images in the right panels. The bottom right panels are labeled counterparts of the raw images in the corresponding top right panels. The common stem cell precursors for the protophloem sieve element (PPSE) and MPSE cell files are labeled in red in the left-most assembly. Note the formative division giving rise to the developing PPSE strand (labeled green) and incipient MPSE strand (labeled yellow). Size bars are 10 micrometer. (C) Distance of the first visibly differentiated cell from the quiescent center, for different root tissues. Box plots display 2nd and 3rd quartiles and the median, bars indicate maximum and minimum.

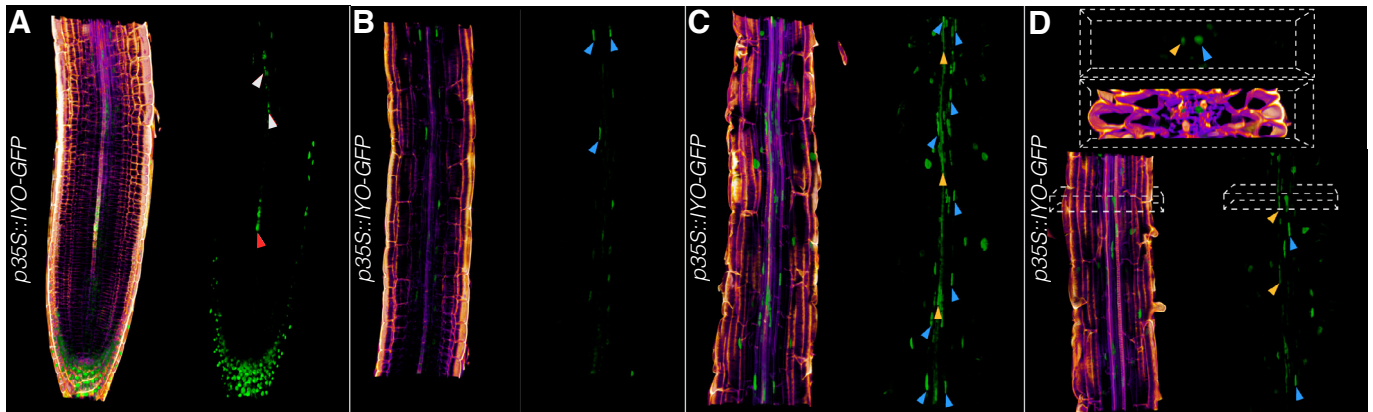


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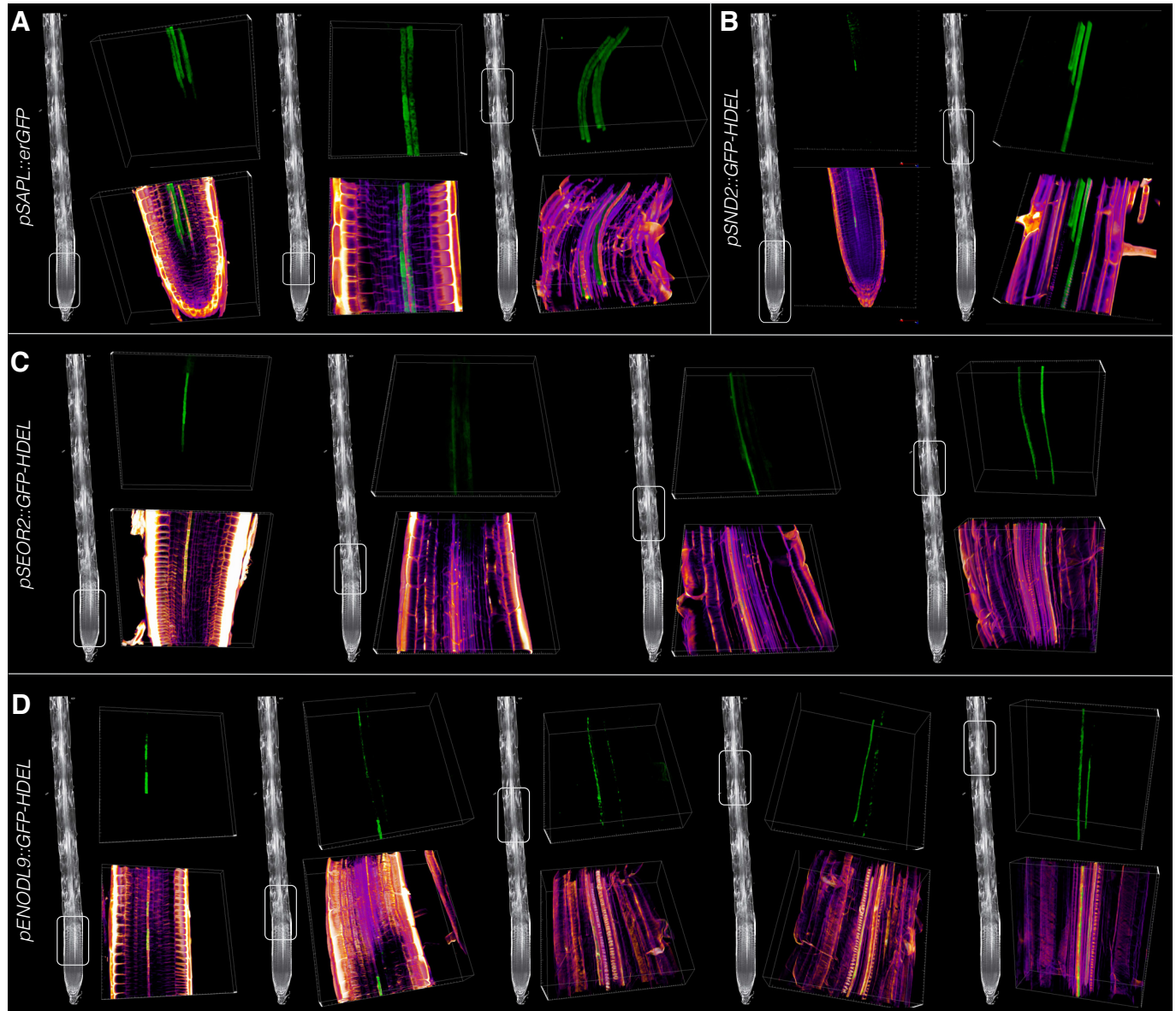


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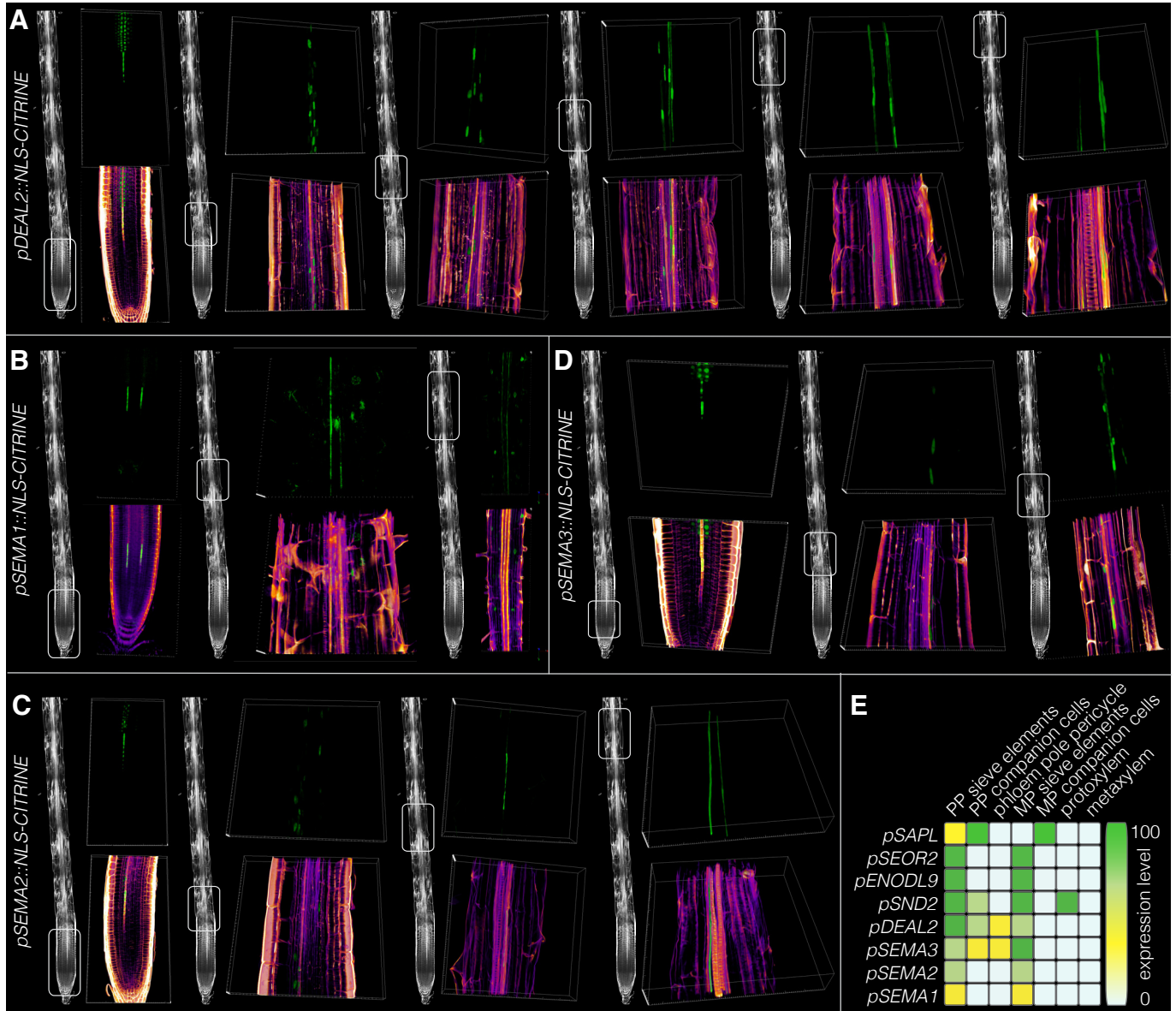


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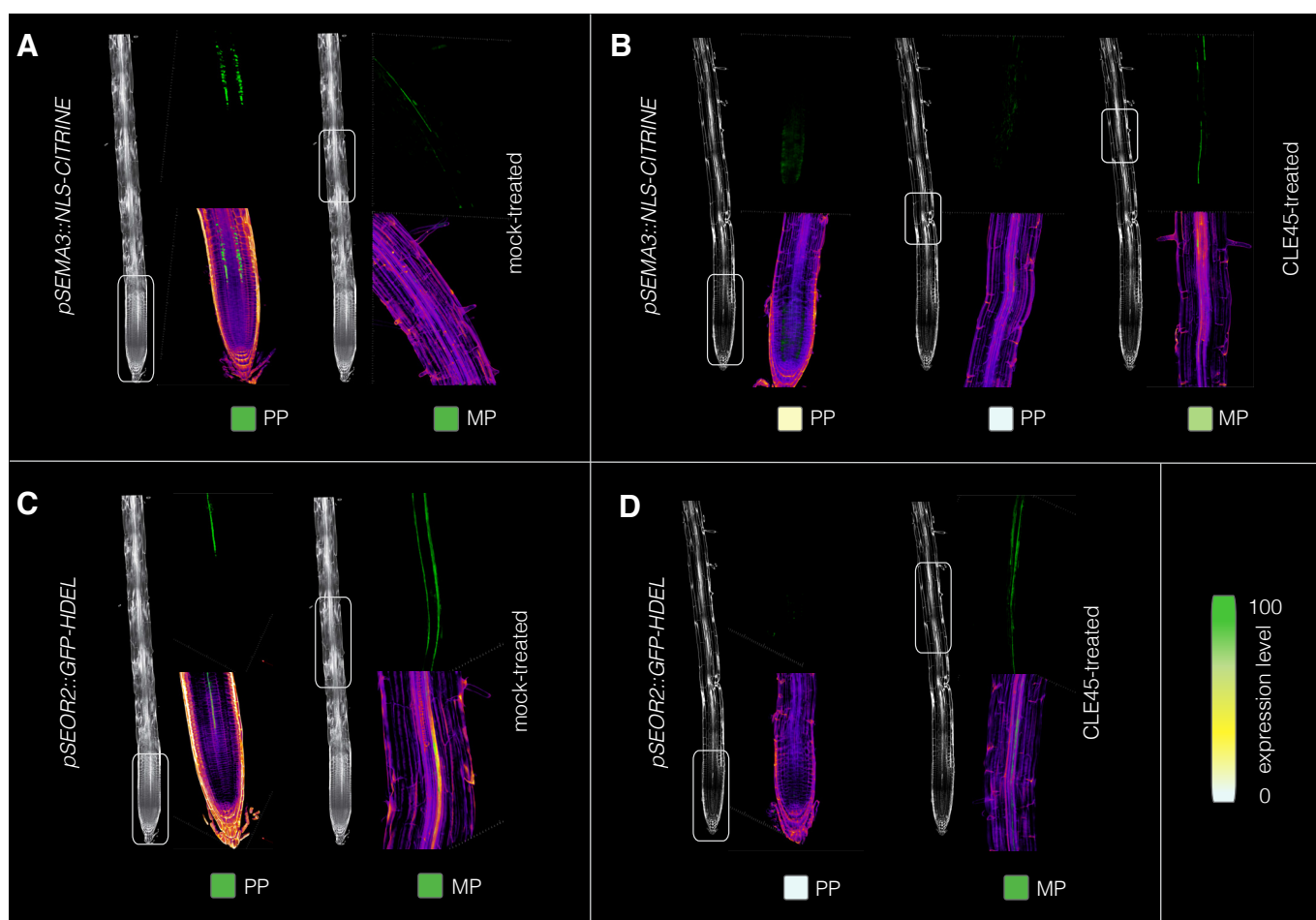


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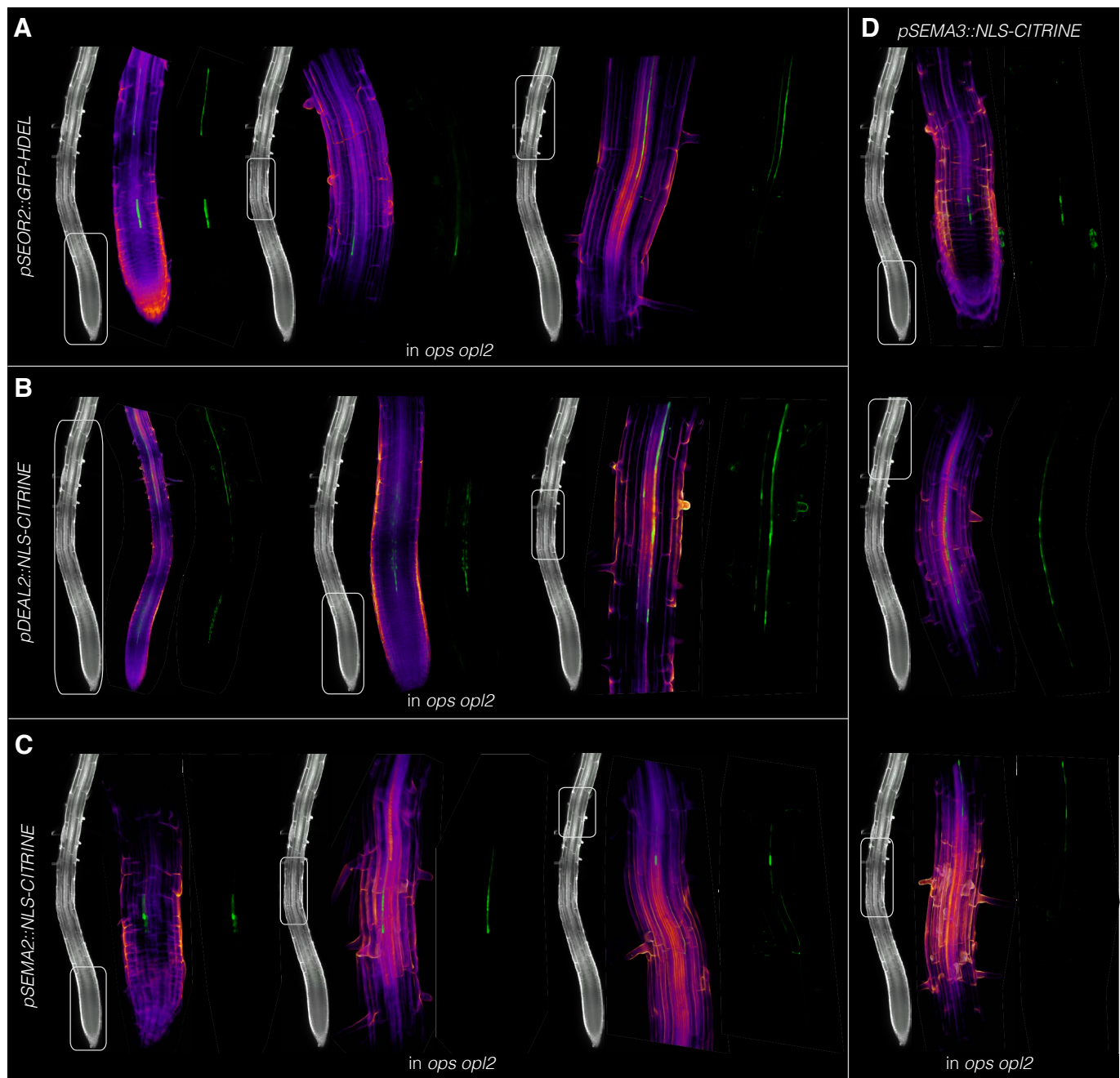


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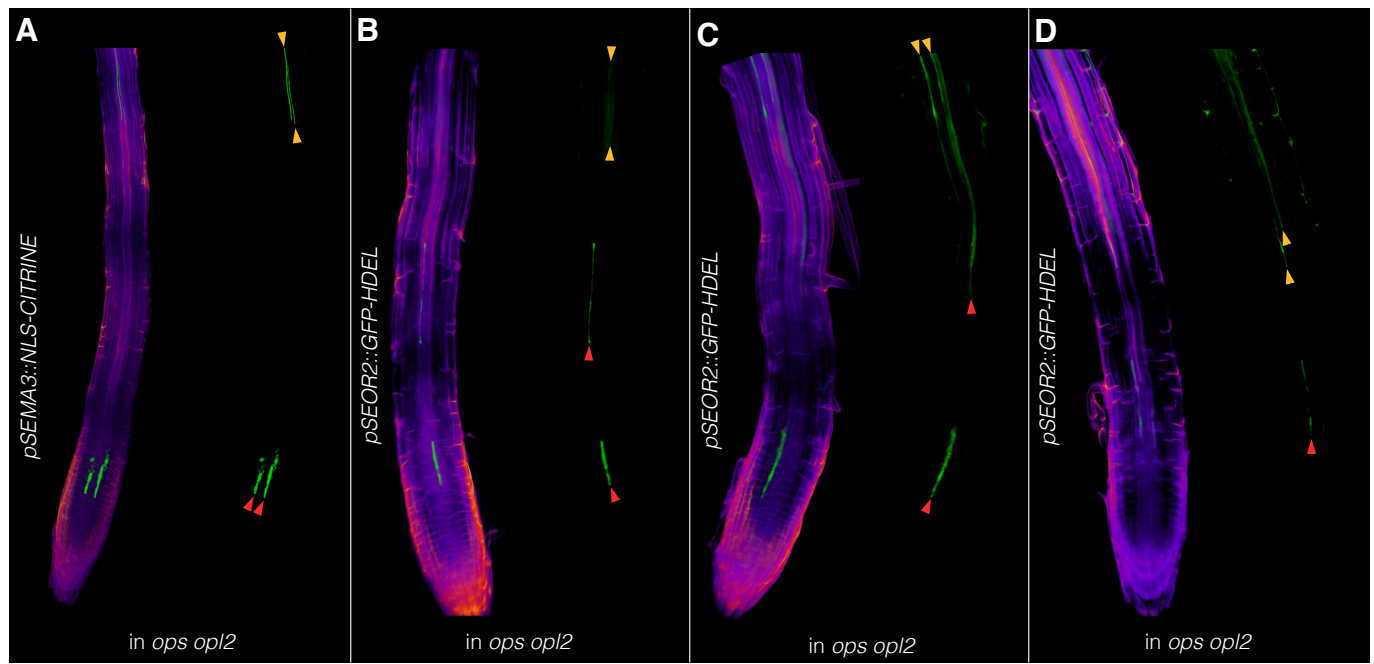


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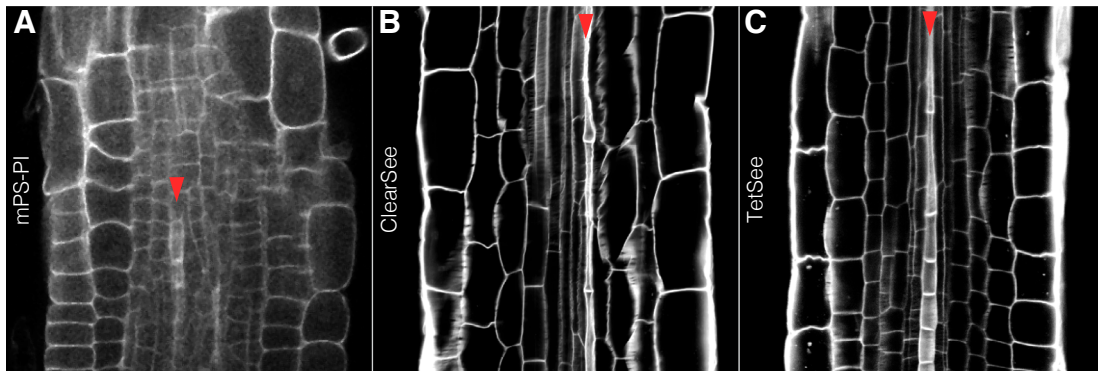


Fig. S1. Imaging of elongating root cells using different fixation protocols. (A-C) Confocal microscopy, optical sections of 7-day-old CCFW-stained root meristems (white fluorescence) fixed with different protocols as indicated. Red arrowheads point out elongating PPSEs.

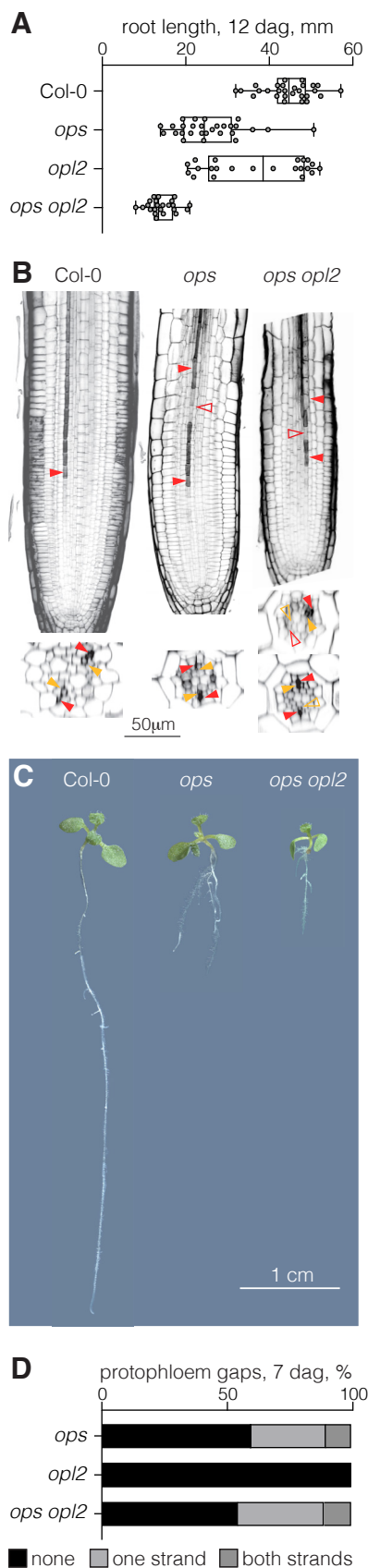


Fig. S2. Root phenotypes of *ops opl2* double mutants. (A) Primary root length of 12-day-old seedlings of indicated genotypes. Box plots display 2nd and 3rd quartiles and the median, bars indicate maximum and minimum. (B) Confocal microscopy, longitudinal and horizontal optical cross sections of 7-day-old CCFW-stained root meristems (black fluorescence). Full red arrowheads point out normally differentiating PPSEs, open red arrowheads point out cells in the PPSE file that fail to differentiate ("gap cells"). (C) Images of 12-day-old seedlings of the indicated genotypes. (D) Frequency of PPSE cell files with gap cells in root meristems of the indicated genotypes (n=20-30).

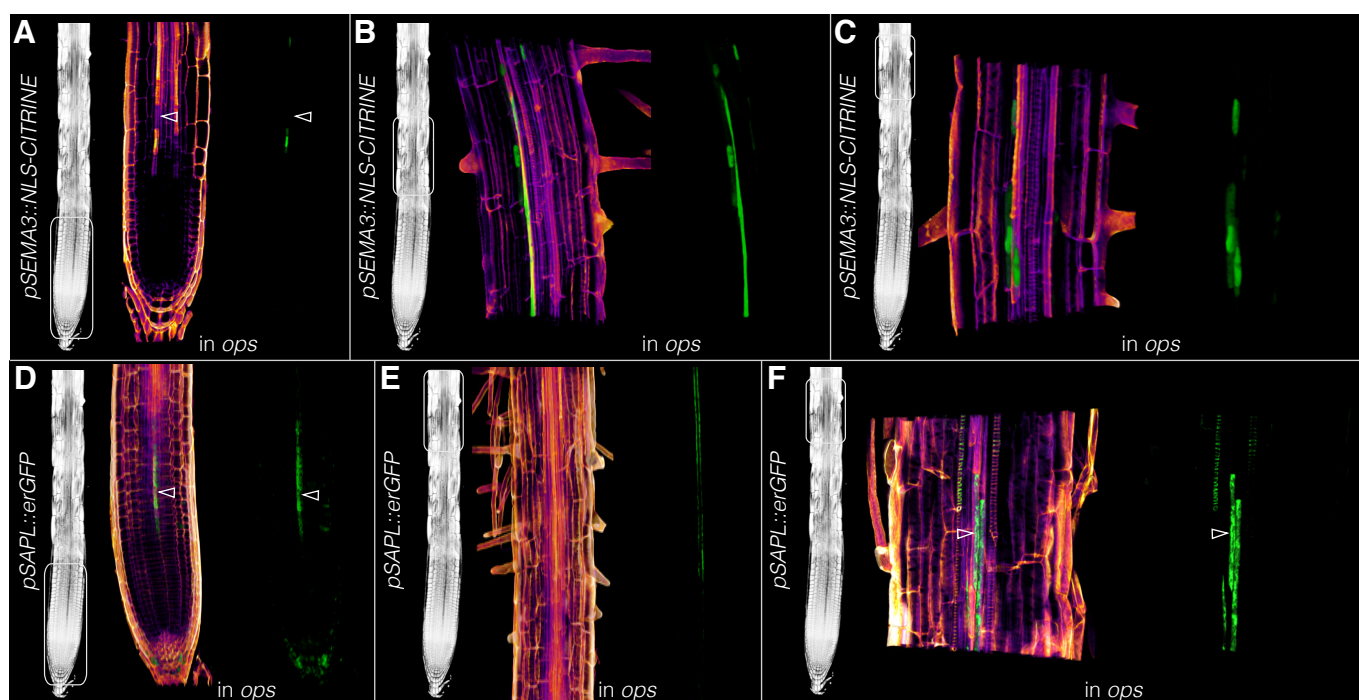


Fig. S3. Phloem reporter gene expression in *ops* single mutants. (A-F) 3D renderings of confocal image stacks, focused on the vasculature of 7-day-old CCFW-stained roots that express the indicated reporter genes (green fluorescence) in *ops* single mutant background. Left overview panels (generic *ops* root) indicate the approximate positions of the magnified images in the right panels. Center panels: CCFW-GFP fluorescence composite images; top panels: GFP fluorescence only. Note that for better viewing of details, images are not always to the same scale. Open arrowheads in (A), (D) and (F) point out cells in the PPSE file that fail to differentiate ("gap cells).

Table S1

5' to 3' oligonucleotide sequences

oligos with attB4/1r extensions					
oligo_name	sequence	gene	name	target	
pAT2G44000_attB4_1F	ggggacaactttgtatagaaaagttgtGCTTGTATGCTGCTGGACA	AT2G44000	LEA hydroxylprolin		promoter
pAT2G44000_attB1r_1R	ggggactgctttttgtacaaactgtCATTCCCTCAGAGATTGTTT	AT2G44000			
pAT5G50120_attB4_1F	ggggacaactttgtatagaaaagttgtCGTGTTTCCCCACTTCTA	AT5G50120	WD40 Transducin-like		promoter
pAT5G50120_attB1r_1R	ggggactgctttttgtacaaactgtGGATTCTGAAATGTATCATTTTTG	AT5G50120			
pAT2G35585_attB4_1F	ggggacaactttgtatagaaaagttgtGGTGACGATCGGAGGATA	AT2G35585	SEMA1		promoter
pAT2G35585_attB1r_1R	ggggactgctttttgtacaaactgtGAAATACCAACCATGCTATTGAA	AT2G35585			
pAT3G26350_attB4_1F	ggggacaactttgtatagaaaagttgtGATAGCGAAGCGAGTTACGG	AT3G26350	SEMA3		promoter
pAT3G26350_attB1r_1R	ggggactgctttttgtacaaactgtCATTAGATGAAGAAGCTAAAAGCA	AT3G26350			
pAT5G45320_attB4_1F	ggggacaactttgtatagaaaagttgtCTTCTGAAGCGCAGCTTCT	AT5G45320	LEA hydroxylprolin		promoter
pAT5G45320_attB1r_1R	ggggactgctttttgtacaaactgtACGAGAAGCAACTCGGGCA	AT5G45320			
pAT3G63050_attB4_1F	ggggacaactttgtatagaaaagttgtTTCCGTGGAAGTGGTGC	AT3G63050	B-cell receptor associated 31 family		promoter
pAT3G63050_attB1r_1R	ggggactgctttttgtacaaactgtATCATTGTCTCTCTGCATCG	AT3G63050			
pAT1G61760_attB4_1F	ggggacaactttgtatagaaaagttgtTTAATTTGTTGGCCATT	AT1G61760	SEMA2		promoter
pAT1G61760_attB1r_1R	ggggactgctttttgtacaaactgtAGAATCGACCTGTGTGCA	AT1G61760			
pAT2G17260_attB4_1F	ggggacaactttgtatagaaaagttgtTTTCCGATTGATTTTCC	AT2G17260	Glutamate receptor 2		promoter
pAT2G17260_attB1r_1R	ggggactgctttttgtacaaactgtCATGGACAACCCAGCAGTG	AT2G17260			
pAT2G14620_attB4_1F	ggggacaactttgtatagaaaagttgtTGCCTATGGCTGAAAAGTCC	AT2G14620	XTH10		promoter
pAT2G14620_attB1r_1R	ggggactgctttttgtacaaactgtCATTATTAAGATGTTGAGGTTGAG	AT2G14620			
pAT4G21310_attB4_1F	ggggacaactttgtatagaaaagttgtTGAGGGTGACCATCAAAACA	AT4G21310	DUF1218 domain protein		promoter
pAT4G21310_attB1r_1R	ggggactgctttttgtacaaactgtCCCTACATCTCCGCCATT	AT4G21310			
pAT2G37610_attB4_1F	ggggacaactttgtatagaaaagttgtGGAGGAGGAAGGAAGATTG	AT2G37610	SMR12		promoter
pAT2G37610_attB1r_1R	ggggactgctttttgtacaaactgtTACGTTTCCAATCTCCATGTG	AT2G37610			
pAT1G73040_attB4_1F	ggggacaactttgtatagaaaagttgtAATTAGGGATGCGTTGCTTG	AT1G73040	Mannose/lectin binding family protein		promoter
pAT1G73040_attB1r_1R	ggggactgctttttgtacaaactgtCATGAGTCGTCTGTGTTTT	AT1G73040			
pAT1G06490_attB4_1F	ggggacaactttgtatagaaaagttgtCGTTGACGCAGCTTATCAAGT	AT1G06490	Glucan synthase like 7		promoter
pAT1G06490_attB1r_1R	ggggactgctttttgtacaaactgtCATAATAGTGATCAATTTCAAATTC	AT1G06490			
pAT5G04890_attB4_1F	ggggacaactttgtatagaaaagttgtccagtttcaactccgttttg	AT5G04890	RTM2		promoter
pAT5G04890_attB1r_1R	ggggactgctttttgtacaaactgtCATtatttaattacttcttctctc	AT5G04890			
pAT3G49380_attB4_1F	ggggacaactttgtatagaaaagttgtTGATGAGAAATTTATCTGTTTTGGA	AT3G49380	IQD15		promoter
pAT3G49380_attB1r_1R	ggggactgctttttgtacaaactgtCAAGATCGATCAACCTCGTCT	AT3G49380			
oligos with attB1/2 extensions					
oligo_name	sequence	gene	name	target	
pAT4G28500_attB1_1F	ggggacaagttgtacaaaaagcaggcttcgaccaccaatgaaaaca	AT4G28500	SND2		promoter
pAT4G28500_attB2_1R	ggggaccactttgtacaagaagctgggtCATgtttgtgtccctaagtt	AT4G28500			
pAT3G06172_attB1_1F	ggggacaagttgtacaaaaagcaggctcagctggagaacttacaatacaaa	AT3G06172	SEOR2		promoter
pAT3G06173_attB2_1R	ggggaccactttgtacaagaagctgggtTTGAAAGCGTTGGCCAT	AT3G06173			
pAT3G20570_attB1_1F	ggggacaagttgtacaaaaagcaggctTctcagattagttggcctttt	AT3G20570	ENODL9		promoter
pAT3G20570_attB2_1R	ggggaccactttgtacaagaagctgggtGTGTAGGAATAGAGTGGAGCTAGA	AT3G20570			