1 **Short title: Microdomain coordination between neighbors in plants**

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6 **Title: CASP microdomain formation requires cross cell wall stabilization of**
7 **domains and non-cell autonomous action of LOTR1**

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28 **Summary**

29 Efficient uptake of nutrients in both animal and plant cells requires tissue-
 30 spanning diffusion barriers separating inner tissues from the outer
 31 lumen/soil. However, we poorly understand how such contiguous three-
 32 dimensional superstructures are formed in plants. Here, we show that
 33 correct establishment of the plant Casparian Strip (CS) network relies on
 34 local neighbor communication. We show that positioning of Casparian Strip
 35 membrane domains (CSDs) is tightly coordinated between neighbors in
 36 wild-type and that restriction of domain formation involves the putative
 37 extracellular protease LOTR1. Impaired domain restriction in *lotr1* leads to
 38 fully functional CSDs at ectopic positions, forming “half strips” LOTR1
 39 action in the endodermis requires its expression in the stele. LOTR1
 40 endodermal expression cannot complement, while cortex expression
 41 causes a dominant-negative phenotype. Our findings establish *LOTR1* as a
 42 crucial player in CSD positioning acting in a directional, non-cell-
 43 autonomous manner to restrict and coordinate CS positioning.

44

45 **Keywords**

46 Endodermis, Casparian Strips, extracellular diffusion barriers, membrane
 47 domains, coordination, cell ablation

48 Introduction

49 Plants mine the surrounding soil for water and dissolved minerals to
 50 sustain their growth and to complete their life cycle. Likened to inverted
 51 guts, roots evolved crucial epithelial functions — selective uptake and
 52 diffusion barriers — in order to generate and sustain body homeostasis in
 53 variable and harsh environments. In gut epithelial cells, the required
 54 separation of outside lumen and inside tissues is achieved through the
 55 formation of tight junctions. These specialized membrane domains form
 56 ring-like domains between the apical (gut lumen) and basal (blood stream)
 57 sides. Close adhesion of both adjacent plasma membranes is achieved by
 58 tight interaction of occludins, claudins, and cadherins, organized into tight
 59 and adherens junctions, forming impermeable and mechanically-resistant
 60 barriers. In plants, a functionally similar diffusion block is achieved by
 61 Casparian strips (CS), highly localized impregnations of the cell wall, the
 62 plant's extracellular matrix, in root endodermal cells.

63 Similar to tight/adherence junctions, a specialized membrane domain
 64 (Casparian Strip membrane Domain, CSD) forms a precise ring in
 65 transversal and anticlinal membranes of elongated endodermal cells. This
 66 domain acts as a molecular fence that separating the endodermal plasma
 67 membranes into peripheral (outer) and central (inner) domains,
 68 highlighted by the polar distribution of nutrient transporters (Julien
 69 Alassimone, Naseer, and Geldner 2010; Bao et al. 2019; Ma et al. 2007;
 70 Takano et al. 2010). CASPARIAN STRIP DOMAIN PROTEINS (CASPs) are
 71 specifically targeted to this domain and form a stable matrix for the
 72 subsequent lignification machinery (Roppolo et al. 2011; Lee et al. 2013;
 73 Hosmani et al. 2013; Kalmbach et al. 2017; Barbosa, Rojas-Murcia, and
 74 Geldner 2019). Lignification of the primary cell wall of CSs extends through
 75 the entire apoplast including cross-linkage to the middle lamella. This is
 76 illustrated in cell wall digestions leaving only the resistant lignified tissues
 77 of CSs and xylem visible as a fishnet-like matrix (Enstone, Peterson, and Ma
 78 2002).

79 The CS and tight/adherens junctions must form a contiguous network in
80 order to fulfill their role as functional diffusion barriers. However, we still
81 poorly understand how such a supracellular structure is coordinated within
82 the endodermal cell layer. Although we are ignorant of the mechanism, the
83 precisely opposing localization of CSDs evidences that endodermal cells
84 coordinate the positioning of their membrane domains across the cell wall
85 space. In epithelial cells, adherens junctions are first initiated by cell-to-cell
86 contact mediated by nectins and cadherins and kept in place by firm
87 attachment to the actin cytoskeleton (Rajasekaran et al. 1996; Itoh and
88 Bissell 2003). In plant cells, their cell wall matrices separate neighboring
89 cell by at least 100-200 nm, which prevents such a direct interaction of
90 membrane bound proteins. Thus, a more complex positioning mechanism
91 that requires local signals from directly adjacent neighbors could ensure
92 coordination of CSDs between neighbors.

93 Many components necessary for correct CS formation have been identified
94 in recent years, but few show impacts on CS positioning *per se*. Mutation of
95 components in the recently discovered barrier surveillance mechanism
96 causes interrupted, but correctly localized CSDs (Pfister et al. 2014; Doblas
97 et al. 2017; Nakayama et al. 2017; J Alassimone et al. 2016). Loss of the
98 endodermal differentiation transcription factor MYB36 abolishes the
99 formation of the entire CSD while disruption of CS cell wall localised ESB1
100 causes unstable, but still correctly localised domains (Kamiya et al. 2015;
101 Liberman et al. 2015; Hosmani et al. 2013). The recently published *lotr2*
102 mutant revealed the importance of a member of the exocyst complex,
103 *EXO70A1*, in mediating targeted secretion of CASP proteins to the pre-
104 established CSD (Kalmbach et al. 2017). However, the fragmented domains
105 visible in this mutant are non-functional and unstable. Thus, based on
106 current data, it is unclear whether formation of functional CSDs at ectopic
107 positions is even possible.

108 Here, we report that the recently discovered *lotr1* mutant forms fully
109 functional CSDs outside the endodermal-endodermal cell interface in. We
110 establish that neighbouring endodermal cells are absolutely required to

111 form stable and continuous CSDs and we highlight close coordination of
112 CSD microdomains during the early stages of domain formation. In
113 addition, we demonstrate that *LOTR1* is crucial for restriction of the CSD
114 and that it acts independently of any known pathway for CSD formation.
115 We show that this new type of putative cell wall protease is expressed in
116 the stele, represents a novel, non-cell-autonomous and directional
117 signalling component that controls CSD stability in the endodermis. Thus,
118 we demonstrate that signalling, both within and between cell layers is
119 crucial for coordinating CS positioning.

120

121 **Results**

122 *Ectopic CASP deposits in lotr1 are fully functional CSDs*

123 During their initial characterization of the *lotr1* mutant, Li et al. 2017
124 reported a novel ‘patchy’ phenotype, where large patches of the Casparian
125 strip membrane domain marker CASP1::CASP1-GFP occurred outside of the
126 regular median CS position. Further analysis revealed an apoplastic delay
127 phenotype attributed to large gaps that could be observed in the CS upon
128 lignin stain. We performed a more detailed analysis of the domain marker
129 CASP1::CASP1-GFP in this mutant background to search for disruptions in
130 the CSD that could explain these gaps in lignification. Confirming the earlier
131 findings (Li et al. 2017), we found large patches of CASP1-GFP occurring in
132 endodermal membranes that were never observed in wildtype plants (Fig.
133 1B). Whereas previously reported to occur only facing pericycle cells, we
134 found similar patches to also occur towards the outer cortex (Fig. 1B, Fig.
135 S1A), although quantification revealed a clear preference for the
136 endodermal-pericycle interface (Fig. S1B). In addition to these patches, we
137 found disruptions of the central CSD which occurred primarily at cell
138 corners with nearby ectopic CASP deposits (Fig. 1B), coherent with the
139 reported gaps in CS lignin. The overall correctly positioned and largely
140 continuous central CSD in *lotr1* suggests that the core-localization
141 mechanism for the CSD is still functional. We speculate that the change in
142 cell shape and cell walls at junctions might favor patch formation in *lotr1*. If

143 patch formation starts simultaneously with initial focalization of CASPs into
 144 micro-domains (known as the string-of-pearls stage), nearby ectopic CASP
 145 deposits might inhibit the growth of the central domain leading to the
 146 observed large discontinuities. In order to better understand the nature
 147 and origin of these patches, we followed CASP deposition throughout
 148 endodermal differentiation. In both wildtype and *lotr1*, CASP1-GFP
 149 aggregated into aligned micro-domains in the cell's median (Fig. S1C).
 150 During this initial focalization step, we observed occurrence of ectopic
 151 CASP islands in *lotr1*, indicating a role of LOTR1 during this early step of
 152 defining the CS location. Ectopic CASP deposits grew over time and were
 153 able to fuse into larger patches, similarly to the central micro-domains
 154 combining into a continuous ring. We did not observe degradation of these
 155 patches nor formation of new patches once the central domain was fused,
 156 confirming that these patches are stable once formed and their
 157 establishment is restricted to the short developmental window in which
 158 the cells determine their CS location. Consequently, LOTR1 likely acts in
 159 this early phase as an inhibitor of ectopic domain formation. We then
 160 investigated whether these patches represent fully functional domains, i.e.
 161 whether they are able to guide lignification enzymes to these sites.
 162 Deposition of lignin can be easily identified in electron microscopy, where
 163 CSs are visible as a clearly defined, homogenous stretch of cell wall
 164 spanning the entire apoplastic space (Roppolo et al. 2011; Fujita et al.
 165 2020) (Fig. S1D). Ultra-structure analysis of *lotr1* revealed similar cell wall
 166 modifications in pericycle-facing endodermal membranes (Fig. 1D). To our
 167 surprise, and in contrast to regular CSs, this ectopic lignin patch was
 168 restricted to the endodermal part of the cell wall, stopping precisely at the
 169 middle lamella, effectively creating half of a Casparian Strip. Such a strict
 170 spatial confinement of lignification on a nanometer scale is quite surprising
 171 since monolignols and ROS, both essential for lignin polymerization, are
 172 thought to be mobile in cell walls and lignin polymerization itself not to be
 173 under direct enzymatic control. To our knowledge, such a strictly restricted
 174 lignification encompassing only half of a thin primary has not been
 175 observed previously. Our finding therefore suggests a very local and

176 directed lignin polymerization in the CS, where each endodermal cell is
177 responsible for lignifying their part of the cell wall to build a functional CS
178 barrier. A functional CSD is also characterized by a tight attachment of the
179 underlying membrane, which is revealed in EM by the mild plasmolysis of
180 the plasma membrane introduced through sample preparation, and which
181 is absent at the CSD. Perfectly matching the cell autonomous nature of the
182 ectopic patches, tight attachment of the endodermal plasma membrane,
183 but not the pericycle plasma membrane was observed.

184 In order to further support our hypothesis that *lotr1* ectopic patches are
185 fully functional domains, we compared consecutive electron micrograph
186 sections with and without potassium permanganate (KMnO₄) staining to
187 check for lignification. Depositing electron dense material by reaction with
188 lignin sidechains, KMnO₄ highlights lignin presence as darkening of cell
189 walls in Casparian strips and protoxylem vessels (Stein, Klomparens, and
190 Hammerschmidt 1992; Yamashita et al. 2016). Ectopic patches in *lotr1*
191 displayed KMnO₄ staining on the endodermal side of the half-strips, while
192 the opposite pericycle cell walls were unstained (Fig. 1E). This correlated
193 with CS-typical cell wall appearance (more homogenous, less electron-
194 dense, slightly thickened) observed in unstained consecutive cuts,
195 confirming lignin presence at these ectopic domains. Notably, several
196 samples displayed CS-type cell wall morphology with accompanying
197 membrane attachment and lignification throughout the entire endodermal
198 cell corner, stretching from the median endodermal-cell interface all the
199 way to the endodermal-pericycle cell walls (Fig. S1E). Although ectopic
200 lignification is a hallmark of many Casparian Strip mutants, this was not
201 described for *lotr1*. Furthermore, enhanced lignification itself does not
202 result in firm membrane attachment outside the regular CS. Consequently,
203 this points to a drastically enlarged CSD indicative of an impaired ability to
204 restrict CSD growth in *lotr1*, agreeing with a presumed role of LOTR1 as
205 inhibitor of domain formation.

206 The regular CSD recruits a multitude of enzymes required for restricted
207 monolignol polymerization to form the CS. The tightly restricted lignin

208 accumulation at ectopic *lotr1* patches suggested similar recruitment of
 209 these enzymes. Indeed, when we co-expressed the CSD marker CASP1-GFP
 210 together with typical members of this lignin machinery in *lotr1* to confirm
 211 their recruitment to ectopic CSDs. Endodermis specific PEROXIDASE 64
 212 (PRX64) and LACCASE 3 (LAC3) displayed a clear co-labelling in both central
 213 and ectopic domains while NADPH oxidase RbohF, the main generator of
 214 ROS for monolignol polymerization, was also found to be present, although
 215 it did not specifically accumulate there (Fig. 1F). Finally, we analyzed the
 216 localization of dirigent-domain protein ESB1 in *lotr1*. Although it was
 217 previously reported to be absent from ectopic deposits (Li et al. 2017). We
 218 found ESB1 to accumulate at the sites of ectopic CASP1-deposition when
 219 expressed as ESB1-mCherry under its native promoter together with
 220 CASP1-GFP. Dirigent-domain proteins are thought to be a key component
 221 in restricting and directing lignin polymerization in the cell wall (Hosmani et
 222 al. 2013). Our results of ESB1 localization therefore agree with PRX64 and
 223 LAC3 labelling, as well as the tight restriction of lignin at these patches,
 224 proving these ectopic formations to be fully functional, but ectopically
 225 formed CSDs.

226 *Focalization of CASPs is coordinated between cells*

227 The fact that an ectopic CSD patch towards a non-endodermal neighbor
 228 can only induce formation of a half-strip suggested that two neighboring
 229 endodermal cells absolutely need to align their respective CSDs in order to
 230 lignify the entire CS cell wall space. To ensure this, a mechanism would
 231 need to be in place that promotes formation of a CSD at places where a
 232 neighboring cell is forming one and/or inhibits CSD formation in the
 233 absence of a neighboring CSD. We therefore investigated the initial
 234 focalization of CASP proteins at high resolution. When properly aligned
 235 along the z-axis, we were able to identify two distinct membrane signals
 236 present between two neighboring endodermal cells (Fig. 2A,C). Strikingly,
 237 at the string-of-pearls stage, CASP1-GFP intensity along the length of both
 238 signals already showed a strong positive correlation ($R^2 = 0.535$, $p = 1.612$
 239 $\times 10^{-9}$) (Fig. 2B), indicating coordination of CSD formation across the cell wall

240 already at this stage. To validate our assumption that these paired signals
 241 represent the signals of neighboring cell membrane domains, we checked
 242 the position of CS cell wall protein ESB1 and cell wall tracer Propidium
 243 Iodide (Fig. 2C,D). In contrast to CASP1-GFP, ESB1-mCherry localized in a
 244 single line in between the observed CASP-labelled membranes in both early
 245 and late CSDs (Fig. 2C). To further confirm that these GFP signals truly
 246 represented separate membranes, we decided to employ cell-specific laser
 247 ablation to destroy one of the cells, arguing that this should remove only
 248 one half of the paired signal. Indeed, upon ablation, GFP signal was
 249 specifically lost in the ablated cell whereas the adjacent signal of the intact
 250 neighboring cell was unchanged (Fig. 2D,E). Together, our data
 251 demonstrate the observed double signals to be adjacent CSDs of
 252 neighboring endodermal cells, whose initial formation is tightly
 253 coordinated between cells. We then investigated whether currently known
 254 mutants such as *sgn1*, *sgn3* and *esb1* would display defects in CASP
 255 microdomain coordination (Fig. S2A). However, all these mutants showed
 256 wildtype like coordination between adjacent CSDs. Interestingly, *lotr1* also
 257 showed well-coordinated deposition of micro-domains at the Casparian
 258 strip position. Therefore, while LOTR1 appears to be necessary to restrict,
 259 unpaired, ectopic domain establishment, its loss-of function does not seem
 260 to affect the process of coordination at the correct central position where
 261 the future CS between neighboring cells is forming. This is corroborated by
 262 the lack of CS half-strips at the regular median position between
 263 endodermal cells in *lotr1* (Fig. S2A).

264 *Endodermal cells require a neighboring cell to stabilize CASPs*

265 The close correlation of CASP micro-domains in neighboring endodermal
 266 membranes could be explained by an unknown feedback control
 267 mechanism that stabilizes microdomains upon presence of neighboring
 268 domains and de-stabilizes them in their absence. Such a mechanism would
 269 immediately guide CSD formation towards endodermis-endodermis
 270 surfaces. In order to test this model, we ablated individual endodermal
 271 cells in the elongation zone, prior to their transition to differentiation. This

272 enabled us to compare CSD establishment with and without a functional
 273 neighbor in the same cell. Initially, we observed CASP deposition in a string-
 274 of-pearls-typical pattern in membranes facing living and dead cells alike
 275 (Fig. 3A,D). However, only membranes facing intact neighbors were able to
 276 form a continuous strip, whereas domains towards ablated cells did not
 277 progress past the string-of-pearls stage. When we ablated all adjacent cells,
 278 the single intact cell was still able to express and focalize CASPs but was
 279 unable to establish any continuous domains (Fig. 3C). We also noticed a
 280 strong difference in signal intensity between membranes facing living and
 281 dead cells, where GFP signal increased rapidly in CSDs towards intact
 282 neighbors, but not ablated cells (Fig. 3B). This discrepancy is likely a result
 283 of an inability to recruit additional CASP proteins to the initially formed
 284 micro-domains. This suggested a control point during CSD establishment
 285 where a neighboring signal is required to progress from the string-of-pearls
 286 stage and commit to formation of a CS. We had to exclude that isolating
 287 endodermal cells from their neighbors by ablation might cause stresses
 288 that influence overall endodermal differentiation and not just a
 289 destabilization of CASP recruitment to the membrane. We therefore used a
 290 CASP1 transcriptional reporter, driving a nuclear fluorescent protein. Upon
 291 ablation of all surrounding endodermal cells a similar CASP promoter
 292 activity compared to non-affected cells was observed, indicating that
 293 progress of differentiation *per se* was not halted in the isolated cell (Fig.
 294 S3A). To rule out possible general effects on secretion or localization of
 295 plasma membrane proteins, we then ablated cells in double marker lines
 296 expressing CASP1-GFP and the generic SYP122-3xmCherry plasma
 297 membrane marker, both under the control of the CASP1 promoter. Again,
 298 this clearly established that isolated cells still express from the CASP1
 299 promoter. Moreover, they accumulate SYP122 on all cell sides, in contrast
 300 to CASP1, which accumulates only at plasma membrane domains facing a
 301 living neighbor (Fig. 3D).

302 We then thought to confirm our findings by means that do not involve
 303 destruction of neighboring cells. To do so we generated inducible

304 complementation lines for MYB36, a key transcription factor of
 305 endodermal differentiation (Lieberman et al. 2015; Kamiya et al. 2015).
 306 *myb36* loss-of-function mutants are unable to activate expression of key
 307 CSD proteins, e.g. CASPs, and consequently lack a CSD. Using very low level
 308 of inducer, we generated sporadic MYB36 activation to enable some, but
 309 not all cells to start differentiation and initiate CS formation. Expectedly,
 310 we observed a sporadic, patchy expression pattern of CSD domain marker
 311 CASP1-GFP upon induction, with some samples displaying expression in
 312 isolated, single cells, while others showed cell groups with a near complete
 313 CSD network (Fig. 3E). Confirming our ablation results, we found that
 314 isolated cells were able to deposit CASP1-GFP in typical micro-domains but
 315 were incapable of forming a stable and continuous CSD, similar to what
 316 was observed using cell ablation (Fig. 3E, left). If two adjacent cells started
 317 to deposit CASPs, we observed an increase in GFP signal only in the domain
 318 between these cells, followed by fusion of the initial micro-domains into a
 319 continuous strip (Fig. 3E, right). The domains not facing a CASP1 expressing
 320 neighbor did not progress beyond a string-of-pearls stage and eventually
 321 lost the CASP1-GFP signal (Fig. 3F, S3B). We conclude that wildtype plants
 322 appear to require signals from neighbors to form stable microdomains and
 323 commit to a CS. *lotr1* is currently the only known mutant that can form
 324 functional microdomains in the absence of neighbors. We therefore asked
 325 whether *lotr1* might be able to fuse micro-domains at the side facing the
 326 dead neighbor into continuous strips. Yet, when we ablated cells in *lotr1*,
 327 we observed a similar disappearance of the central CSD facing the ablated
 328 cell (Fig S3C). One explanation could be that LOTR1 function is redundant
 329 for destabilization of unpaired domains in the correct, central position, but
 330 is required at surfaces facing non-endodermal neighbors. Indeed, we did
 331 observe formation of stable, *lotr1*-typical patches on the side facing its
 332 dead neighbor, although they occurred in membranes facing living
 333 pericycle cells.

334 *LOTR1 defines a novel pathway controlling CASP domain positioning*

335 While no other knock-out mutant resembles *lotr1*, overstimulation of the
 336 SCHENGEN (SGN) pathway by external application of CIF2 peptide, leads to
 337 ectopic formation of CASP domains somehow resembling the phenotype of
 338 *lotr1*. The CIF2 peptide is perceived by the Leucine-rich repeat receptor
 339 kinase SGN3 (also called GSO1), a key receptor controlling a novel
 340 apoplastic barrier surveillance pathway (Doblas et al. 2017; Nakayama et
 341 al. 2017). A complete absence of SGN3 activity in knock-out mutants leads
 342 to an incomplete fusion of otherwise correctly positioned CASP micro-
 343 domains. In order to test, whether *lotr1* ectopic patches are due to an
 344 overactive SGN pathway, we generated double knockouts of *lotr1* in
 345 combination with *sgn1*, *sgn2* or *sgn3*. Single mutants *sgn1* and *sgn2*
 346 showed sporadic disruptions in their central CSD while *sgn3* displayed
 347 more numerous holes in comparison, agreeing with previously published
 348 data (J Allassimone et al. 2016; Doblas et al. 2017) (Fig. 4A). Double mutants
 349 displayed *lotr1*-typical ectopic deposition of CASP1-GFP, as well as
 350 discontinuities of the central domain. Although discontinuities in the
 351 central CSDs were visible in *lotr1* itself, these occurred mostly at cell
 352 junctions, while *sgn*-typical interruptions were present throughout the
 353 length of the CSD. Both types of disruptions were found in the respective
 354 double mutants. Moreover, no significant difference between the double
 355 and single mutants was observed in blockage of apoplastic tracer PI, a test
 356 for CS functionality (Fig 4C). This clear additivity of phenotypes suggests
 357 that LOTR1 and SGN mutants are involved in separate pathways. It
 358 certainly excludes a model whereby ectopic patch formation of *lotr1* is due
 359 to an overactive SGN pathway. We also tested a possible connection to
 360 ESB1. Again, *lotr1 esb1* double mutants displayed a addition of parental
 361 phenotypes with an *esb1*-typical string-of-pearl-stage central CSD and
 362 *lotr1*-typical ectopic CASP deposits and showed no enhanced apoplastic
 363 barrier phenotype, indicating that LOTR1 also acts independently of *esb1* in
 364 CS domain formation.

365 The model of SGN3-CIF1/2 integrity signaling predicts enhanced
 366 lignification of endodermal cell corners and an early onset of suberization

whenever the diffusion barrier is broken in such a way that CIF peptides cannot be restricted anymore. The previously described enhanced suberin phenotype in *lotr1* is consistent with such a barrier-defect induced activation of the SGN pathway, due to its large corner gaps in the CS domain. The previously reported large gaps in lignification of CSs (Li et al., 2017), fit the discontinuous CSDs in this mutant. Presence of KMnO₄-stained lignin throughout the endodermal cell corners found in some samples during our ultra-structural analysis (Fig. S1E) resembled SGN3-induced ectopic lignification. To unambiguously demonstrate ectopic lignin deposition outside the CASP1-labelled domain, we employed a novel clearing protocol that enables co-observation of fluorescent markers, e.g. CASP1-GFP, and lignin specific stains, such as Basic Fuchsin (Ursache et al. 2018) (Fig. S4A). As described, in addition to the central CS, lignin accumulates at ectopic CASP1-deposits in *lotr1* (FIG 4B). However, cell corner lignification not associated with CASP1 domains was observed at sites of gaps in the CSD, but not at places where the CSD was continuous. This nicely supports the recently proposed model in which SGN3-stimulation can induce local lignification with subcellular precision (Fujita et al. 2020). Consistently, enhanced lignin deposition in cell corners was not present in the *lotr1 sgn3* double mutant, confirming that this ectopic lignin is caused by activation of the barrier surveillance mechanism. *lotr1*-typical patches, by contrast, were still lignified in the double mutant, supporting the concept of two separate pathways controlling lignin deposition in the endodermis: A developmental lignification pathway building up CS-type lignin and the SGN3-mediated ectopic lignin deposition that compensates in case of barrier defects. Exogenous application of CIF2 peptide further demonstrated that the SGN3-pathway is functional in *lotr1* (Fig. 4D). In the presence of CIF2, both genotypes formed ectopic lignin at cell corners.

LOTR1 is broadly expressed in the root

Due to a lack of available expression data in public databases (*LOTR1* was not included on the ATH1 microarrays), it was crucial to generate a

reporter line. We therefore placed a nuclear-localized tdTomato under the control of a 3 kb *LOTR1* promoter fragment. We observed strong promoter activity in the root meristem in nearly all tissue layers and cells, including QC and columella, with the highest expression found in the stele (FIG 4E). Very low expression was found in the epidermis and ground cell lineage, with promoter activity visible in cortex/endodermal initials and their endodermis daughter cells, but not in the cortex daughter. In older meristematic cells, *LOTR1* expression further reduced in epidermis and endodermis, whereas stele cells continued to show strong promoter activity. This pattern continued past the elongation zone, where stele expression was still strong with some residual signals found in epidermal and endodermal cells. Inside the stele itself, *LOTR1* was specifically absent in xylem precursor and xylem-adjacent procambium cells, whereas pericycle, phloem precursor and phloem-adjacent procambium cells displayed strong expression (FIG 4E). Recently published single-cell RNA sequencing results showed strong expression of *LOTR1* in clusters corresponding to phloem, stele, xylem, and root cap cells, confirming our promoter activity analysis (Ryu et al. 2019; Denyer et al. 2019). Despite this broad expression pattern, *lotr1* mutants displayed only phenotypes related to endodermal differentiation, i.e. ectopic CASP deposition and disrupted CSDs causing compensatory lignification and suberin deposition, which themselves are likely responsible for the previously observed delay in lateral root development and low-calcium-sensitivity phenotype (Li et al. 2017). Consequently, if the expression pattern points to a much broader function for *LOTR1* outside of endodermal differentiation, these additional functions are probably redundantly fulfilled some of the many homologs of *LOTR1* (Fig. 4F).

LOTR1 codes for a putative cell wall protein with a predicted signal peptide, a putative auto-inhibitory domain (Neprosin-AP), and a Neprosin domain, recently identified as novel proline protease domain in an enzyme found in the digestive fluids of pitcher plants (Rey et al. 2016; Schrader et al. 2017). We found 42 other proteins in the Arabidopsis proteome with a domain

structure like LOTR1, i.e. having both Neprosin-AP and Neprosin domains, although subsequent phylogenetic analysis showed considerable variation among the family members. Average identity towards LOTR1 among this “HOMOLOGS-OF-LOTR1” (HOLO) family was only 38 % ($\pm$ 12) with the three closest homologs standing out at $\sim$ 70 % (Table S1). Phylogenetic clustering identified 5 subgroups (A:E) with instances of gene-duplication events visible especially in group C, where 8 homologs were found in consecutive loci on chromosome 4, highlighting the variable nature of this family (Fig. 4F). Nevertheless, putative orthologs of LOTR1 can be identified in both mono- and eudicot species, indicating that, although some family members seem to evolve rapidly, LOTR1 has a deep conservation of function, expected for a regulator of CS formation.

While 34 of the putative homologs showed predicted signal peptides for apoplastic localization, we also found 5 proteins in the family predicted to have a single N-terminal transmembrane domain with their active domain outside in the apoplast (Fig. 4F, Table S1). Thus, the HOLO family are interesting candidates for proteolytically processing membrane or cell wall proteins relevant for domain establishment. Single-cell RNAseq expression profiles of *LOTR1*’s four closest homologs partially overlapped in cell clusters for QC/meristem (At1g23340, At1g70550, At5g56530), root cap (At1g10750, At1g70550), phloem (At1g70550, At5g56530), and xylem (At1g10750, At23340, At1g70550, At56530) (Ryu et al. 2019), explaining the lack of any meristematic phenotypes in *lotr1*. However, while 8 separate mutants with this unique patchy phenotype identified in the *LORD-OF-THE-RINGS* screen all turned out to be alleles of *LOTR1*, none of its homologs were found, indicating they likely have separate roles.

Mature and active Neprosin has been reported to consist only of its functional domain (PF03080) (Rey et al. 2016), presumably undergoing processing during or after secretion. However, only full-length protein was found in functional, full-length complementation lines (Fig. S4C), suggesting that LOTR1 might retain its putative auto-inhibitory Neprosin-AP domain. Expression of artificially truncated versions of LOTR1 consisting of only its

463 Neprosin domain or only its putatively auto-inhibitory Neprosin-AP domain
464 resulted in extensive intracellular labelling and absence of
465 complementation (Fig. S4D), supporting that LOTR1 does not undergo post-
466 processing.

467 As many of the Neprosin-containing proteins are still annotated as putative
468 carboxyterminal proteases, we investigated potential homologies towards
469 such enzymes. Database searches identified *E.coli* protein mepS, a cell wall
470 protease required for cell wall expansion, as a potential candidate.
471 Although displaying only 20 % identity to LOTR1's Neprosin domain,
472 structural comparison revealed that three crucial amino acids in the
473 catalytic center of mepS, one cysteine and two histidines, are present in
474 LOTR1, its three closest homologs, as well as Neprosin itself (Fig. S5A,B).
475 Expression of a full-length LOTR1 version with putative active-site residues
476 C255 and C260 mutated to alanine showed apoplastic localization similar
477 to unmutated LOTR1, but was unable to complement the *lotr1* phenotype
478 (Fig. S4D). This suggests an important functional role for these amino acids
479 in LOTR1 and supports a protease function for this enzyme class in
480 *Arabidopsis*.

481 *Tissue specific complementation indicates a non-cell autonomous action for*
482 *LOTR1.*

483 The strong expression pattern of LOTR1 suggests a function in the inner
484 stele tissues and a non-autonomous action of LOTR1 on endodermal
485 differentiation. With a potential protease function an action on the equally
486 stele-expressed CIF peptides was conceivable, but inconsistent with our
487 genetic analysis. In order to establish the site of LOTR1 action, we
488 generated LOTR1 fusion proteins and were able to complement the loss-of-
489 function phenotype with LOTR1 N-terminal (behind the signal peptide), but
490 not C-terminal fusion proteins, indicating that a free C-terminus is
491 important for its functionality (Fig. 5A). Interestingly, the *lotr1-8* allele,
492 where a late STOP-codon truncated the protein by just 16 aa leads to a
493 knock-out phenotype, further suggesting an important role of the c-
494 terminal extremity. In agreement with the promoter activity, fluorescence

of the fusion construct was mainly visible in the apoplastic space between stele cells, with outer tissues showing very low levels of accumulation (Fig. 5A).

To determine if the very low expression observed in the endodermis is nonetheless responsible for LOTR1 function, we generated lines driving LOTR1 expression from stele-, endodermal-, or cortex-specific promoters in the *lotr1* mutant background. We found continuous CSDs and lack of patches only when expressed from the stele specific SHORTROOT (SHR) and CIF2 promoters, but not from endodermal SCR, CASP1, and ELTP, nor cortex specific C1 promoters (Fig. 5C). Covering early, intermediate, and late endodermal development, the lack of complementation by endodermal promoters demonstrated that LOTR1 was not acting in the endodermis to fulfil its role in CSD formation. Peculiarly, expression of the same construct under the ubiquitous 35S CaMV promoter did also not seem to complement the underlying *lotr1* phenotype. Several scenarios could explain this finding: LOTR1 function could require exclusive expression in the stele, although its native promoter showed some weak outer tissue activity. Alternatively, over- and misexpression of the construct might lead to the same patchy *lotr1* phenotype, but through dominant interference. We therefore created an inducible complementation construct, where the 3kb *LOT1* promoter fragment was fused to the estrogen-inducible artificial XVE transcription factor, with a subsequent minimal 35S promoter driving expression of the native gene (Zuo, Niu, and Chua 2000; Siligato et al. 2016). This allows control of expression strength of the target genes. Upon induction of this construct in the *lotr1* background, we observed a full complementation of the phenotype even under strong induction conditions (Fig. 5B). Control plants without inducer showed the typical phenotype. Thus, expression strength was not causing the seeming absence of complementation in the 35S lines, leaving mis- expression in outer tissues as a probable cause for the observed phenotype.

We therefore tested whether ubiquitous expression via 35S would lead to a dominant-interference phenotype in wildtype. We outcrossed all generated lines to wildtype CASP1-GFP plants to test for genetic dominance of the transformed constructs. Previously complementing constructs LOTR1::LOTR1, SHR::LOTR1, and CIF2::LOTR1 continued to display a wild-type CSDs and no occurrence of *lotr1* typical patches in the F1 generation (Fig. 5C). Expectedly, wild-type phenotypes were also observed for the non-complementing, endodermis-specific SCR and CASP1 driven lines. Remarkably, not only expression driven by 35S, but also from cortex-specific C1 and endodermal/cortex-expressing ELTP promoters caused ectopic CASP1-GFP patches like those observed in uncomplemented *lotr1* in the F1 progeny. We confirmed this dominant phenotype in the following segregating F2 generations, where all plants harboring ELTP, C1, or 35S constructs showed patch formation. Only 7/37 (ELTP), 9/42 (C1), and 8/45 (35S) plants displayed wildtype like continuous CSDs and no patches in the respective F2 populations. Since the dominant phenotype was not easily distinguishable from the normal *lotr1* loss-of-function phenotype, our observed ratios (18.9 %, 21.4 %, 17.7 %) matched the expected 3/16 (18.75 %) Mendelian segregation ratio for a wildtype phenotype (non *lotr1*-homozygous, plus absence of dominant transgene). Apoplastic barrier functionality tests in these lines confirmed our observations (Fig. 5D).

548

549 Discussion

Establishment of a functional apoplastic barrier relies on the correctly localized deposition of lignin at the median position between endodermal cells (Naseer et al. 2012; Geldner 2013). Although many components involved in this process have been identified in the last decade, we still poorly understand the mechanism by which these cells achieve the precise formation of Casparian Strips in such a way that a single, thin, continuous ring of lignin is formed in between endodermal cells. In our current work, we provide new insights into this mechanism by demonstrating strict

coordination of CASP deposition in membranes of adjacent endodermal cells. Independent of any known CS mutant, this coordination pathway appears to allow a local initial pairing of forming CASP micro-domains, across the cell wall of neighboring endodermal cells, which promotes their stabilization and eventual fusion. This results in the necessity for having a functional, differentiating endodermal cell neighbors in order to form a continuous CS. Without a neighboring cell in the same developmental state, wild-type endodermal cells abort CS development after the initial formation of CASP microdomains, which eventually disappear. In our current understanding, CASPs are initially secreted in a non-localized fashion (Roppolo et al. 2011), yet rapidly form aligned CASP micro domains. This is initiated by a specific exocyst complex, defined by subunit EXO70A1, whose accumulation at the site of the future CS precedes CASP accumulation and is necessary for their targeted secretion to this domain (Kalmbach et al. 2017). Accordingly, *exo70a1* mutants display a high number of non-localized and non-functional, unstable CASP microdomains. What mechanism could account for the selective stabilization of CASPs in domains facing intact neighbors of the same differentiation state, but not towards dead, undifferentiated cells or cells of a different cellular identity? One explanation would postulate the existence of a stabilizing short-range signal, produced by CASPs domain that can only act *in trans*, not *in cis*. However, such an exclusive *in trans* action is difficult to conceive with small, released molecules able to diffuse across the cell wall space. It would be much easier to conceive with filaments, reaching across the cell walls between domains, but there is no evidence for the existence of such filament-like structures. Alternatively, a cumulative threshold for a short-range, stabilizing signal produced by the microdomains can be postulated, that could only be reached when both cells produce it. However, such a model would require a very fine-tuned thresholding. Although a promising candidate for a factor involved in this coordination process, our detailed analysis of the *lotr1* mutant demonstrates that it does not affect the coordination of the properly localized micro-domains between cells, nor does it allow for their formation without neighbors. Nevertheless, absence

of LOTR1 activity clearly allows for formation of non-paired, stable, and fully functional microdomains, oriented towards non-endodermal neighbors. Therefore, it is most likely responsible for inhibiting sporadic micro-domain formation throughout the endodermal membrane when CASPs are starting to focalize from their ubiquitous distribution in the plasma membrane into aligned micro-domains. The peculiar formation of Casparian half-strips in *lotr1*, further indicates that lignin deposition into the CS cell wall is separately performed by both contributing cells, which would make a tight coordination of neighboring CASP domains all the more critical for achieving a fully sealed cell wall space. Restricted mostly to the stele in mature regions of the root, we provide evidence that this putative cell wall protease might act on a target originating outside of its own expression profile. *lotr1* could only be complemented using stele-specific promoters and neither endodermal, cortex nor ubiquitous expression leads to complementation. Importantly we discovered that wildtype plants displayed a dominant *lotr1*-like phenotype with ectopic patches, disrupted CSDs, and a corresponding delay in PI block when LOTR1 was mis-expressed in the cortex. How could ectopic expression of a putative cell wall protease in the cortex interfere with the action of the same protease in its wild-type expression domain in the stele? The most parsimonious explanation we can conceive is that a LOTR1 substrate is produced in the outer cortical tissues and has to diffuse into the stele for activation by stele-expressed LOTR1 (Fig. 6). Expression of LOTR1 in the cortex would lead to a premature activation of its substrate in the cortex and not allow it to reach the stele. It would thus deplete the endogenous LOTR1 of its substrate and cause the observed dominant loss-of-function phenotype. This explanation thus draws a “reverse” SGN3/CIF pathway model where a cortex-specific substrate passes across the endodermal cell layer to be activated by a stele-specific enzyme complex, destabilizing CASPs and preventing domain formation at ectopic positions.

621

622 Acknowledgments

623 We wish to thank the Central Imaging Facility (CIF) and the Electron
 624 Microscopy Facility (EMF) of the University of Lausanne for their technical
 625 expertise. We thank Baohai Li and Takehiro Kamiya (Tokyo University) for
 626 initial discussions on the project and exchange of material. We also thank
 627 Hiroko Uchida and Marie Barberon for their graphical work. This work was
 628 supported by an ERC Consolidator Grant (GA-N°: 616228—ENDOFUN) and
 629 two SNSF grants (CRSII3_136278 and 31003A_156261) to N.G., and a
 630 Federation of European Biochemical Sciences Postdoctoral Long-Term
 631 Fellowship to P.M..

632 **Author contributions**

633 AK, PM, LK, and NG conceived and designed the project. AK, PM, and DDB
634 performed experiments and AK and PM performed image quantification.
635 AK and NG wrote the manuscript and all authors revised the manuscript.

636

637 **Declaration of Interests**

638 The authors declare that they have no conflict of interest.

639 Figure legends

640 Fig. 1: Ectopic domain formation in *lotr1*.

641 A Schematic representation of endodermal maturation with stages of
 642 Casparian Strip (CS) development (green). Elongated endodermal cells
 643 focalize CASP1-GFP into aligned micro-domains which are subsequently
 644 fused into a continuous ring around each endodermal cells.

645 B CASP1-GFP localization in wildtype and *lotr1* with focus on patch
 646 localization. Images depict maximum intensity projections of z-stack
 647 images. 3D reconstruction image depicts region indicated in *lotr1* overview
 648 image, section view images are annotated by yellow (top) and blue
 649 (bottom) arrows. Overview images scale bars: 20 μm ; 3D reconstruction &
 650 section views scale bars: 10 μm .

651 C 3D schematic for visualization of median endodermal cell section with
 652 annotated cell faces.

653 D TEM section of *lotr1* obtained at 2 mm from root tip. Overview of section
 654 with focus on regular CS and adjacent ectopic patch. Closeup indicated by
 655 dashed black line in overview image; en = endodermis, pe = pericycle, co =
 656 cortex, scale bars = 2 μm .

657 E Consecutive TEM sections at 2 mm from root tip, with and without
 658 KMnO_4 staining, position indicated in (D). Dark deposits indicate electron-
 659 dense MnO_2 precipitation caused by reaction with lignin. Note that in the
 660 case of ectopic patches, the staining is restricted to the endodermal side of
 661 the cell wall; en = endodermis, pe = pericycle, interspaced white line
 662 depicts extend of CS-like cell wall morphology, scale bar = 1 μm .

663 F Localization of enzymes necessary for CS lignification in wildtype Col-0
 664 and *lotr1*, pictures depict cells as seen in (C). Cellulosic cell walls are stained
 665 with Calcofluor White and depicted in white in overlay images, scale bar =
 666 10 μm .

667

668 Fig. 2: CASP deposition is coordinated between neighboring endodermal 669 cells.

670 A Double membrane phenotype visible with CASP1::CASP1-GFP during
 671 string-of-pearls stage of CS development, scale bars depict 10 μm . Red and
 672 grey arrows indicate separate membranes measured in (B).

673 B Comparison of GFP intensity between adjacent membranes indicated in
 674 (A). Pixel intensity was measured along each membrane and relative GFP
 675 intensity was adjusted to mean intensity of each membrane. Correlation of

676 original intensities (R-squared) was determined by fitting of a linear model
677 with indicated probability of fit (p-value).

678 C Localization of CSD marker CASP1-GFP in comparison to cell wall protein
679 ESB1-mCherry at early and mature CSs, scale bar depicts 5 μ m.

680 D,E Double membrane phenotype of CASP1-GFP before and after ablation
681 of one adjacent endodermal cell in early (D) and late (E) CSs. Cell walls (red)
682 are stained with Propidium iodide (PI), yellow arrows indicate intensity
683 profiles measured in F-I; asterisks indicate ablated cell, scale bars = 5 μ m.

684 F-I Quantification of CASP1-GFP and PI intensity before and after cell
685 ablation. Intensity was quantified in a 7-pixel wide line indicated in (D) and
686 (E).

687

688 **Fig. 3: Endodermal neighbors are required for proper CSD establishment**

689 A Endodermal cell ablation leads to instability of the CSD in membranes
690 facing dead cells. Endodermal cells were ablated 3-5 cells prior to onset of
691 CASP1-GFP (white) expression and followed over indicated time spans. First
692 image depicts overview after ablation with cells outlined with PI; # depicts
693 remaining live endodermal cell, dashed yellow line depicts cell outlines of
694 ablated cells, arrow indicates onset of CASP expression, dashed green and
695 red rectangles indicate quantification areas shown in (B), scale bar = 20
696 μ m.

697 B Quantification of GFP fluorescence over time in membranes facing live
698 (green) and dead (red) adjacent endodermal cells. Intensity depicted in
699 relation to maximum total GFP intensity.

700 C Ablation of all adjacent endodermal cells prevents fusion of CSDs.
701 Endodermal neighbors were ablated at timepoint 0 and CASP1-GFP
702 expression (white) followed over time in remaining cell (#), PI (red)
703 highlighted cell outlines and confirmed destruction of cells; yellow arrow
704 indicates initial CASP microdomains, scale bar = 50 μ m.

705 D CASP instability in membranes facing ablated cells is independent from
706 CASP expression. Cells ablated 3-5 cells prior to onset of CASP expression,
707 cell outline depicted with dashed yellow line, arrow indicates stable CASP
708 formation, scale bar = 100 μ m.

709 E CASP fusion occurs only if both neighboring cells reach expression
710 threshold. Endodermal differentiation was induced via 0.5 μ M β -estradiol
711 and CASP1-GFP expression followed over the next 12 h. Yellow arrows
712 indicate CASP deposits in the membrane, dashed green and red rectangles
713 indicate areas for quantification shown in (F).

714 F Quantification of GFP fluorescence over time in membranes reaching
715 local stability threshold (green) and those below the threshold (red).

716

717 **Fig. 4: LOTR1 restricts CSD establishment through independent pathway.**

718 A CASP1-GFP fluorescence of known CS mutants in wildtype and *lotr1*
719 background. Yellow arrows indicate parental CS mutant phenotype while
720 blue arrows highlight *lotr1* specific phenotypes; scale bar = 20 μ m.

721 B Enhanced lignification in *lotr1* is SCHENGEN-dependent. Ectopic lignin
722 (magenta) accumulates in *lotr1* at disruptions of the CASP1-GFP (green)
723 marked CSD. Lignification of the central domain and ectopic patches is
724 unaffected by disruption of *SGN3*, scale bar = 20 μ m.

725 C Apoplastic barrier phenotypes of *lotr1* double mutants. Formation of
726 apoplastic barrier was determined by penetrance of apoplastic tracer PI to
727 the stele; n = 10, significance determined by one-way ANOVA and separate
728 groups identified with Tukey-Kramer test.

729 D SCHENGEN-pathway is unaffected in *lotr1*. Wildtype and *lotr1* seedlings
730 were treated for 24 h with 100 μ M synthetic CIF2 peptide and lignin
731 accumulation (magenta) outside the regular CSD domain (green) analysed.
732 Cell walls were stained with Calcofluor White, yellow arrows indicate
733 ectopic lignin accumulation, scale bar = 5 μ m.

734 E LOTR1 expression indicates additional roles. Promoter activity highlighted
735 by tdTomato fluorescence in 5 day old seedlings; cells outlined by
736 Propidium iodide (white); mid-view regions depicted below highlighted by
737 continuous rectangles (a,b) and section views indicated by dash-dotted
738 lines (c,d), inlay shows colour profile of *LOTR1* expression intensity. a
739 Single-plane section through root meristem region, arrow indicates cortex-
740 daughter cell. b Single-plane section after cell elongation, arrows indicate
741 expression in endodermis (top arrow) and epidermis (bottom arrow). c
742 Maximum-intensity projection of section view of z-stack through meristem
743 region indicated in top picture. d Maximum-intensity projection of section
744 view of z-stack through mature region indicated in top picture; * denotes
745 endodermal cell lineage, scale bars: *top*: 100 μ m; *a,b*: 20 μ m; *c,d*: 50 μ m.

746 F Phylogenetic analysis of *HOMOLOGS-OF-LOTR1* (HOLO) family in
747 *Arabidopsis thaliana*. Homologs of LOTR1 in *A.thaliana* were identified and
748 grouped into five subfamilies by phylogenetic distance, LOTR1 (At5g50150)
749 highlighted in red, underlined genes have predicted N-terminal
750 transmembrane domains.

751 **Fig. 5: Stele-specific LOTR1 restricts CSD establishment through a**
 752 **cortex-expressed, mobile target.**

753 A Complementation of *lotr1* phenotype. N-terminal fusion construct
 754 expressed under native *LOTR1* promoter complements ectopic CSD
 755 phenotype. Scale bars: *left* 100 μ m, *section views* 20 μ m.

756 B Overexpression of *LOTR1* in wild-type and *lotr1* backgrounds does not
 757 affect complementation. CASP1-GFP phenotype of seedlings grown for 5
 758 days on control or 5 μ M β -estradiol; scale bar: 20 μ m.

759 C Mis-expression of LOTR1 in cortical cells causes dominant loss-of-
 760 function phenotypes. CASP1-GFP phenotypes of seedlings expressing
 761 LOTR1-complementation construct used in (A) under indicated promoters.
 762 Activity of used promoter indicated in schematics below; blue arrows
 763 indicate *lotr1*-like phenotypes; EP = epidermis, CO = cortex, EN =
 764 endodermis, ST = stele; scale bars = 20 μ m.

765 D Apoplastic barrier phenotypes of tissue specific complementation lines in
 766 wildtype (white) and *lotr1* (grey) background. Cells after onset of
 767 elongation counted until penetrance of PI was blocked. n = 10, significance
 768 determined via one-way ANOVA and independent groups identified via
 769 Tukey-Kramer post-hoc test.

770 **Fig. 6: Speculative model of LOTR1 function**

771 **Wild-type:** LOTR1 is a predicted protease (*scissors*), experimentally
 772 determined to be expressed in the stele and to localize in the cell wall. We
 773 speculate that LOTR1 cleaves a cortex-derived substrate (*grey, dark-red*
 774 *coffee bean, inactive*), activating it in the stele (*red half-bean, active*). This
 775 substrate then inhibits ectopic CASP-domain formation (*green*) at the stele-
 776 facing endodermal surface by unknown means. ***lotr1* mutant:** Absence of
 777 LOTR1 would not allow activation of the ectopic CASP-domain inhibitor in
 778 the endodermal, stele-facing apoplast, leading to the observed formation
 779 of ectopic, stable CASP-domain predominantly at the stele-facing side of
 780 the endodermal surface. **LOTR1 cortex mis-expression:** This model explains
 781 the observation that cortical mis-expression of LOTR1 dominantly
 782 interferes with wild-type LOTR1 action, if it would precociously cleave and
 783 activate the LOTR1 substrate, not allowing it to reach the stele to be
 784 activated by wild-type LOTR1. This would lead to the observed, similar
 785 phenotype than the *lotr1* knock-out.

786

787

788 **STAR Methods**

789 *Plant material and growth conditions*

790 All experiments were performed in the *Arabidopsis thaliana* Columbia-0
 791 ecotype. T-DNA insertion lines obtained from NASC: *lotr1-10*
 792 (SALK_051707) (Li et al. 2017), *sgn3-3* (SALK_043282) (Pfister et al. 2014),
 793 *sgn1-2* (SALK_055095C) (J Alassimone et al. 2016), *sgn2-2* (SALK_009847)
 794 (Doblas et al. 2017); *myb36-2* (GK- 543B11) (Kamiya et al. 2015), *esb1-1*
 795 was kindly shared by Prof. David Salt's group (Hosmani et al. 2013), and the
 796 *lotr1-1* allele was identified in the *LOTR*-screen (Li et al. 2017). Inducible
 797 pUBQ10>>XVE::MYB36 were obtained from the TRANSPLANTA collection
 798 (N2102512 and N2102513) and crossed to CASP1::CASP1-GFP. If not
 799 otherwise stated, plants were grown as follows: Seeds were surface
 800 sterilized and sown on half-strength Murashige-Skoog medium (Duchefa)
 801 solidified with 0.8 % plant agar (Duchefa). After stratification for 2 days at 4
 802 °C, seeds were germinated in growth chambers with long-day conditions
 803 (16 h light/8 h dark) and temperature cycling between 22 °C/19 °C
 804 (day/night). 5 days after germination, seedlings were directly analysed or
 805 cleared and stained with ClearSee as described (Ursache et al. 2018). For
 806 long-term imaging, agar blocks with seedlings were cut and transferred
 807 into microscope chamber slides (Marhavý and Benková 2015; Marhavý et
 808 al. 2016) and equal volume of double-concentrated β -estradiol added
 809 before imaging.

810 *Plasmid construction*

811 A 3 kb promoter fragment of *LOTR1* was generated by 5'-
 812 aacaggtctcgacctctttctatctgtttgtacctaagt-3' and 5'-
 813 aacaggtctcatgttaactagagaatgtacggcttgttt-3' and cloned via Eco31I-
 814 restriction sites into GreenGate entry module vector pGGA000
 815 (Lampropoulos et al. 2013). Genomic fragment of *LOTR1* coding sequence
 816 was amplified with 5'-
 817 aacaggtctcaggctcgatgTCAGCTATTCATCTTAAAAACCAAACTTCA and 5'-
 818 aacaggtctcaCTGAAGGACACCTTGGGTTCCG-3' and cloned into pGGC000.
 819 mScarlet-I was amplified from pmScarlet-I_C1 (Bindels et al. 2016)

820 obtained from Addgene and secretion peptide was added by tandem PCR
 821 amplification with 5'-
 822 gctctttccctctatctcctgcccaatccagccactagtATGGTGAGCAAGGGCGAG-3' (FW1)
 823 and 5'-
 824 aacaGGTCTCaAACAAatgaaagccttcacactcgctctcttcttagctctttccctctatctcctg-3'
 825 (FW2) and 5'-aacaGGTCTCaAGCCCTTGTACAGCTCGTCCATGC-3' (RV) and
 826 cloned via Eco31I sites into pGGB000. Final destination clones were
 827 obtained by subsequent GoldenGate reaction with pGreenII based
 828 pGGZ003 and transformed into *Arabidopsis* using floral dip.

829 *Confocal imaging*

830 Imaging was performed on Leica SP8 or Zeiss LSM880 microscopes,
 831 excitation and emission as follows (excitation, detection): Calcofluor White
 832 (405 nm, 425 – 475 nm), GFP (488nm, 500 – 550 nm),
 833 mCherry/tdTomato/mScarlet-I (563 nm, 580 – 650 nm), PI (563 nm, 580 –
 834 650 nm), basic Fuchsin (563 nm, 580 – 650 nm).

835 *PI assay*

836 PI was performed as described in Lee et al. 2013 with following changes:
 837 Seedlings were incubated in 10 µg/ml PI dissolved in water for 5 min and
 838 imaged immediately afterwards. Number of endodermal cells determined
 839 after onset of elongation (defined as first endodermal cell with length to
 840 width ratio of at least 2, i.e. at least twice as long than wide).

841 *Transmission electron microscopy (TEM)*

842 5-day-old *Arabidopsis* seedlings were fixed in glutaraldehyde solution
 843 (EMS, Hatfield, PA) 2.5% in 100 mM phosphate buffer (pH 7.4) for 1 hour at
 844 room temperature. Then, they were post-fixed in osmium tetroxide 1%
 845 (EMS) with 1.5% of potassium ferrocyanide (Sigma, St. Louis, MO) in
 846 phosphate buffer for 1 hour at room temperature. Following that, the
 847 plants were rinsed twice in distilled water and dehydrated in ethanol
 848 solution (Sigma) at gradient concentrations (30% 40 min; 50% 40 min; 70%
 849 40 min; two times (100% 1 hour). This was followed by infiltration in Spurr
 850 resin (EMS) at gradient concentrations (Spurr 33% in ethanol, 4 hours;

851 Spurr 66% in ethanol, 4 hours; Spurr two times (100% 8 hours) and finally
852 polymerized for 48 hours at 60°C in an oven. Ultrathin sections 50 nm thick
853 were cut transversally at 2mm ± 0.2 mm from the root tip, on a Leica
854 Ultracut (Leica Mikrosysteme GmbH, Vienna, Austria) and two consecutive
855 sections were picked up on a nickel slot grid 2X1 mm (EMS) coated with a
856 polystyrene film (Sigma).

857 *Lignin staining with permanganate potassium (KMnO₄) using TEM*

858 Visualization of lignin deposition around Casparian strip was done using
859 permanganate potassium (KMnO₄) staining (Hepler, Fosket, and Newcomb
860 1970). The first section was imaged without any post-staining. Then the
861 sections were post-stained using 1% of KMnO₄ in H₂O (Sigma, St Louis, MO,
862 US) for 45min and rinsed several times with H₂O. Then the second section
863 of the grid was imaged. Micrographs were taken with a transmission
864 electron microscope FEI CM100 (FEI, Eindhoven, The Netherlands) at an
865 acceleration voltage of 80kV and 11000X magnifications (pixel size of
866 1.851nm), with a TVIPS TemCamF416 digital camera (TVIPS GmbH,
867 Gauting, Germany) using the software EM-MENU 4.0 (TVIPS GmbH,
868 Gauting, Germany). Panoramic were aligned with the software IMOD
869 (Kremer, Mastronarde, and McIntosh 1996).

870 *Cell ablation*

871 Endodermal cells were ablated using a MaiTai-SpectraPhysics laser at 800
872 nm integrated into a Zeiss LSM880 microscope. A ROI outlining the cells to
873 ablate was used and cells were ablated with 2 % power and 0.8 µs pixel
874 dwell time. Cell destruction was confirmed using transmitted light and PI
875 staining.

876 *Western blot*

877 Roots of 5-day old seedlings grown on ½ MS medium were cut and
878 weighted before shock-freezing in liquid nitrogen. Samples were then
879 homogenized using TissueLyser II (Qiagen). Proteins were then denatured
880 by addition of 3x (v/w) 1x NuPAGE LDS sample buffer + 50 mM DTT
881 (Invitrogen, NP0007) and heating for 5 min at 70 °C. Cell debris was

882 removed by centrifugation at 1'000 *g* for 5 min before separation of
 883 proteins on pre-cast 12 % Bis-Tris SDS-PAGE gels (iD PAGE, Eurogentec).
 884 After electrophoresis, proteins were transferred onto PVDF membrane
 885 using Pierce FastBlotter G2 semi-dry blotting. Membranes were then
 886 blocked (5 % skim milk in TBS) for 1 h before incubation with anti-RFP
 887 antibody (1:1000, Chromotek 6G6) overnight at 4 °C. After washing the
 888 membrane three times with 1x TBS + 0.1 % Tween (TBS-T), blot incubated
 889 for 2 h at room temperature with anti-mouse-HRP antibody (1:5000).
 890 Finally, after washing blot three times with TBS-T, HRP activity was
 891 detected using SuperSignal West Femto Kit (Thermo Scientific) and GE
 892 ImageQuant LAS 500.

893 *Phylogenetic analysis*

894 Homologs of LOTR1 were identified by searching the *Arabidopsis thaliana*
 895 proteome for proteins containing both Neprosin-AP (PF14365) and
 896 Neprosin domains (PF03080). Sequence similarity was compared using
 897 MUSCLE (EMBL-EBI, Madeira et al. 2019). Phylogenetic analysis was done
 898 using the NGPhylogeny.fr online-tool with PhyML+SMS settings. Final tree
 899 was assembled and annotated with iTOL online tool (Letunic and Bork
 900 2019). Conserved residues were identified using pre-aligned sequences and
 901 the online-weblogo tool from the University of Berkeley
 902 (<https://weblogo.berkeley.edu/logo.cgi>) (Crooks et al. 2004).

903 *Statistical analysis*

904 Statistical analysis was done in R software (R Core Team, 2013)
 905 (<https://www.r-project.org/>). For multiple group comparisons, one-way
 906 ANOVA was performed, and significantly different groups identified with
 907 Tukey-Kramer post-hoc test.

908

909 **Fig. S1: Establishment of ectopic domain formations in *lotr1*.**

910 A Localization of ectopic deposition of CASP1-GFP (green). Section view of
911 z-stack through roots 5-10 cells after onset of protoxylem differentiation,
912 cell wall stained with Calcofluor White (white) and crops shown on top
913 outlined in overview pictures below (dashed yellow line); scale bar = 50
914 μm , c = cortex, e = endodermis, p = pericycle.

915 B Quantification of ectopic CASP1-GFP patches. Number and position of
916 ectopic patches were determined per cell and depicted as ratio of patches
917 facing pericycle cells vs total patch number, maximum of 3 cells counted
918 per seedling, $n_{\text{total}} = 11$.

919 C Deposition of CASP1-GFP in *lotr1*. CASP1-GFP fluorescence depicted
920 throughout CSD formation in *lotr1*; scale bars = 5 μm . Kymograph image at
921 bottom illustrates fluorescence over time in line indicated above, individual
922 patch formation visible as independent pyramid-like structures (yellow
923 arrows).

924 D TEM section of wildtype at 2 mm. Closeup indicated by dashed black line
925 in overview image, en = endodermis, pe = pericycle, scale bars = 2 μm .

926 E Consecutive TEM sections at 2 mm from root tip, with and without
927 KMnO_4 staining. Dark deposits indicate electron-dense MnO_2 precipitation
928 caused by reaction with lignin; en = endodermis, pe = pericycle, black
929 arrows indicate CS-like cell wall with attached plasma membranes, white
930 arrows highlight plasmolyzed membranes detached from the cell wall,
931 scale bar = 1 μm .

932

933 **Fig. S2: CASP1-GFP membrane coordination.**

934 Double membrane phenotype in CS mutants, scale bars = 5 μm . R^2 value
935 depicts Spearman rho correlation coefficients of fluorescence of 5 pixel
936 wide lines following each membrane.

937

938 **Fig. S3: Cell ablation impacts CS establishment.**

939 A CASP1 promoter activity highlighted by nls-GFP (green) after ablation of
940 all neighboring cells prior to onset of expression, PI (red) outlines cells and
941 confirmed cell destruction. Scale bar = 50 μm .

942 B Endodermal differentiation was induced via 0.5 μM β -estradiol and
943 CASP1-GFP expression (white) followed over the next 12 h. Red arrows
944 indicate unstable CASP deposits in the membrane, scale bar = 50 μm .

945 C CSD formation in wildtype and *lotr1*. Single endodermal cells ablated 3-4
946 cells prior to onset of CASP expression, * depicts ablated cell, scale bar = 50
947 um.

948

949 **Fig. S4: Investigation of putative post-translational processing in LOTR1.**

950 A Lignin accumulation (magenta) in wildtype and *sgn3* with CSDs labelled
951 by CASP1-GFP (green).

952 B Schematic view of LOTR1 with position of identified loss-of-function
953 alleles indicated.

954 C Processing of LOTR1. Root protein extracts separated via SDS-PAGE and
955 LOTR1-fragments detected by anti-RFP antibody (Chromotek 6G6). Note
956 that C-terminal fusion construct LOTR1::LOTR1-mCherry does not
957 complement the *lotr1* phenotype, but shows processing/degradation
958 fragments. Both weak (#1) and strong (#2) C-terminal complementation
959 lines lack processing/degradation fragments.

960 D Complementation analysis of the *lotr1* CASP1-GFP phenotype using
961 truncated versions consisting of only LOTR1's Neprosin domain (Neprosin),
962 the putative autoinhibitory Neprosin-AP domain (Neprosin-AP), or full-
963 length LOTR1 with conserved cysteines C255 and C260 mutated
964 (LOTR1^{C255A, C260A}), scale bars = 50 µm.

965

966 **Fig. S5: Neprosin domain analysis in the HOLO family.**

967 A Conservation of Neprosin domain throughout the HOLO-family, including
968 LOTR1. Putative catalytic triade amino acids aligning with *E.coli* mepS
969 marked with red arrows.

970 B Sequence alignment of *E.coli* mepS, Neprosin, LOTR1 and its closest
971 homologs At1g23340 (HOLO2), At1g70550 (HOLO3), and At1g10750
972 (HOLO4)

973

974

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