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Architecture and permeability of post-cytokinesis plasmodesmata lacking cytoplasmic sleeves

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34 Plasmodesmata are remarkable cellular machines responsible for the controlled
35 exchange of proteins, small RNAs and signalling molecules between cells. They are lined
36 by the plasma membrane (PM), contain a strand of tubular endoplasmic reticulum (ER),
37 and the space between these two membranes is thought to control plasmodesmata
38 permeability.

39 Here, we have reconstructed plasmodesmata 3D ultrastructure with an unprecedented
40 level of 3D information using electron tomography. We show that within
41 plasmodesmata, ER-PM contact sites undergo substantial remodelling events during cell
42 differentiation. Instead of being open pores, post-cytokinesis plasmodesmata present
43 such intimate ER-PM contact along the entire length of the pores, that no inter-
44 membrane gap is visible. Later on, during cell expansion, the plasmodesmata pore
45 widens and the two membranes separate, leaving a cytosolic sleeve spanned by tethers
46 whose presence correlates with the appearance of the intermembrane gap. Surprisingly,
47 the post-cytokinesis plasmodesmata allow diffusion of macromolecules despite the
48 apparent lack of an open cytoplasmic sleeve, forcing the reassessment of the
49 mechanisms that control plant cell-cell communication.

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60 INTRODUCTION

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62 Plasmodesmata are membrane-lined channels that cross the plant cell wall and
63 allow the exchange of molecules between virtually all plant cells. Plasmodesmata are
64 required for coordinated plant growth and development, plant defence signalling¹⁻¹¹
65 and are also exploited by viruses to spread from cell-to-cell and systemically throughout
66 the plant^{12,13}.

67 Plasmodesmata are characterised by the apposition of two membranes: the plasma-
68 membrane (PM) which lines the plasmodesmal pore and a strand of tubular
69 endoplasmic reticulum (ER), which is tightly constricted into a rod-like structure known
70 as the desmotubule¹⁴⁻¹⁶. These two plasmodesmal membrane compartments are highly
71 specialised and contain specific sets of proteins and lipids, both of which are critical for
72 proper function^{7,8,17-22}. Inherent to their structure, plasmodesmata constitute a
73 specialised type of membrane contact site (MCS), a general term describing areas of
74 close (10-30 nm) apposition between two membranes²³⁻²⁵. In yeast and human cells
75 MCS are well established sites for inter-organelle signalling, non-vesicular lipid
76 exchange and calcium homeostasis²⁶⁻²⁹. In plasmodesmata, the function of ER-PM
77 contacts remains an enigma³⁰ and signalling between the two membranes is still
78 speculative. In current models however, the gap between the two membranes, the
79 cytoplasmic sleeve, defines the space available for molecular trafficking, governing the
80 size exclusion limit (SEL) of the pores. Plasmodesmata symplastic connectivity is
81 strongly regulated in space and time. Their SEL can be modulated in response to
82 biotic/abiotic stresses but also varies depending on the cell type and stage of tissue
83 differentiation^{7-9,31-36}. In any cases, the structural plasticity of plasmodesmata is
84 assumed to be critical to adjust symplastic connectivity through the regulation of ER-PM
85 spacing^{2,36,37}. However, how plasmodesmata channels are built and organised within the
86 narrow space between the ER and the PM, and how ER-PM spacing affects cell-to-cell
87 connectivity remains little understood. While spectacular advances have been made
88 over the last decade in imaging supramolecular structures such as the nuclear pore
89 complex³⁸, we currently have no data on the 3D structure of plasmodesmata in higher
90 plants. Past studies have greatly contributed to models of plasmodesmata
91 ultrastructure^{14,39,40} but they were based on 2D transmission electron micrographs
92 where no depth (z-axis) and no information on the true 3D organisation of membrane

93 contacts within plasmodesmata was available. Yet, in depth understanding of
94 plasmodesmata architecture and how it relates to intercellular connectivity is critical to
95 understand their mechanisms of action.

96 Here we used electron tomography to gain access to the ultrastructure of
97 plasmodesmata with an unprecedented level of 3D information and shed light on the
98 structural plasticity of their ER-PM junctions. By acquiring multiple snapshots of
99 plasmodesmata at different stages we reconstructed the structural dynamics of their
100 architecture from their biogenesis to later maturation events. We unexpectedly show
101 that within the plasmodesmal pores, ER-PM contacts undergo extensive remodelling,
102 which varies from very tight contacts to intermembrane gaps of about 10 nm, spanned
103 by spokes. Differences in ER-PM connections set apart two plasmodesmata
104 morphotypes, which occurrence correlates with tissue growth and differentiation. Type
105 II display archetypal organisation, with a cytoplasmic sleeve spanned by spoke-elements,
106 and correspond to “mature” plasmodesmata. Contrary to the textbook model, we show
107 that these “spokes” are insensitive to F-actin polymerization inhibitor drugs, suggesting
108 they may not be related to the cytoskeleton. In addition to the archetypal
109 plasmodesmata, we observed a second morphotype (Type I), which occurs in post-
110 cytokinesis walls and unexpectedly presents such a tight contact between the ER and the
111 PM that no visible intermembrane space remains. Despite the lack of visible cytoplasmic
112 sleeve, these plasmodesmata are surprisingly capable of non-targeted movement of
113 macromolecules such as GFP. Transition from Type I to Type II plasmodesmata is
114 correlated with cell differentiation and tissue growth. Based on our data we propose
115 that membrane-tethering elements control plasmodesmata MCS maturation and define
116 different functional states of the plasmodesmata channels.

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125 **RESULTS**

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127 **ER-PM spacing within plasmodesmata is regulated during tissue differentiation in**
128 **root tips.**

129 To analyse the ultrastructural organisation of plasmodesmata during tissue
130 differentiation, we first focused on Arabidopsis root tip columella (COL), a tissue
131 involved in gravitropism and soil excavation⁴¹⁻⁴³. COL cells offered excellent
132 plasmodesmata preservation after cryo-fixation and freeze-substitution but also
133 unequivocal traceability of cell lineage where plasmodesmata modification from early
134 formation to later maturation stages can be easily traced. The columella is organised
135 into several cell layers (Fig. 1a); the mitotically active COL cell initials (CCI), situated
136 immediately below the root quiescent center (QC), which divide periclinally providing
137 primary plasmodesmata on their division walls and supplying new COL cell layers⁴⁴
138 (identified as C1 for the inner-most layer, followed by C2, C3 etc. where the outer-most
139 layer (C0) ultimately sloughs off⁴⁵). Unlike the CCI, the COL cells from C1 to C0 are
140 unable to divide and undergo drastic cell elongation. This tissue therefore offers an
141 excellent framework to track down potential modification in plasmodesmata
142 architecture during cell differentiation.

143 Root tissues from one-week-old seedlings were high-pressure-frozen and freeze
144 substituted to stay as close as possible to native conditions, then processed for electron
145 tomography. The outermost cell layers of the COL (from C2 to C4) featured
146 plasmodesmata with an archetypal ultrastructural organisation (n = 15; Fig. 1d). All
147 presented a central desmotubule visible as an electron-opaque rod and an electron-
148 lucent cytoplasmic sleeve spanned by multiple spoke-like tethers connecting the
149 desmotubule to the PM (Fig. 1d, yellow arrows). These plasmodesmata had an average
150 diameter (PM-PM; inner leaflets) of 39.3 ± 9.8 nm, while the desmotubule had a
151 diameter of 19.15 ± 2.5 nm, and they traversed thick cell walls (Fig. 1e,f) and could
152 display branched morphology (data not shown). On the opposite boundary, at the CCI
153 interfaces, newly established plasmodesmata were of drastically different appearance.
154 They presented a grainy electron dense interior with surprisingly no detailed internal
155 features and no visible cytoplasmic sleeve (n = 20; Fig. 1b). They traversed the very thin
156 CCI walls (79.1 ± 44.5 nm; Fig. 1e) and their average diameter (PM-PM; inner leaflets)
157 was significantly smaller (23.2 ± 5.4 nm) than plasmodesmata encountered in

158 differentiated COL cells (Fig. 1f). Although not readily visible, nearly all plasmodesmata
159 observed (n = 18 out of 20) presented cortical ER entering the pores in the form of an
160 electron-dense rod suggesting that despite their tiny size they contained a desmotubule
161 (Fig. 1b, black arrowheads). When comparing the size of the desmotubule in archetypal
162 COL plasmodesmata, to the diameter of the CCI pores, we found no significant difference
163 (Fig. 1f), suggesting that these non-canonical plasmodesmata presented an
164 unconventional and underrated organisation with a very close ER-PM apposition within
165 the entire length of the pores. For clarity, we called plasmodesmata with no visible
166 cytoplasmic sleeve Type I (CCI) and Type II when both the ER-PM electron-lucent gap
167 and the spoke elements were present (from C2 to C4). A transitional stage, between
168 Type I and Type II, was visible at the C1 cell layer (Fig. 1c). While Type I plasmodesmata
169 predominate at the CCI/C1 cell interface, the opposite transverse cell wall (the C1-C2
170 interface) displayed plasmodesmata with intermediate structural organisation and a
171 partially “opened” cytoplasmic sleeve (n = 15). At this stage however, spoke-like tethers
172 were difficult to distinguish.

173 Altogether our results indicate that ER-PM contacts within plasmodesmata may be
174 differentially regulated during tissue differentiation in COL cells and reveal unexpected
175 ultrastructural organisation of plasmodesmata in the CCI where no visible electron-
176 lucent cytoplasmic sleeve remains between the two tightly apposed membranes.

177

178 **Type II plasmodesmata membrane tethers control ER-PM gaps and are actin-** 179 **independent.**

180 The archetypal Type II plasmodesmata are characterised by the presence of
181 spoke-like tethers that appeared as fine, electron-dense strands bridging the
182 membranes across the cytoplasmic sleeve (Fig. 2a-c). In COL cells, the spokes were
183 numerous and regularly spaced, with an average of 8 ± 3 nm (n= 8 plasmodesmata
184 tomograms, 112 tethering elements measured) between the tethers and often the
185 position of the tethers on opposite sides of the desmotubule matched up (Fig. 2a,b
186 yellow arrow). Their length varied from 4 nm to 20 nm with a mean value of 9.7 ± 3.3
187 nm (Fig. 2d). Most tethers consisted of single, unbranched filaments, but V- and Y-
188 shaped tethers were occasionally observed, in which case the two branches could either
189 connect to the PM or to the desmotubule (Fig. 2e). Spokes of similar appearance and

190 length were also observed in Arabidopsis cultured cell plasmodesmata (Fig.2d; see also
191 Fig. 3b).

192 The spoke appearance was concomitant with the transition from Type I to Type II,
193 suggesting that these structures may be involved in controlling the spacing between the
194 desmotubule and the PM. In root COL cells but also in Arabidopsis cultured cells,
195 transitioning or Type II plasmodesmata sometimes presented subsections of the
196 cytoplasmic sleeve gap devoid of spokes (Suppl. Fig. 1 and Fig. 3b) that seemed larger
197 and looser when compared to cytoplasmic sleeve gaps spanned by spokes. To quantify
198 this difference, we measured the cytoplasmic sleeve gaps in subsections of Type II pores
199 harbouring cytoplasmic sleeve gaps spanned by or devoid of spoke-like tethers. The
200 distance between the two membranes was maintained at around 9.6 ± 2.44 nm when
201 the spokes were present whereas this distance nearly doubled, and was more variable,
202 when the spokes were absent 18.38 ± 7.26 nm (Fig. 2f; see also similar results for
203 cultured cells). Our data therefore suggests that there is a direct correlation between the
204 presence of spoke-like tethers and the control of ER-PM spacing.

205 In conventional models of plasmodesmata, the spokes are often depicted as
206 myosin molecules that would tether the two membranes by binding with F-actin
207 imbedded in the desmotubule⁴⁶⁻⁵¹. This model was built upon immunological data⁵²⁻⁵⁴
208 and functional cell-to-cell communication tests^{49,55}. If true, disturbing the delicate
209 synthesis/degradation balance of F-actin homeostasis could affect plasmodesmata
210 structure and more especially the spokes elements. In this regard, Arabidopsis roots
211 were treated with drugs altering F-actin polymerization (Latrunculin B and Cytochalasin
212 D) and processed for tomography. Efficiency of the treatments was checked using the
213 actin markers fimbrin actin binding domain 2-green fluorescent protein (35S::GFP-
214 fABD2-GFP)⁵⁶. We also used the Golgi markers, N-acetylglucosamine1 (NAG1)⁵⁷ and the
215 SNARE protein MEMBRIN12 (MEMB12)⁵⁸ to test their mobility after drug treatment as
216 the cytosolic streaming of Golgi vesicles is known to heavily rely on the actin network⁵⁹
217 (Supplementary movie 2). Despite alteration of the actin filament network (Fig. 2g-i),
218 the spokes were still observed spanning the cytoplasmic sleeve (Fig. 2j,k) and we saw no
219 significant differences in the pore dimensions or in the length distribution of the spoke-
220 elements (Fig. 2d,l).

221 We therefore concluded that the spokes may control ER-PM spacing within the
222 plasmodesmata pores but their nature remains unclear. The fact that treatments with F-

223 actin polymerization inhibitors did not affect plasmodesmal ultrastructure suggests the
224 spokes could be stable actin elements or not cytoskeleton related¹⁵.

225

226 **Post-cytokinesis plasmodesmata display very close appositions between the** 227 **desmotubule and the PM**

228 A surprising outcome of Type I plasmodesmata organisation in the root CCI was
229 the absence of a visible cytoplasmic sleeve. These plasmodesmata were only present in
230 the mitotically active CCI and transition to Type II occurred rapidly across a single cell
231 interface. To get more information about these non-canonical plasmodesmata, we next
232 turned to Arabidopsis liquid cultured cells which are actively dividing, contain primary
233 plasmodesmata⁶⁰ (Suppl. Fig. 3) and do not undergo cell differentiation.

234 An initial survey revealed the presence of both Type I and II plasmodesmata (Fig.
235 3a-b). Similar to the CCI, Type I plasmodesmata had a grainy appearance as if the pores
236 were filled with electron dense material throughout the entire channel, with no
237 cytoplasmic sleeve visible nor apparent sub-elements (Fig. 3a). Close examination of
238 tomograms revealed the presence of ER membranes entering the pores (15 out of 17),
239 supporting the presence of a desmotubule (Fig. 3c, yellow arrow; Supplementary movie
240 4). Type II plasmodesmata in cultured cells displayed a visible cytoplasmic sleeve (Fig.
241 3b) but in contrast with COL plasmodesmata, it was only occasionally spanned by spoke-
242 like elements, and often presented subsections with direct contact between the
243 desmotubule and the PM (Suppl. Fig. 5; Supplementary movies 6-7). Similar to COL cells,
244 Type I plasmodesmata also had a significantly smaller diameter than Type II (23 ± 2.6
245 nm versus 37 ± 7.2 nm, respectively) (Fig. 3d). They also displayed a remarkably
246 constant diameter (PM-PM; inner leaflets) along their entire length, which never varied
247 by more than 5 nm. This contrasted with Type II, whose diameter could range from 25
248 nm to more than 40 nm within a single channel (Fig. 3e). Altogether our data support
249 the view that Type I plasmodesmata present a desmotubule tightly apposed against the
250 PM along the entire length of the pores and that these non-canonical plasmodesmata are
251 not unique to CCI cells.

252 We next investigated whether Type I and Type II plasmodesmata in cultured cells
253 were in open or closed configurations, or whether these morphotypes corresponded to
254 two populations with distinct internal features. In order to visualise dynamics of
255 plasmodesmata ultrastructure during tissue growth, we screened Arabidopsis cultured

256 cells at four, six and thirteen days after sub-culturing. At four days old, when the cells
257 were at the beginning of the linear growth phase, we observed a majority of
258 plasmodesmata with “opaque” appearance (77% against 23%), similar to CCI
259 plasmodesmata. With cell ageing, the relative proportion of Type I and Type II was
260 reversed and the majority of the pores had apparent cytoplasmic sleeves (72% of Type
261 II at six-day-old) (Fig.3 f-g). This transition happened relatively quickly, between days
262 four and six of cell culture. Consistent with these data, quantitative analyses showed that
263 Type I plasmodesmata were preferentially associated with thin (101 ± 48 nm),
264 presumably newly formed cell walls, whereas Type II appeared in thicker (202 ± 78 nm),
265 presumably older, cell walls (Fig. 3h).

266 Our data suggest that Type I and Type II plasmodesmata correspond to two
267 distinct morphotypes whose appearance is not only correlated with tissue
268 differentiation but also cell ageing and/or cell wall modification. Type I occur in post-
269 cytokinesis plasmodesmata and display unconventional structural features
270 characterised by intimate membrane contact between the desmotubule and the PM
271 along the entire length of the channels.

272

273 **ER-PM contacts are likely to be established during cell plate biogenesis and may**
274 **lead to Type I plasmodesmata.**

275 Our data suggest that newly formed plasmodesmata can exhibit very close ER-PM
276 appositions but it is not clear whether such intimate membrane contacts arise during
277 the pore formation or are established post-cytokinetically. We therefore captured
278 plasmodesmata biogenesis events in meristematic epidermal root cells (Fig. 4). As
279 reported in previous studies^{61,62} the earliest traceable event of plasmodesmata
280 formation corresponded to ER membranes perforating the cell plate during the planar
281 fenestrated sheet stage (Fig. 4a-f). At this early stage, the gaps within the fenestrated
282 membrane network were still large (57.3 ± 19.7 nm, n = 8) compared to plasmodesmata
283 diameter and the traversing ER strands were non-constricted with apparent lumen
284 (23.5 ± 4.8 nm, n = 8). However, regardless of the non-constricted appearance of the ER,
285 intimate ER-PM contacts were already evident at this stage (Fig. 4d-f, red arrows). As the
286 gaps within the fenestrated sheet became narrower, the ER bilayers appressed and tight
287 ER-PM contacts on both sides of the ER were more prominent (Fig. 4g-l).

288

289 Our observations are consistent with an ER-PM attachment occurring in early
290 stages of plasmodesmata biogenesis, before fenestrae closure and ER “entrapment”.

291

292 **Type I plasmodesmata with no visible cytoplasmic sleeve allows non-selective**
293 **molecule diffusion.**

294 Current models for cell-to-cell trafficking postulate that there is a direct link
295 between ER-PM spacing and plasmodesmata permeability^{2,36,37,49}. Surprisingly, our data
296 revealed that newly formed plasmodesmata (Type I) are narrow with no apparent
297 cytoplasmic sleeve. We therefore evaluated their permeability.

298 First, we focused on the CCI/C1 interface and used fluorescence recovery after
299 photobleaching (FRAP) after loading the cells with carboxyfluorescein diacetate (CFDA),
300 a membrane permeant fluorophore that is cleaved by intracellular esterases, yielding a
301 membrane impermeant form of the probe⁶³ (Fig. 5a-c). We bleached a row of cells
302 including the CCI (see Fig. 5a), the QC and adjacent lateral root cap cells to minimize
303 recovery from lateral cells and isolate the CCI-C1 interface. After photobleaching we
304 observed a fast recovery within the CCI suggesting that the probe was able to rapidly
305 diffuse (half time recovery was of 9.7 seconds; n = 10), through the CCI/C1 interface,
306 which contain only Type I plasmodesmata (Fig. 5c).

307 As CFDA has an estimated Stokes radius of only 0.61 nm⁶⁴, we next investigated
308 the cell-to-cell diffusion of GFP, which has a Stokes radius of 2.82 nm⁶⁵. To do so, we
309 used Arabidopsis plants expressing GFP under the control of the phloem specific
310 promoter SUC2⁶⁶. In such lines, GFP expression driven by the SUC2 promoter serves as a
311 marker for non-targeted macromolecular movement^{9,11} as it is expressed in the phloem
312 companion cells (Fig. 5d, green) and then diffuses in the neighbouring tissues when
313 plasmodesmal permeability allows it. GFP fluorescence measurements show that GFP
314 was able to diffuse into the root tip including the CCI cells that are exclusively
315 surrounded by Type I plasmodesmata (Fig. 5e,f). Additionally, we measured a gradual
316 decrease of fluorescence from the CCI to the outermost columella tiers (2 fold reduction;
317 Fig. 5f), where only Type II plasmodesmata are found.

318 We could also observe that the transversal walls of root epidermal cells in the
319 meristematic and division zone exclusively harboured Type I plasmodesmata (Fig.5g; n
320 = 12). To confirm whether or not macromolecules can diffuse through Type I
321 plasmodesmata, we used photo-activable GFP (35S::PA-GFP) and specifically activated

322 the GFP in one cell and monitored its spread in neighbouring cells. Within few minutes
323 cytoplasmic GFP fluorescence was apparent in neighbouring epidermal cells, indicating
324 symplastic movement across the apico-basal walls.

325 Our results demonstrate that although Type I plasmodesmata have no apparent
326 cytoplasmic sleeve, they can nevertheless allow cell-to-cell movement of
327 micromolecules and macromolecules.

328 **DISCUSSION**

329 The structural analysis of plasmodesmata channels is a formidable challenge
330 given their nanoscopic size, location in the cell wall and dynamic nature. Using electron
331 tomography of high pressure-frozen, near-native plasmodesmata, we resolved their
332 ultrastructure at an unprecedented level of 3D detail. Our results revealed unforeseen
333 architectural changes during cellular differentiation and tissue growth, with
334 considerable modification in the internal organisation of these specialised membrane
335 junctions. Based on our observation, we propose a model where archetypal
336 plasmodesmata (Type II), harbouring a cytoplasmic sleeve and spoke-elements, derive
337 from the unconventional Type I plasmodesmata established during cell plate formation.
338 Contrarily to the archetypal model, Type I plasmodesmata present a remarkably close
339 apposition between the desmotubule and the PM to such extent that no visible
340 intermembrane space remains. Type I plasmodesmata we observed resemble, in their
341 ultrastructure and size, pores previously reported in various species such as *N.*
342 *tabaccum*, *A. pinnata* and *B. oleracea*, suggesting these may in fact be common
343 structures^{14,39,67}. Such proximity between membranes is uncommon amongst MCS
344 structures, where the intermembrane spacing usually varies from 10 to 30 nm^{23,25,68} and
345 most likely membrane-bridging complexes are required to maintain this minimum gap⁶⁹.
346 Based on our measurements we propose that a ~2-3 nm wide electron-dense protein
347 meshwork, associated with the desmotubule/PM interfaces, stabilises the contact and
348 perhaps prevents the membranes from repelling each other as such an intimate
349 membrane apposition is thermodynamically unstable⁶⁹. Why are the two membranes in
350 such close contact in Type I plasmodesmata remains to be elucidated. One possibility is
351 that it could favour direct and rapid exchanges of molecules for the establishment of
352 specialised membrane domains during early stages of plasmodesmata formation. We
353 observed Type I plasmodesmata in young walls of Arabidopsis cultured cells, CCI and
354 epidermal root cells, and we propose that these may actually be the predominant
355 morphotype in young/meristematic tissues. As a matter of fact when going back to
356 previous TEM reports on plasmodesmata structure, pores that resemble Type I
357 plasmodesmata have been observed, very often in similar young, meristematic, sink
358 tissues where walls are more likely to be newly divided^{14,34,39,40,52,70,71}. Only later in cell
359 development, the gap between the ER and the PM enlarges to form a cytoplasmic sleeve
360 leading to the archetypal Type II plasmodesmata. As seen in the root, this “opening” of

361 the sleeve can occur quickly, on the opposite cell walls of a single cell. Our model depicts
362 a desmotubule detaching from the PM, first in limited areas and then along the entire
363 length of the channel. In advanced stages of plasmodesmata maturation, the
364 desmotubule is centrally positioned and spokes are numerous and regularly spaced.
365 These correspond to the canonical model of plasmodesmata ultrastructural organisation
366 and have been observed many times in previous TEM studies although not with that
367 level of detail^{14,36,39,40,72,73}.

368 A great variety of MCS exist in yeast, animal and plant cells, many of which have
369 been shown to be controlled by tethering proteins or protein complexes⁷⁴ that can
370 appear as electron-dense filament on electron micrographs⁶⁸. These tethering elements
371 physically and functionally connect the two opposing membranes. Tether-like structures
372 are clearly visible in Type II plasmodesmata in the form of spoke-like elements, of about
373 9 nm in length, contacting the desmotubule and the PM. Their appearance coincides with
374 the opening of the cytoplasmic sleeve and the adjustment of the intermembrane gap.
375 This indicates the plasmodesmata tethers/spokes are likely to control ER-to-PM spacing
376 throughout the pore maturation. At this stage it is not clear whether these spokes are
377 already present at early stages of plasmodesmata formation, then unfolded. These
378 plasmodesmata spokes have been observed before by classic TEM and have been
379 thought to be F-actin-associated proteins such as myosin VIII⁴⁶⁻⁵⁴. However, our data
380 suggest that membrane tethering at Type II plasmodesmata is not sensitive to
381 destabilization by F-actin polymerization inhibitor drugs. We nevertheless cannot
382 completely rule out that actin in plasmodesmata is unattainable by the drugs.
383 Latrunculin B and Cytochalasin D are 0.4 and 0.5 kDa respectively and most certainly
384 pass through plasmodesmata as compounds with similar molecular weights, such as
385 carboxyfluorescein, do⁷⁵. This suggests that the drugs can indeed physically pass
386 through the pores and reach putative F-actin. However, plasmodesmata may contain
387 very stable actin-associated structures that are not affected by the inhibitors of actin
388 polymerization in our experimental conditions. Nevertheless, our results corroborate
389 the alternative model introduced in 2011 by Tilsner et al. ¹⁵ questioning the presence of
390 F-actin within the plasmodesmal pores due to sterical constraints and conveying a
391 model where spokes are cytoskeleton-independent molecules. In any case, the identity
392 of these plasmodesmata MCS tethers currently remains unanswered.

393 Unexpectedly, our data revealed that narrow, newly formed Type I
394 plasmodesmata with no apparent cytoplasmic sleeve nonetheless enable fast small
395 molecule diffusion and even non-selective macromolecule trafficking between cells
396 which appears counterintuitive based on their morphology. These results are however
397 consistent with previous data showing that i) young and meristematic sink tissues with
398 newly divided cell walls harbour plasmodesmata reminiscent of the Type I
399 morphology^{14,34,39,40,70,71,76}, and ii) Such young, sink or meristematic tissues often display
400 higher symplastic connectivity than more mature (source tissues), allowing for instance
401 free GFP movement from cell-to-cell, whereas source tissues do not^{34,35,66,77,78}. This
402 raises the question of how macromolecules can move through Type I plasmodesmata
403 with such tight cytoplasmic sleeves. Regardless of the answer, our work forces a re-
404 consideration of how trafficking is achieved in newly formed Type I plasmodesmata.

405 Altogether, our findings show that ER-PM contacts within plasmodesmata are
406 dynamic and differentially regulated during tissue development and populated by either
407 different tethering molecules and/or different tether conformations at various stages of
408 plasmodesmata maturation. From our data it is clear the PM and desmotubule are
409 always in intimate connection within the pores either through very tight membrane
410 contacts or through spoke-like tethering elements. Similar to other MCS, the function of
411 ER-PM may primarily be the exchange of molecules between the two organelles^{23,24,30},
412 which would then affect plasmodesmata function. In this context, the strikingly different
413 ER-PM connections in Type I and Type II plasmodesmata may have profound
414 implications for intermembrane exchanges and the regulation of plant cell-cell
415 communication.

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426 **MATERIAL & METHODS**

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428 **Biological material and growth conditions**

429 Six day-old *Arabidopsis (Columbia)* root tips were grown vertically under greenhouse
430 conditions on solid medium composed of *Murashige and Skoog* (MS) medium including
431 vitamins (2.15g/L), MES (0.5g/L) and plant-Agar (7g/L), pH 5.7. Growth conditions
432 were set at 22°C in a greenhouse with a long day 16h photoperiod (100µE/m/s).
433 *Arabidopsis (Landsberg erecta)* culture cells were cultivated as described in ²² under
434 constant light (20µE/m/s) at 22°C. Cells were used for experimentation at various ages
435 ranging from four to thirteen-day-old (always mentioned in experiments).

436 For the establishment of a growth curve 5 independent liquid cultures were grown in
437 the same conditions. 2mL of culture was sampled every day and the fresh weight was
438 monitored (without the growth medium).

439

440 **Cryofixation and freeze-substitution**

441 200µm deep and 1.5 mm wide copper platelets were rapidly filled with either fresh
442 cultured cell clusters or sectioned seedling root tips (approximately 1 mm in length).
443 These platelets are beforehand coated with 1% phosphatidyl-choline, hexadecene and
444 the bottom is covered with a 50 µm thick aclar disk. Additional BSA 20% was also added
445 in the platelet as a cryoprotectant filler. The prepared platelets containing the samples
446 were then frozen with an EMPACT1 high pressure freezer (*Leica*, Vienna, Austria). The
447 platelets were then transferred at -90°C into an AFS 2 freeze-substitution machine
448 (*Leica*, Vienna, Austria) and incubated into a cryosubstitution mix: glutaraldehyde 0.5%,
449 osmium tetroxide 2%, uranyl acetate 0.1% and water 1% in pure acetone. The
450 incubation is only carried out at very low temperature (-90°C) for 48h. Then a
451 progressive raise of the temperature of 3°C/h is initiated until -50°C is reached. The
452 cryofixation mix is then carefully and thoroughly removed by 3 consecutive pure
453 acetone washes followed by 3 pure ethanol washes. This very low temperature staining
454 procedure produces a fine membrane staining allowing an improved contrast and
455 resolution, suitable for the observation of nanometric details in electron tomography. To
456 improve embedding, the samples were then carefully removed from the copper platelets
457 and consecutively incubated in HM20 *Lowicryl* resin (*Electron Microscopy Science, EMS*)
458 solutions of increasing concentration (dilutions done in pure ethanol): 25% and 50% (2

459 hours each), 75% (overnight), 100% (twice for 2 hours) and a final 4 day incubation in
460 HM20 100% under UV light. The two first days of incubation were done at -50°C then
461 temperature was quickly risen up to +20°C for the last two days. The use of such an
462 electron lucent resin allowed us to reduce electronic scattering (hence noise) caused by
463 resin-electron interaction, thus improving the x, y, z resolution.

464

465 **Electron microscopy acquisitions**

466 Cylindrical moulds (*Leica*, Vienna, Austria) were used to produce the blocks primarily
467 because they made the production of longitudinal root sections easier.
468 Sections were collected with an *Ultracut S* (*Leica*, Vienna, Austria). The sections used
469 ranged from 90 nm to 180 nm depending on the volume to acquire. To prevent grid bars
470 from blocking the image, 2x1mm oval slot grids filmed with formvar and carbon coated
471 (*Electron Microscopy Science, EMS*) were used for section collection. Prior to observation,
472 the grids are coated on both sides with 5 nm gold fiducials (essentially old immunogold
473 secondary antibodies at a dilution ranging from 1/20 to 1/100 or a 1:1 mixture of 0.5%
474 BSA and 5 nm colloidal gold solution from *BBI solution EM-GC5*) for the subsequent
475 alignment step.

476 Observations were carried out on a FEI TECNAI Spirit 120kV electron microscope
477 equipped with a -70 to +70° tilting goniometer. A tomography optimised single tilt
478 specimen holder (*Fichione instruments, model-2020*) was also used to improve the tilting
479 range. The tilt series of longitudinal views of plasmodesmata were acquired at
480 magnifications ranging from x30,000 to x56,000, with images taken each degree. The
481 *batch mode* special feature of the FEI 3D-explore tomography software allowed us to
482 designate objects of interests, which were then acquired *via* an automated tilt series
483 data collection process. This technique improved the throughput of our electron
484 tomography workflow allowing us to acquire up to 10 tilt series overnight.

485

486 **Tomogram reconstruction**

487 The raw 4k by 4k pixels tilt series collected need to be aligned before reconstruction.
488 For this manner, two strategies were used : fiducial-less alignment with TomoJ, an
489 ImageJ plug-in^{79,80} and fiducial alignment with eTomo, a graphic interface allowing the
490 use of the IMOD tilt series processing package Etomo
491 (<http://bio3d.colorado.edu/imod/>). With Etomo, 20 to 30 fiducials in the field of view

492 were used to correctly align all images. Good tilt series usually yielded errors of ≈ 1.2
493 pixels and below. Aligned stacks were binned two times before reconstruction to make
494 data handling easier. Reconstruction was performed either by using the weighed back-
495 projection or the SIRT algorithm (15 to 20 iterations with default parameters) of Etomo
496 or the OSART iterative algorithm of TomoJ (100 iterations, 0.01 as relaxation coefficient,
497 update every 5 images). Optional 2D filtering of the aligned stack performed prior to
498 reconstruction with the default parameters of Etomo (0.35 cut-off and 0.05 sigma role-
499 off) efficiently filtered the highest frequencies, rendering less noisy tomograms when
500 needed. This extra filtering step allowed the SIRT algorithm of Etomo to yield more
501 contrasted tomograms.

502 Combination of tomograms in the case of dual-tilt axis tomography was performed using
503 Etomo with default parameters. Both single tilt tomograms were generated as described
504 above. The cell wall region was eventually excluded from the processing as it increased
505 the correlation error scores because of its lack of electron-dense features necessary for
506 the patch correlation step.

507

508 **Image segmentation and tomogram analysis**

509 Manual segmentation of tomograms was performed with 3dmod and allowed us to
510 visualise and appreciate the organisation of the sub-elements in a 3D space. Additionally,
511 an efficient way of segmenting structures was to accurately outline their main contours
512 throughout the volume using the interpolation tool (*drawing tools* IMOD package
513 developed by Andrew Noske) and then generate an isosurface of the structure.

514 Systematic measurements were taken on all relevant raw tomograms (without filters
515 applied to minimize measurement errors), comprising width of the desmotubule,
516 plasmodesmata channel, cell wall thickness at the pores, length of the spokes if visible
517 etc. Pore width, cytoplasmic sleeve space and spoke length were measured relative to
518 the inner leaflets of the PM (facing the symplasm). The desmotubule was always
519 measured in sections where it was clearly distinguishable and at its largest. Inter-spoke
520 spacing, shape of spokes were also accounted for in relevant tomograms where spokes
521 were clearly distinguishable.

522

523

524

525 **TEM plasmodesmata Type I and II screening method**

526 For Fig. 3 panels h and g, fractions of Type I and type II plasmodesmata were assessed
527 by TEM in order to raise statistically significant numbers. This was possible because
528 Type I and Type II plasmodesmata could be discriminated by classic TEM (Suppl. Fig. 8).
529 To avoid potential sampling artefacts, a stereology based approach inspired from the
530 dissector method^{81,82} was used. Two to three biological replicates were used for
531 plasmodesmata screening in culture cells for each time point.

532 Counting was done on 90 nm serial sections. Preliminary measures showed that only
533 20% of the plasmodesmata spotted on a reference section n could be followed on the
534 contiguous lookup section $n+1$, meaning that 80% of the plasmodesmata spotted on a
535 single section were “resolved”. The counting was therefore performed on sections n and
536 $n+2$ to avoid any kind of double counting therefore giving more weight to larger pores.
537 Plasmodesmata were considered resolved when the PM bilayers were clearly
538 distinguishable.

539

540 **Drug treatment**

541 10mM/DMSO stock solutions of actin destabilizing agents latrunculin B (*Calbiochem*,
542 1mg) and cytochalasin D (*Sigma-aldrich*, 1mg) were used at a working concentration of
543 50 μ m (dilution in liquid MS medium) for 1h30min. Controls contained diluted DMSO
544 only.

545

546 **Permeability measurements**

547 *FRAP/CFDA*: Plasmodesmal permeability assessments were made using FRAP on six
548 day-old Arabidopsis root tips co-stained with CFDA (50 μ g/mL) and Propidium iodide.
549 Roots were incubated in an aqueous CFDA solution for 5 minutes, then successively
550 washed out in 3 water baths and mounted with propidium iodide in water for imaging.
551 Acquisitions were made on a Zeiss LSM 880 equipped with an Argon laser (excitation
552 488 nm) and a 40x apochromate 1.30 oil-objective. In order to optimize the frame rate,
553 cropping of the scanned area was done very consciously by limiting the height of the
554 scanned area as much as possible and enlarging it in order to decrease the scanning time
555 (\approx 90 ms at max scanning speed) while having access to the background, and
556 neighbouring cells relative to the photobleached region. To assess the permeability
557 specifically at the CCI-C1 interface, the photobleaching region consisted in a rectangle

558 encompassing CCI, QC cells and the surrounding initial cells, while recovery was
559 monitored in the CCI only. This allowed us to isolate and measure fluorescence recovery
560 in a unidirectional fashion. The FRAP routine consisted in 10 images pre-bleach at 20%
561 laser power and max scanning speed (reaching ≈ 90 ms per image), ten iterations of
562 photobleaching with 100% of 488 nm laser where pixel dwell time was increased to a
563 value of $\approx 1\mu\text{s}/\text{pixel}$. ≈ 400 post-bleach images were then acquired in order to reach the
564 stationary phase of the fluorescence recovery with the same parameters than the pre-
565 bleach images. The recovery profiles were accounted for noise and then double
566 normalized and set to full scale mode (pre-bleach is set to 1 and first image post-bleach
567 is set to 0) as described by Kote Miura in his online *FRAP-teaching module* (EAMNET-
568 FRAP module, <https://www.embl.de>). Plotting and curve fitting was done using
569 GraphPad Prism (*GraphPad Software, Inc*).

570 *pSUC2::GFP root* : Regions of interest of same dimensions were used to measure the
571 fluorescence intensity in multiple regions of six day-old *pSUC2::GFP* roots (vasculature,
572 CCI and C1-CO columella layers) using confocal microscopy. The fluorescence intensities
573 were then normalized relative to the intensity measured in the root vasculature.
574 Background noise was subtracted using wild type roots. Co-staining with propidium
575 iodide allowed an easy visualization of the cellular organization of the root tip, thus
576 allowing precise fluorescence intensity measurements in the different cells of interest.

577 *Photoactivation in the root epidermis using 35S::PA:GFP lines* : Six days old *35S::PA:GFP*
578 arabidopsis roots were imaged using a Zeiss LSM880 confocal laser scanning
579 microscope with 63x oil lens. Propidium iodide was excited at 488nm with 10% of laser
580 power and fluorescence collected at 590-650 nm. PA-GFP was activated at 405nm with
581 3% of laser power and fluorescence emission collected at 505-550 nm with 10% 488 nm
582 laser power. Photoactivation was done in single epidermal cell of the meristematic zone,
583 where type I plasmodesmata were observed on the transversal walls. Both GFP and
584 propidium iodide fluorescence were acquired every five minutes during 25 minutes.
585 Quantification was done using Zenblue 2012 (Zeiss) in the activated and neighbouring
586 cells (proximal and proximal+1) to assess GFP diffusion through apico-basal walls.
587 Fluorescence intensity in the different cells was expressed as a percentage of total
588 fluorescence (activated, proximal, proximal+1).

589

590

591 **Data availability**

592 The data that support the findings of this study are available from the corresponding
593 author upon request

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- 791

792 **FIGURE LEGENDS**

793

794 **Figure 1. Plasmodesmata ER-PM contact site morphology evolves during tissue**
795 **development in root tips.**

796 (a) Schematic representation of Arabidopsis root tip cellular organisation, in which the
797 colours depict cell lineage. Plasmodesmal ultrastructure was studied in the Columella
798 tiers in light red (yellow boxes), from the C1 to C4 layer and in the COL cell initials in
799 red (CCI, yellow asterisks). (b-d) Tomographic slices of representative plasmodesmata
800 from the CCI (b), C1-C2 (c) and C3-C4 (d) interfaces, respectively 0.56, 0.56 and 0.49 nm
801 thick, and their associated 3D segmentation highlighting the changes in ER-PM
802 architecture within the pores during COL cell differentiation. In CCI, appressed ER is
803 seen entering the plasmodesmal pores (black arrowheads) and fills the entire canal (b).
804 With tissue differentiation the cytoplasmic sleeve becomes gradually visible as the gap
805 between the desmotubule and PM expands and become populated by spoke-like tethers
806 (d, yellow arrows). In the C1/C2 transition zone there is no clearly identifiable spoke-
807 elements in the cytoplasmic sleeve, only amorphous material (c, red). (e) Difference in
808 cell wall thickness in CCI, C1-C2 and C3-C4 cells (Dunn's multiple comparison test ****
809 $P < 0.0001$). $n = 94$ for CCI, $n = 27$ for C1-C2 and $n = 79$ for C3-C4. (f) Average diameter of
810 plasmodesmata (PM-PM, inner leaflets) and desmotubules in CCI, C1-C2 and C3-C4 cells
811 (Dunn's multiple comparison test, * $P < 0.05$, *** $P < 0.001$). $n = 20$, $n = 15$ and $n = 15$
812 plasmodesmata tomograms for CCI, C1-C2 and C3-C4, respectively. CCI: columella cell
813 initials; CS: cytoplasmic sleeve; Dt: desmotubule; ER: endoplasmic reticulum; QC:
814 quiescent centre; PM: plasma membrane.

815

816 **Figure 2. The spoke-like tethering elements of Type II plasmodesmata correlate**
817 **with ER-PM spacing and are not sensitive to F-actin polymerization inhibitor**
818 **drugs**

819 (a-c) 1.24 nm thick tomographic slices depicting the typical and regular arrangement of
820 the spoke-like tethering elements (yellow arrows) in Type II plasmodesmata, in the root
821 columella (C2-C4). (b) Close-up view of the plasmodesma squared in (a). (c) Manual
822 segmentation of (b). (d) Density representation of the spoke length, measured in COL
823 cells and cultured Arabidopsis cells (non-treated, Latrunculin B, LaB and Cytochalasin D,
824 CytD treated for roots and non treated for cultured cells). In either condition, there is a

825 peak density for spokes of about 9 nm in length. (e) Three close-up views of V/Y shaped
826 spokes (red arrowheads) oriented towards the PM (top; middle) and the desmotubule
827 (bottom). Scale bars = 5 nm. (f) Plot representation of the cytoplasmic sleeve gaps
828 (desmotubule – PM distance) measured in subsections of transitioning or Type II pores
829 with or without spokes. Both in roots and cultured cells the presence of spokes stabilizes
830 the width of the intermembrane gap and keeps it at an average of 9.6 ± 2.44 nm whereas
831 without the spokes this gap is 18.38 ± 7.26 nm (Mann-Whitney two tailed test, ****
832 $P < 0.0001$) $n = 31$ and $n = 65$ measurements in cell and root plasmodesmata respectively.
833 (g-l) Type II plasmodesmata in COL cells (C2-C4) are not altered by F-actin-
834 polymerization inhibitor drugs. (g-i) Maximum projections of confocal stacks taken in
835 columellas of *Arabidopsis* marker lines fimbrin actin binding domain 35S::GFP-fABD2-
836 GFP. In control conditions (g), cells show a dense, reticulated actin network, while after
837 1h30 hour treatment with 50 μ M Cytochalasin D (CytD, h) or Latrunculin B (LaB, i), the
838 F-actin network is heavily altered. (j-k) 0.56 nm thick tomographic slices of
839 plasmodesmata acquired in the columella of CytD (j) and LaB (k)-treated roots, showing
840 that the spokes are still present. (l) The diameter (PM-PM; innerleaflets) of the pores
841 and the desmotubule width remain unchanged both treatment (Dunn's multiple
842 comparison test, **** $P < 0.0001$, *** $P < 0.001$) $n = 41$ plasmodesmata tomograms for
843 non-treated (NT) condition, $n = 13$ for LaB and $n = 16$ for CytD treated condition. Dt:
844 desmotubule ; PM : plasma membrane.

845

846 **Figure 3. Very tight ER-PM contact in post-cytokinesis plasmodesmata**

847 (a-b) Consecutive tomographic slices, of respectively 1.24 nm and 0.49 nm in thickness,
848 of a Type I plasmodesma (a) and Type II plasmodesma (b, spokes are indicated by
849 yellow dashes) in *Arabidopsis* cultured cells. (c) Type I plasmodesmata are traversed by
850 the ER which becomes appressed just before entering the pores (yellow arrows). Three
851 0.56 nm thick tomographic slices and the corresponding 3D segmentation. (d)
852 Plasmodesmata diameter (PM-PM; inner leaflets) of Type I (23 ± 2.6 nm) and Type II (37
853 ± 7.2 nm), and the desmotubule measured in Type II plasmodesmata (17 ± 2.4 nm). $n =$
854 17 and 22 tomograms for Type I and Type II plasmodesmata, respectively, and $n = 22$ for
855 desmotubule measurements (**** $P < 0.0001$ by Mann-Whitney test). (e) plasmodesmata
856 width at different points along the pores in Type I and II. Measurements (PM-PM; inner
857 leaflets) were taken at the extremities and largest part of the channels. Type I

858 plasmodesmata have a remarkably constant diameter compared to Type II. $n = 17$ and n
859 $= 22$ tomograms for Type I and Type II plasmodesmata, respectively. **(f)** Growth curve of
860 Arabidopsis liquid cultured cells. Black arrows indicate the cell culture ages used for this
861 study (four-, six- and thirteen-day-old). **(g)** Quantification of the relative proportion of
862 Type I and II plasmodesmata in Arabidopsis cultured cells at four, six and thirteen days
863 ($n = 111, 89$ and 22 screened plasmodesmata for four, six and thirteen days old cells).
864 **(h)** Average cell wall thickness in relation to plasmodesmata Type. In four-day-old
865 cultured cells, Type I plasmodesmata are abundant in thin young cell walls whereas
866 Type II plasmodesmata are preferentially associated with thicker, older walls. $n = 69$
867 and 28 for Type I and II plasmodesmata, respectively ($**** P < 0.0001$ by Mann-Whitney
868 test). For **(g)** and **(f)**, plasmodesmata screening was done on 90 nm thick sections by
869 TEM (see M&M for details). CS: cytoplasmic sleeve; Do: days of culture; Dt: desmotubule;
870 ER: endoplasmic reticulum; PM: plasma membrane.

871

872 **Figure 4. Very tight ER-PM contacts are established during cell plate formation.**

873 **(a-f)** 0.46 nm thick tomographic slices, and the associated segmentation show non-
874 appressed ER strands trapped by the fenestrated cell plate, establishing very tight
875 contacts (**d-f**, red arrows and dashed line) at very early stages of plasmodesmata
876 initiation. **(g-l)** 0.56 nm thick (**g, h**) and 0.36 nm thick (**j, k**) tomographic slices depicting
877 the establishment of very tight ER-PM contacts occurring on one end of the forming
878 plasmodesma (**g-i**, red arrows and dashed line) and along its entire length (**j-l**, red
879 arrows and dashed line). CP: cell plate; ER: endoplasmic reticulum; PM: plasma
880 membrane.

881

882 **Figure 5. Molecular trafficking through Type I plasmodesmata**

883 **(a-c)** Plasmodesmata permeability at the CCI/C1 interface monitored by FRAP and CDFA
884 **(a)** Col. 0 root tip co-stained with CFDA and Propidium Iodide. Orange and blue (CCI)
885 boxes indicate regions that were photobleached and where fluorescence intensity was
886 monitored, respectively. **(b)** Representative kymograph of CCI region (blue box in **a**).
887 *Fire* LUT was applied to enhance visualization of the photobleaching and recovery. **(c)**
888 Mean recovery curve with error bars indicating standard deviation (3 independent
889 experiments; 10 successful FRAPs in 10 individual roots), showing rapid recovery of

890 CFDA within the CCI. The one-phase exponential association curve fit ($R^2 = 0.86$)
891 calculated a half-time recovery of 9.7 seconds, a K constant of 0.07 s^{-1} and a Y_{max} of 0.86
892 **(d-f)** Non-targeted diffusion of free GFP in the COL cells using pSUC2::GFP lines. (d)
893 Cartoon of Arabidopsis pSUC2::GFP root. Green cells represent the companion cells
894 where the GFP is expressed. The presence of GFP in other parts of the root is due to
895 diffusion through plasmodesmata. Red and blue colours show cell interfaces harbouring
896 Type I or Type II plasmodesmata, respectively. (e) Confocal slices through Col 0 (left
897 panels) and pSUC2::GFP (right panels) root tips exhibiting GFP signal (green) in the
898 columella. Close up view (yellow boxed regions in upper panels) in the meristematic
899 region show the CCI contours in the propidium iodide channel (white stars in the two
900 bottom panels) reveal the CCI cells, right below the periblem layer containing the
901 quiescent centre. GFP signal is visible in the meristematic area of pSUC2::GFP lines, in
902 contrast to the absence of fluorescence in the Col.0 root tip. (f) GFP fluorescent
903 quantification in the pSUC2::GFP lines in CCI, and C1-C4 COL layers (background was
904 subtracted against Col-0 roots). Intensities are normalized within a given root relative to
905 the intensity in the vascular system (set to 1). ($n = 15$ pSUC2::GFP roots and $n = 10$ Col.0
906 roots in 3 independent experiments). Wilcoxon test was used to compare each cell type
907 to the CCI cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

908 **(g-i)** Photoactivated (PA)-GFP diffusion through Type I plasmodesmata. (g) Two 0.56
909 nm thick tomographic slices of Type I plasmodesmata in transversal walls of epidermal
910 cells in the root meristematic zone. (h) Confocal slices showing PA-GFP signal in
911 photoactivated cell (t_0 ; white asterisk) and reaching the neighbouring cells after t_{25}
912 minutes. Right panel represents a color-coded cartoon. (i) Fluorescence was quantified
913 in the photo-activated (blue) and the adjacent (n proximal, in red and $n+1$ in green) cells.
914 PA-GFP fluorescence in activated cells consistently showed a decrease of intensity over
915 time whereas neighbouring cells ($n, n+1$) showed a concomitant increase in fluorescence.
916 ($n = 15$ roots; 5 independent experiments). Two-tailed Wilcoxon test was used to
917 compare the fluorescence intensity in a given cell over time. The subsequent times
918 points after photoactivation were always tested with t_0 as the reference, for a given cell.
919 ** $P < 0.01$. CCI: columella cell initial; CFDA: carboxyfluorescein diacetate; COL: columella;
920 FRAP: Fluorescence recovery after photobleaching; LUT: look up table.

921

922

923 **Supplementary Fig 1.**

924 (a, b) 0.56 nm thick tomographic slices of plasmodesmata found in the longitudinal cell
925 walls of the C2 cell tier showing transition in their architecture between Type I and Type
926 I with spoke-less central cavities. (c) 0.56 nm thick tomographic slices of a transitioning
927 plasmodesmata at the C2-C3 layers interface. Spokes are starting to appear (white
928 arrowheads) between PM and desmotubule and the cytoplasmic gap is consequently
929 tighter than when spokes are absent. (d) Schematic representation depicting how the
930 measurements were done for figure panel 3f. Measurements were always taken where
931 the width of the gap was at its maximum. Blue double-headed arrow shows a typical
932 measurement done in a spoke-less cavity contributing to the data plotted in panel 3f.
933 Red double headed arrow shows same measurement executed in a cavity spanned by
934 spoke-like tethers. Brown : cell wall ;Green : ER and the derived desmotubule ;Orange :
935 spoke-like tethers.

936

937 **Supplementary movie 2.**

938 Timelapse of Mb12:YFP lines (a-c) and Ng1:GFP lines (d-f) taken at t = 0, 30min and 1h
939 of treatment at 1 image every 5 seconds. Before treatment (a,d) golgi vesicles are
940 numerous small and rapidly moving within the cells. After LaB (b, e) and CytD (c, f)
941 treatment the vesicles were less mobile and tended to aggregate. The decrease in
942 specific signal over time is due to photobleaching. All images were taken at same
943 magnification under same conditions.

944

945 **Supplementary Fig 3.**

946 Cultured cells consist of small cell clusters and mostly contain primary plasmodesmata
947 on contact walls that are formed during cytokinesis^{60,62}.

948 (a) Overview of Arabidopsis cell clusters in transmitted light. (b) 90 nm ultrathin section
949 containing cells on a copper EM hexagonal grid. (c) TEM micrograph of a single stranded
950 plasmodesma (white arrow) inserted in a division wall.

951

952 **Supplementary movie 4.**

953 A plasmodesma with Type I structure in Arabidopsis cultured cells.
954 The movie displays 0.68 nm thick tomographic slices and a 3D rendering of the data
955 shown in Fig. 3c. ER/desmotubule in pale blue and PM in orange. Scale bar = 50 nm

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Supplementary Fig 5.

(a-d) Tomograms of two Type II plasmodesmata from Arabidopsis cultured cells illustrating the fluctuation in the desmotubule to PM gap along the channels and points of very close ER-PM appositions (black arrowheads). The presence of spoke-elements are indicated by yellow arrows. Tomographic slices of 0.56 nm (a) and 0.46 nm (c) in thickness, and their corresponding segmentation (b and d). CS: cytoplasmic sleeve; Dt: desmotubule; ER: endoplasmic reticulum; PM: plasma membrane.

Supplementary movie 6&7.

Plasmodesma Type II in Arabidopsis cultured cells. The movies display 0.56 nm thick tomographic slices and a manual segmentation of the data shown in Supplementary Fig.4. ER/desmotubule in pale blue, PM in orange and tethering elements in red.

Supplementary Fig 8.

Classic TEM micrographies of representative Type I (a, b) and Type II (c, d) plasmodesmata in gray levels and fake *Fire* coloring to highlight the presence or absence of cytoplasmic sleeve.

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1001

1002 **Author contributions**

1003 Electron microscopy and associated-quantitative analyses were done by W.J.N. with the
1004 help of S.T. and L.B.. M.S.G. performed the cell-to-cell connectivity essays with the help of
1005 W.J.N and L.B. F.P.C. and L.B. provided technical support for the FRAP experiments and
1006 with image quantification and acquisition. All statistical analyses were run by W.J.N. and
1007 M.S.G. A.G. and M.F. performed the control tests for latrunculin and cytochalasin
1008 treatments in the roots. The manuscript was written by E.M.B. and W.J.N. with
1009 contributions of L.B., K.O. and J.T. Research was designed by E.M.B.

1010

1011 **Additional information**

1012 Correspondence and requests for materials should be addressed to W.J.N and E.M.B.

1013

1014 **Competing interests**

1015 The authors declare no competing financial interests.

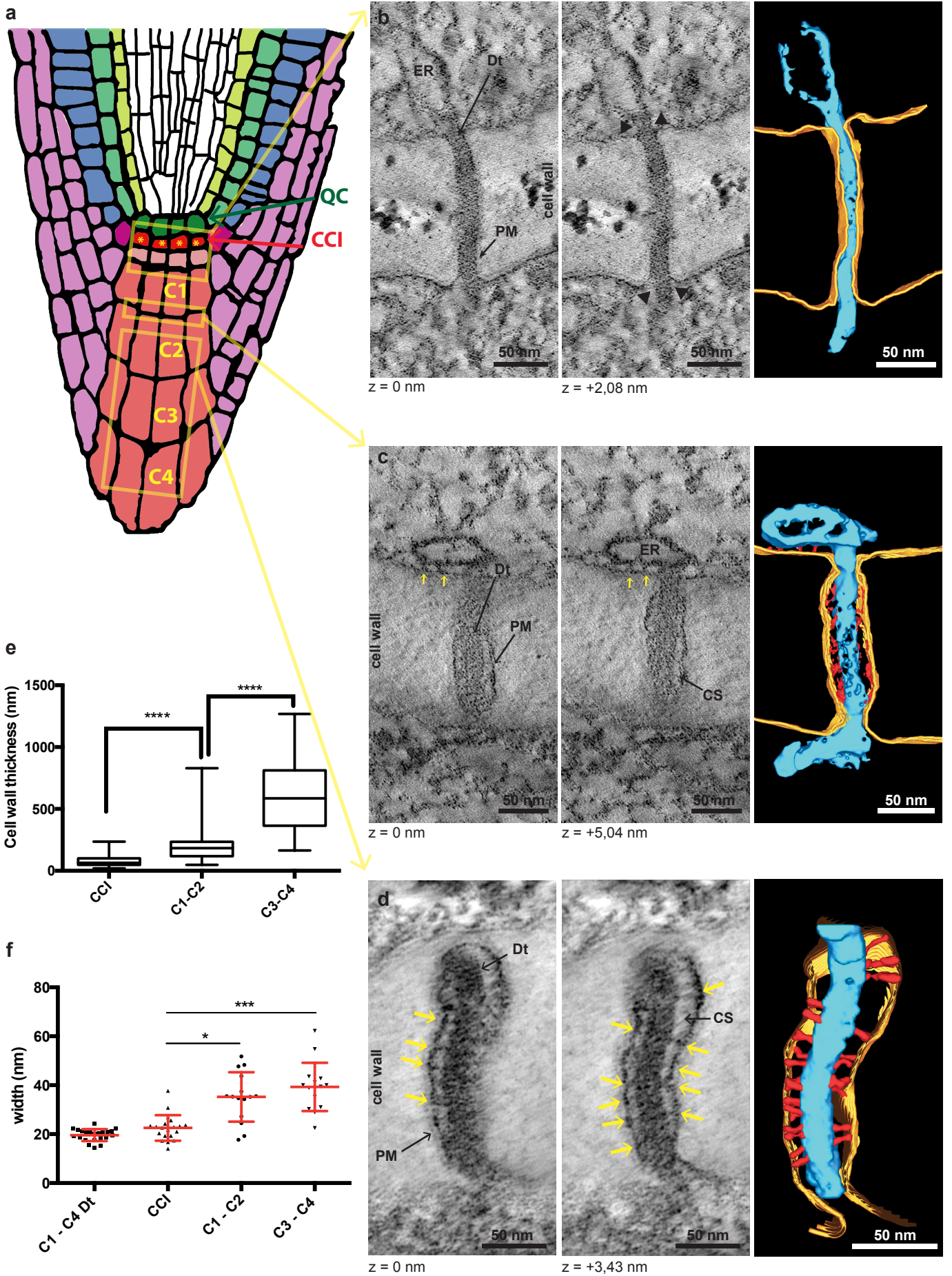


Figure 1. Plasmodesmata ER-PM contact site morphology evolves during tissue development in root tips.

(a) Schematic representation of Arabidopsis root tip cellular organisation, in which the colours depict cell lineage. Plasmodesmal ultrastructure was studied in the Columella tiers in light red (yellow boxes), from the C1 to C4 layer and in the COL cell initials in red (CCI, yellow asterisks). (b-d) Tomographic slices of representative plasmodesmata from the CCI (b), C1-C2 (c) and C3-C4 (d) interfaces, respectively 0.56, 0.56 and 0.49 nm thick, and their associated 3D segmentation highlighting the changes in ER-PM architecture within the pores during COL cell differentiation. In CCI, appressed ER is seen entering the plasmodesmal pores (black arrowheads) and fills the entire canal (b). With tissue differentiation the cytoplasmic sleeve becomes gradually visible as the gap between the desmotubule and PM expands and become populated by spoke-like tethers (d, yellow arrows). In the C1/C2 transition zone there is no clearly identifiable spoke-elements in the cytoplasmic sleeve, only amorphous material (c, red). (e) Difference in cell wall thickness in CCI, C1-C2 and C3-C4 cells (Dunn's multiple comparison test **** $P < 0.0001$). $n = 94$ for CCI, $n = 27$ for C1-C2 and $n = 79$ for C3-C4. (f) Average diameter of plasmodesmata (PM-PM, inner leaflets) and desmotubules in CCI, C1-C2 and C3-C4 cells (Dunn's multiple comparison test, * $P < 0.05$, *** $P < 0.001$). $n = 20$, $n = 15$ and $n = 15$ plasmodesmata tomograms for CCI, C1-C2 and C3-C4, respectively. CCI: columella cell initials; CS: cytoplasmic sleeve; Dt: desmotubule; ER: endoplasmic reticulum; QC: quiescent centre; PM: plasma membrane.

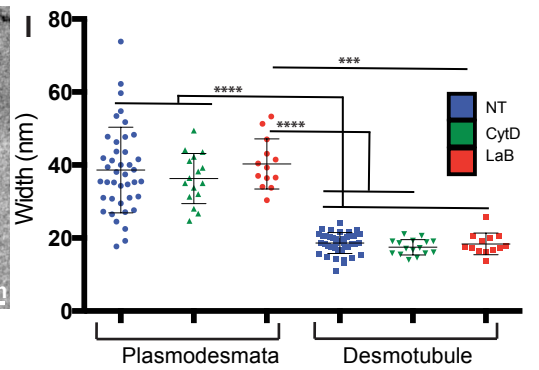
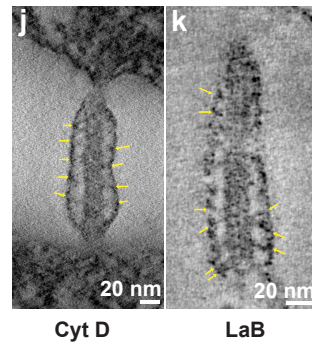
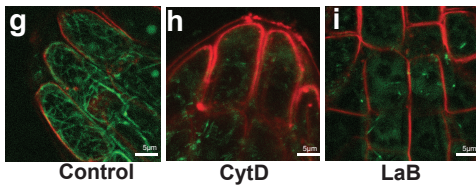
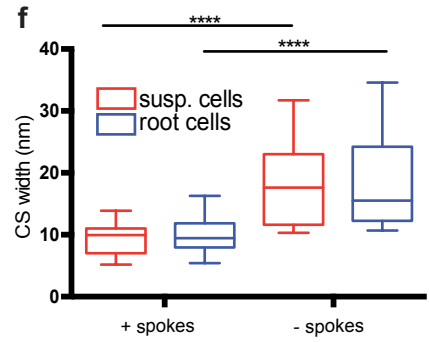
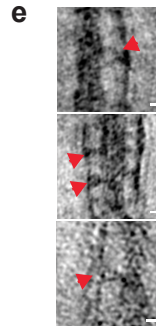
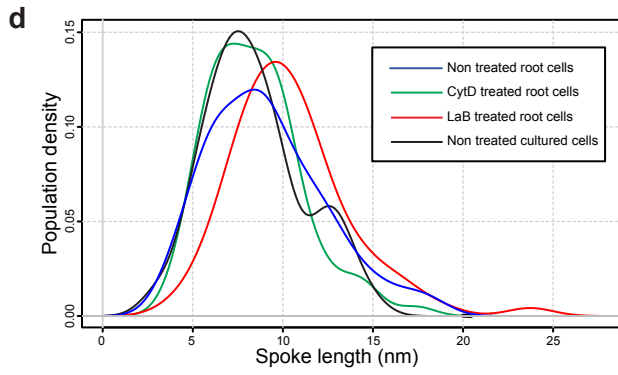
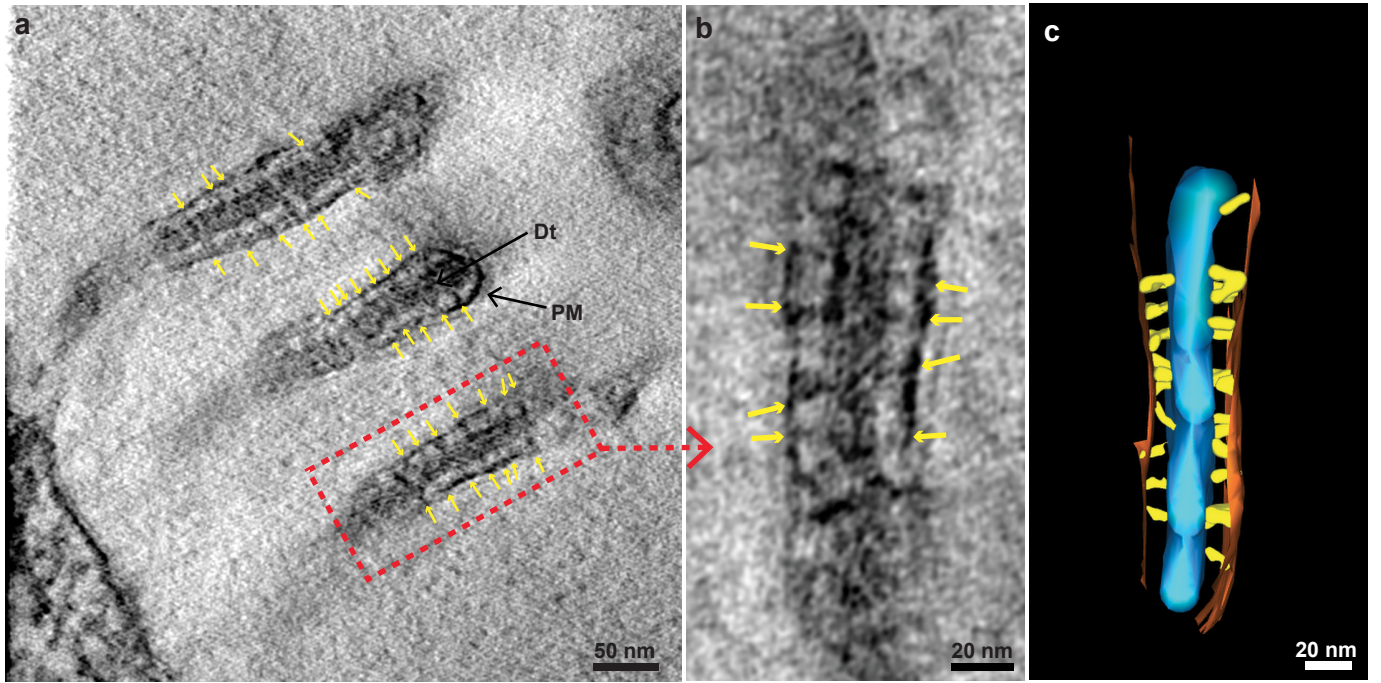
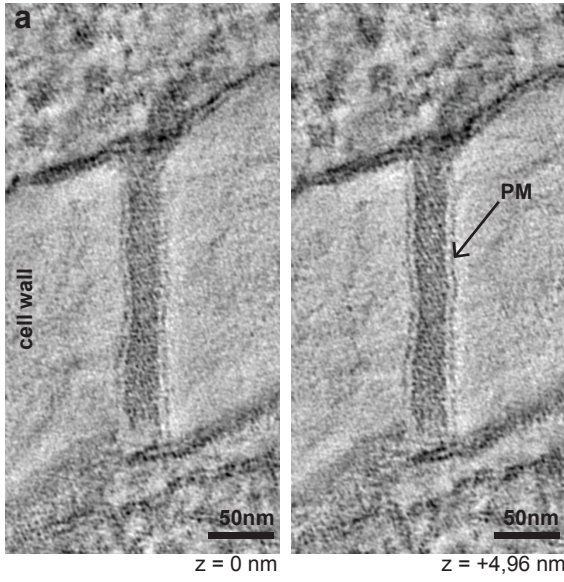


Figure 2. The spoke-like tethering elements of Type II plasmodesmata correlate with ER-PM spacing and are not sensitive to F-actin polymerization inhibitor drugs

(a-c) 1.24 nm thick tomographic slices depicting the typical and regular arrangement of the spoke-like tethering elements (yellow arrows) in Type II plasmodesmata, in the root columella (C2-C4). (b) Close-up view of the plasmodesma squared in (a). (c) Manual segmentation of (b). (d) Density representation of the spoke length, measured in COL cells and cultured *Arabidopsis* cells (non-treated, Latrunculin B, LaB and Cytochalasin D, CytD treated for roots and non treated for cultured cells). In either condition, there is a peak density for spokes of about 9 nm in length. (e) Three close-up views of V/Y shaped spokes (red arrowheads) oriented towards the PM (top; middle) and the desmotubule (bottom). Scale bars = 5 nm. (f) Plot representation of the cytoplasmic sleeve gaps (desmotubule – PM distance) measured in subsections of transitioning or Type II pores with or without spokes. Both in roots and cultured cells the presence of spokes stabilizes the width of the intermembrane gap and keeps it at an average of 9.6 ± 2.44 nm whereas without the spokes this gap is 18.38 ± 7.26 nm (Mann-Whitney two tailed test, **** $P < 0.0001$) $n = 31$ and $n = 65$ measurements in cell and root plasmodesmata respectively.

(g-l) Type II plasmodesmata in COL cells (C2-C4) are not altered by F-actin-polymerization inhibitor drugs. (g-i) Maximum projections of confocal stacks taken in columellas of *Arabidopsis* marker lines fimbrin actin binding domain 35S::GFP-fABD2-GFP. In control conditions (g), cells show a dense, reticulated actin network, while after 1h30 hour treatment with 50 μ M Cytochalasin D (CytD, h) or Latrunculin B (LaB, i), the F-actin network is heavily altered. (j-k) 0.56 nm thick tomographic slices of plasmodesmata acquired in the columella of CytD (j) and LaB (k)-treated roots, showing that the spokes are still present. (l) The diameter (PM-PM; innerleaflets) of the pores and the desmotubule width remain unchanged both treatment (Dunn's multiple comparison test, **** $P < 0.0001$, *** $P < 0.001$) $n = 41$ plasmodesmata tomograms for non-treated (NT) condition, $n = 13$ for LaB and $n = 16$ for CytD treated condition. Dt: desmotubule ; PM : plasma membrane.

Type I plasmodesmata



Type II plasmodesmata

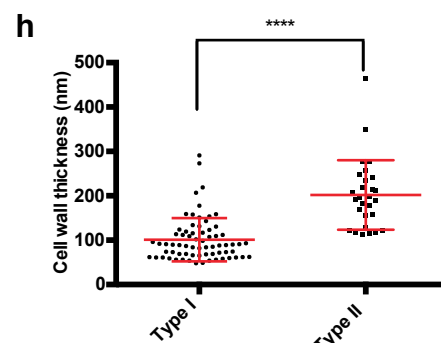
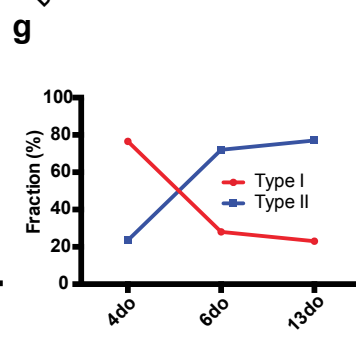
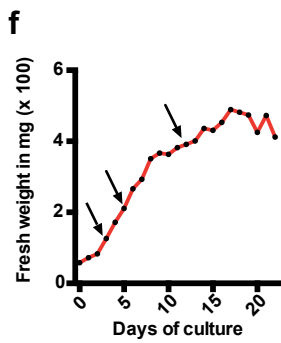
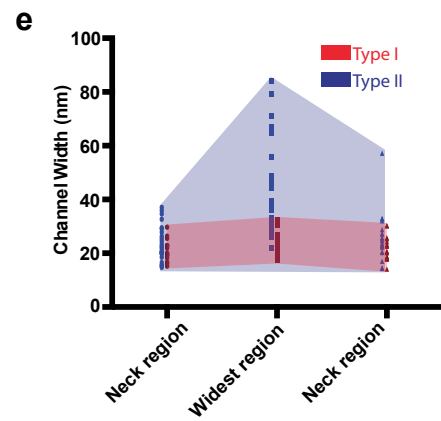
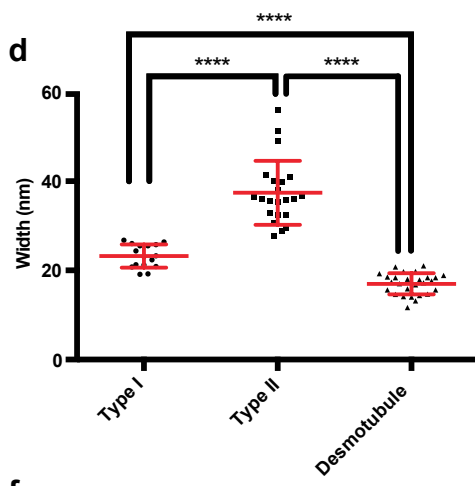
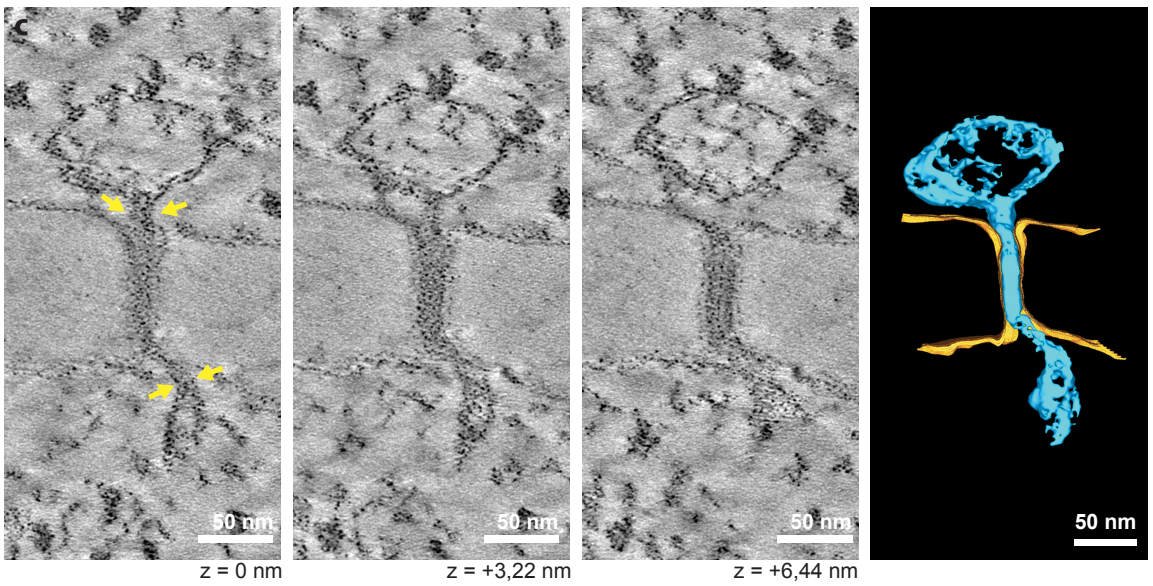
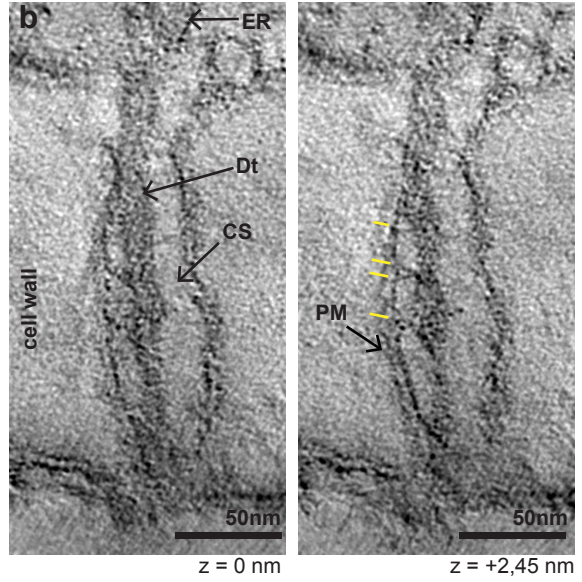
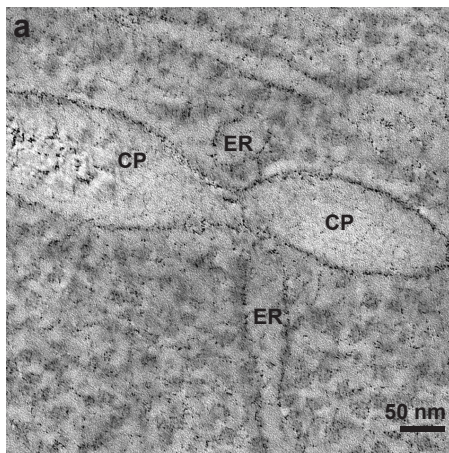
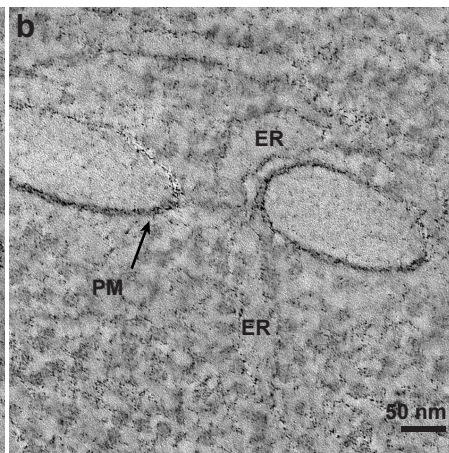


Figure 3. Very tight ER-PM contact in post-cytokinesis plasmodesmata

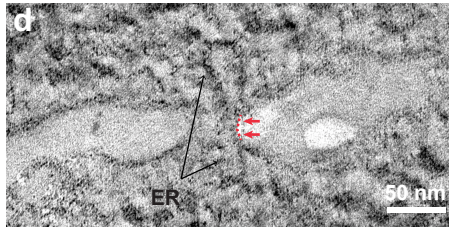
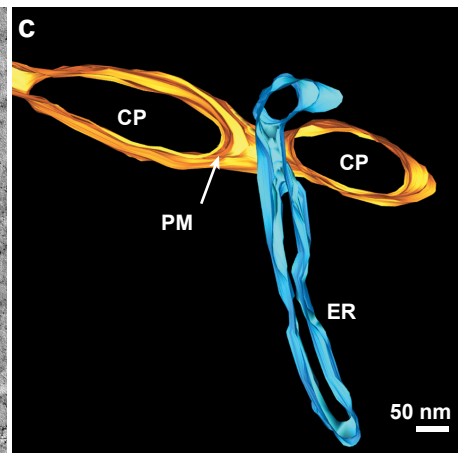
(a-b) Consecutive tomographic slices, of respectively 1.24 nm and 0.49 nm in thickness, of a Type I plasmodesma (a) and Type II plasmodesma (b, spokes are indicated by yellow dashes) in Arabidopsis cultured cells. (c) Type I plasmodesmata are traversed by the ER which becomes appressed just before entering the pores (yellow arrows). Three 0.56 nm thick tomographic slices and the corresponding 3D segmentation. (d) Plasmodesmata diameter (PM-PM; inner leaflets) of Type I (23 ± 2.6 nm) and Type II (37 ± 7.2 nm), and the desmotubule measured in Type II plasmodesmata (17 ± 2.4 nm). $n = 17$ and 22 tomograms for Type I and Type II plasmodesmata, respectively, and $n = 22$ for desmotubule measurements (**** $P < 0.0001$ by Mann-Whitney test). (e) plasmodesmata width at different points along the pores in Type I and II. Measurements (PM-PM; inner leaflets) were taken at the extremities and largest part of the channels. Type I plasmodesmata have a remarkably constant diameter compared to Type II. $n = 17$ and $n = 22$ tomograms for Type I and Type II plasmodesmata, respectively. (f) Growth curve of Arabidopsis liquid cultured cells. Black arrows indicate the cell culture ages used for this study (four-, six- and thirteen-day-old). (g) Quantification of the relative proportion of Type I and II plasmodesmata in Arabidopsis cultured cells at four, six and thirteen days ($n = 111, 89$ and 22 screened plasmodesmata for four, six and thirteen days old cells). (h) Average cell wall thickness in relation to plasmodesmata Type. In four-day-old cultured cells, Type I plasmodesmata are abundant in thin young cell walls whereas Type II plasmodesmata are preferentially associated with thicker, older walls. $n = 69$ and 28 for Type I and II plasmodesmata, respectively (**** $P < 0.0001$ by Mann-Whitney test). For (g) and (f), plasmodesmata screening was done on 90 nm thick sections by TEM (see M&M for details). CS: cytoplasmic sleeve; Do: days of culture; Dt: desmotubule; ER: endoplasmic reticulum; PM: plasma membrane.



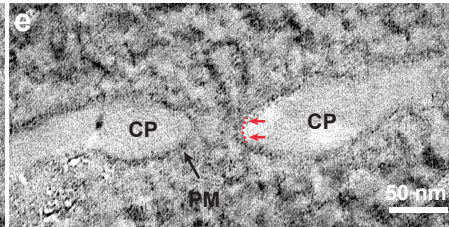
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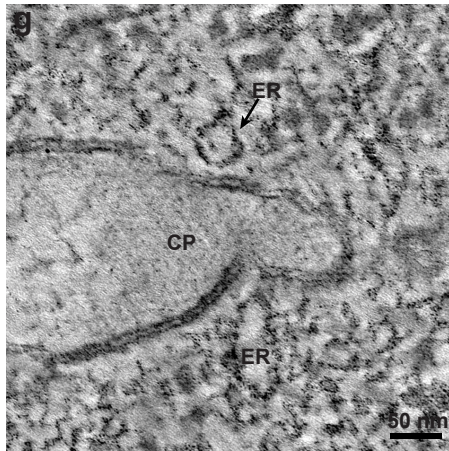
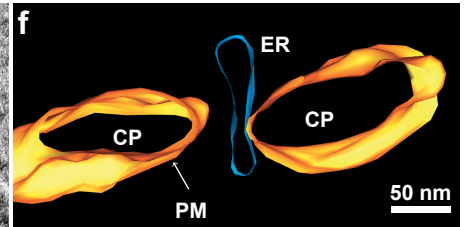
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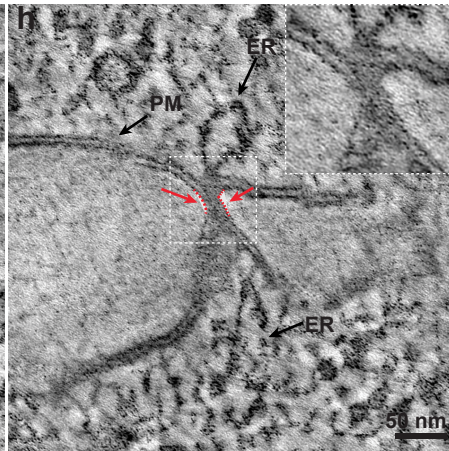
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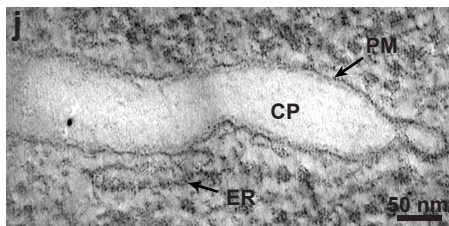
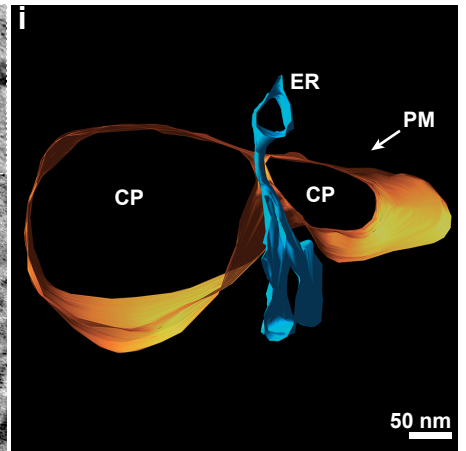
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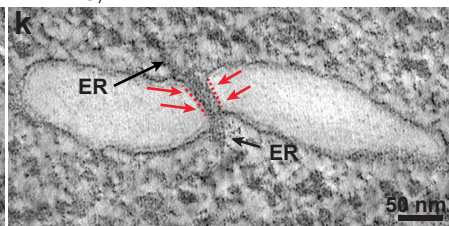
$z = 0$ nm



$z = +16.24$ nm



$z = 0$ nm



$z = +16.56$ nm

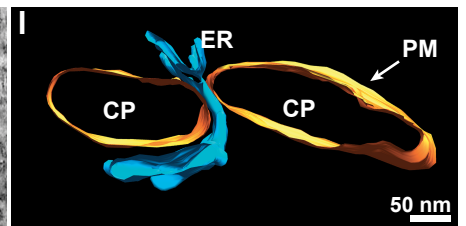


Figure 4. Very tight ER-PM contacts are established during cell plate formation. (a-f) 0.46 nm thick tomographic slices, and the associated segmentation show non-appressed ER strands trapped by the fenestrated cell plate, establishing very tight contacts (d-f, red arrows and dashed line) at very early stages of plasmodesmata initiation. (g-l) 0.56 nm thick (g, h) and 0.36 nm thick (j, k) tomographic slices depicting the establishment of very tight ER-PM contacts occurring on one end of the forming plasmodesma (g-i, red arrows and dashed line) and along its entire length (j-l, red arrows and dashed line). CP: cell plate; ER: endoplasmic reticulum; PM: plasma membrane.

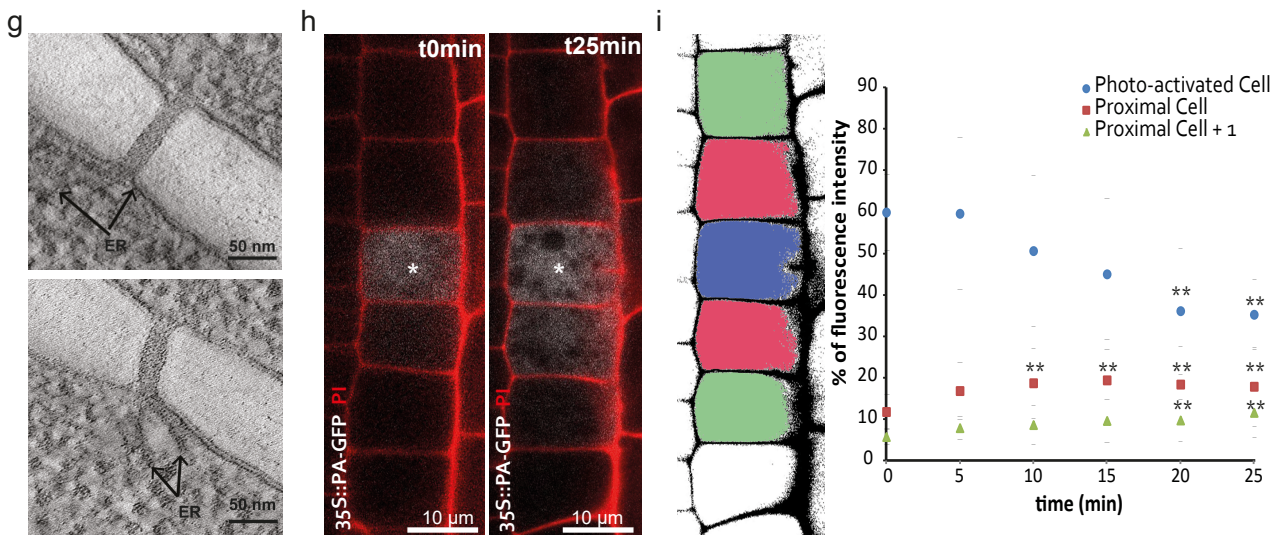
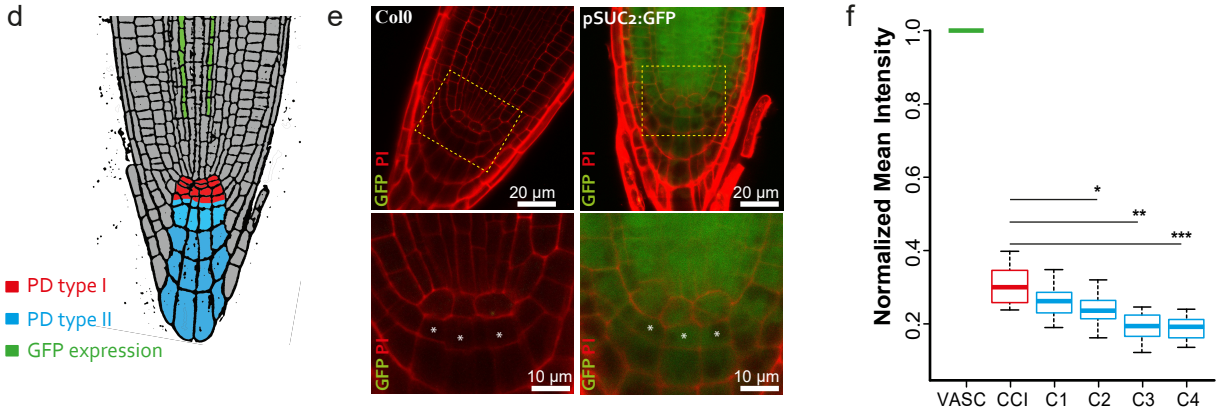
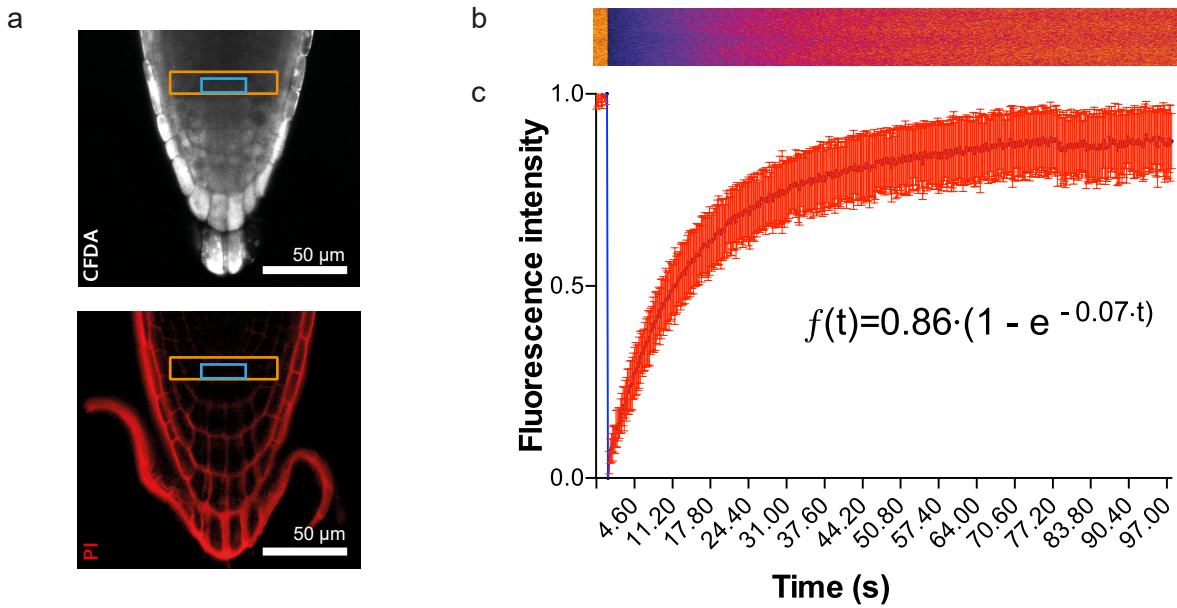


Figure 5. Molecular trafficking through Type I plasmodesmata

(a-c) Plasmodesmata permeability at the CCI/C1 interface monitored by FRAP and CDFA (a) Col. 0 root tip co-stained with CFDA and Propidium Iodide. Orange and blue (CCI) boxes indicate region that were photobleached and where fluorescence intensity was monitored, respectively. (b) Representative kymograph of CCI region (blue box in a). *Fire* LUT was applied to enhance visualization of the photobleaching and recovery. (c) Mean recovery curve with error bars indicating standard deviation (3 independent experiments; 10 successful FRAPs in 10 individual roots), showing rapid recovery of CFDA within the CCI. The one-phase exponential association curve fit ($R^2 = 0.86$) calculated a half-time recovery of 9.7 seconds, a K constant of 0.07 s^{-1} and a Ymax of 0.86

(d-f) Non-targeted diffusion of free GFP in the COL cells using pSUC2::GFP lines. (d) Cartoon of Arabidopsis pSUC2::GFP root. Green cells represent the companion cells where the GFP is expressed. The presence of GFP in other parts of the root is due to diffusion through plasmodesmata. Red and blue colours show cell interfaces harbouring Type I or Type II plasmodesmata, respectively. (e) Confocal slices through Col 0 (left panels) and pSUC2::GFP (right panels) root tips exhibiting GFP signal (green) in the columella. Close up view (yellow boxed regions in upper panels) in the meristematic region show the CCI contours in the propidium iodide channel (white stars in the two bottom panels) reveal the CCI cells, right below the periblem layer containing the quiescent centre. GFP signal is visible in the meristematic area of pSUC2::GFP lines, in contrast to the absence of fluorescence in the Col.0 root tip. (f) GFP fluorescent quantification in the pSUC2::GFP lines in CCI, and C1-C4 COL layers (background was subtracted against Col-0 roots). Intensities are normalized within a given root relative to the intensity in the vascular system (set to 1). ($n = 15$ pSUC2::GFP roots and $n = 10$ Col.0 roots in 3 independent experiments). Wilcoxon test was used to compare each cell type to the CCI cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(g-i) Photoactivated (PA)-GFP diffusion through Type I plasmodesmata. (g) Two 0.56 nm thick tomographic slices of Type I plasmodesmata in transversal walls of epidermal cells in the root meristematic zone. (h) Confocal slices showing PA-GFP signal in photoactivated cell (t_0 ; white asterisk) and reaching the neighbouring cells after t_{25} minutes. Right panel represents a color-coded cartoon. (i) Fluorescence was quantified in the photo-activated (blue) and the adjacent (n proximal, in red and $n+1$ in green) cells. PA-GFP fluorescence in activated cells consistently showed a decrease of intensity over time whereas neighbouring cells (n , $n+1$) showed a concomitant increase in fluorescence. ($n = 15$ roots; 5 independent experiments). Two-tailed Wilcoxon test was used to compare the fluorescence intensity in a given cell over time. The subsequent times points after photoactivation were always tested with t_0 as the reference, for a given cell. ** $P < 0.01$. CCI: columella cell initial; CFDA: carboxyfluorescein diacetate; COL: columella; FRAP: Fluorescence recovery after photobleaching; LUT: look up table.