Current Biology

Role of LOTR1 in nutrient transport through organization of spatial distribution of root endodermal barriers

--Manuscript Draft--

Manuscript Number:	CURRENT-BIOLOGY-D-16-01123R2
Full Title:	Role of LOTR1 in nutrient transport through organization of spatial distribution of root endodermal barriers
Article Type:	Report
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Abstract:	The formation of Casparian strips and suberin lamellae at the endodermis limit the free diffusion of nutrients and harmful substances via the apoplastic space between the soil solution and the stele in roots [1-3]. Casparian strips are ring-like lignin polymers deposited in the middle of anticlinal cell walls between endodermal cells and fill the gap between them [4-6]. Suberin lamellae are glycerolipid polymers covering the endodermal cells and likely function as a barrier to limit transmembrane movement of apoplastic solutes into the endodermal cells [7-8]. However, the current knowledge on the formation of these two distinct endodermal barriers and their regulatory role in nutrient transport is still limited. Here, we identify an uncharacterized gene, LOTR1, essential for Casparian strip formation in Arabidopsis thaliana. The lotr1 mutants display altered localization of CASP1, an essential protein for Casparian strip formation [9], disrupted Casparian strips, ectopic suberization of endodermal cells, and low accumulation of shoot calcium (Ca). Degradation by expression of a suberin-degrading enzyme in the mutants revealed that the ectopic suberization at the endodermal cells limits Ca transport through the transmembrane pathway, thereby causing reduced Ca delivery to the shoot. Moreover, analysis of the mutants showed that suberin lamellae function as an apoplastic diffusion barrier to the stele at sites of lateral root emergence where Casparian strips are disrupted. Our findings suggest that the transmembrane pathway through unsuberized endodermal cells rather than the sites of lateral root emergence mediates the transport of apoplastic substances such as Ca into the xylem.

January 16, 2017

Dear Dr. Anne Knowlton,

Thank you very much for your provisional acceptance on our manuscript entitled "**Roles of LOTR1 in nutrient transport through organizing spatial distribution of root endodermal barriers**" for publication in *Current Biology*. We deeply appreciate three reviewers for understanding of our responses and providing positive comments on the revised version of our manuscript. Our point-by-point responses on the new comments kindly given by the reviewers are listed below. In the followings, the paragraphs in italic are the comments from the reviewers followed by our response. We believe these modifications further improved our manuscript.

Sincerely,

Baohai Li, Takehiro Kamiya and Toru Fujiwara

January 10, 2017

Dear Dr. Anne Knowlton,

Thank you very much for your provisional acceptance on our manuscript entitled "**Roles of LOTR1 in nutrient transport through organizing spatial distribution of root endodermal barriers**" for publication in *Current Biology*. We deeply appreciate three reviewers for understanding of our responses and providing positive comments on the revised version of our manuscript. Our point-by-point responses on the new comments kindly given by the reviewers are listed below. In the followings, the paragraphs in italic are the comments from the reviewers followed by our response. We believe these modifications further improved our manuscript.

Reviewer #1: *The revised version definitely tackled all points of criticism raised in my first review in a satisfactory manner. I consider this manuscript now as a highly original, solid and important contribution to this field. The authors may just consider the following editorial suggestions:*

Comment1*- in general, maybe use "visible lateral root" instead of "lateral root" whenever the term lateral root is used in a developmental context*

Authors' response: Thank you for the suggestion. The term "lateral root" has been replaced with "visible lateral root" in the description of the mature lateral root phenotype in *lcs2-1* mutant throughout the manuscript.

Comment2*- line 46: "degradation" is difficult to understand for someone reading only the abstract, as it is unclear how the degradation was imposed. Could you say "Degradation by expression of a suberin-degrading enzyme..." or something like that?*

Authors' response: Thank you for the correction. "Degradation of suberin" has been changed to "Degradation by expression of a suberin-degrading enzyme" in the abstract.

Comment3*- line 122: "the apoplastic barier function in lcs2-1 is lower..." What is a "lower function"? I think this sentence does not work, please reformulate.*

Authors' response: Thank you for pointing it out. The sentence of "These results indicate that the apoplastic barrier function in *lcs2-1* is **lower** than that of *esb1-1*, but **higher** than that of *sgn3-3* mutant" has been modified into "These results indicate that the apoplastic barrier function in *lcs2-1* is **weaker** than that of *esb1-1*, but **stronger** than that of *sgn3-3* mutant".

Reviewer #2: *The revised manuscript is much improved as a result of author's acting on the majority of constructive comments made by all 3 reviewers. A few points remain to be addressed.*

Comment 1*. It is disappointing that the authors did not decide to include LOTR-GFP data in this paper, given that this data is available (as flagged in their response) as it would avoid the current speculative comments in the Discussion where the authors attempt to describe how LOTR may function to regulate CS deposition. Its inclusion would greatly strengthen the paper.*

Authors' response: We are sorry about that. In the context of our paper, adding this data would not give the readers more information about the function of LOTR1. We believe that it would be more appropriate to include the localization data in the upcoming paper that mainly focused on the LOTR1 protein function.

Comment 2*. The authors state at the end of the results "...that suberin lamellae were aggressively deposited on the surface of lateral root primordia (LPR) and their surrounding endodermal cells". Please delete with the inappropriate underlined phrase.*

Authors' response: Thank you for pointing out this problem. The sentence has been modified as follows: "… that suberin lamellae were firstly deposited on the surface of lateral root primordia (LRP) at younger stages and then spread to their surrounding endodermal cells as the LRP grew"

Reviewer #3*: The manuscript has been improved in response to the reviewers' comments. I suggest one additional point to improve the manuscript as follows.*

In Figure S1 (panel C), the stages of lateral root's development are shown as stage I to stage VIII. The authors cited the Malamy and Benfey paper (1997), in which the classification of these developmental stages was stated, but it would be more friendly for the readers if the authors could briefly explain some key stages among the stages I-VIII.

Authors' response: Thank you for the comment. In the Casparian strips defective mutants, we think the stage IV may be a key stage to be affected, because at this stage the LRP has penetrated the parent endodermal layer [S1]. We have added following sentence to the legend

of Figure S1 to point out the key stage: “At the stage IV, the LRP has penetrated the parent endodermal layer [S1]”

Thank you very much for your reading through the response. We hope these responses and the revision in the manuscript make our paper to be accepted in *Current Biology*.

Sincerely,

Baohai Li, Takehiro Kamiya and Toru Fujiwara

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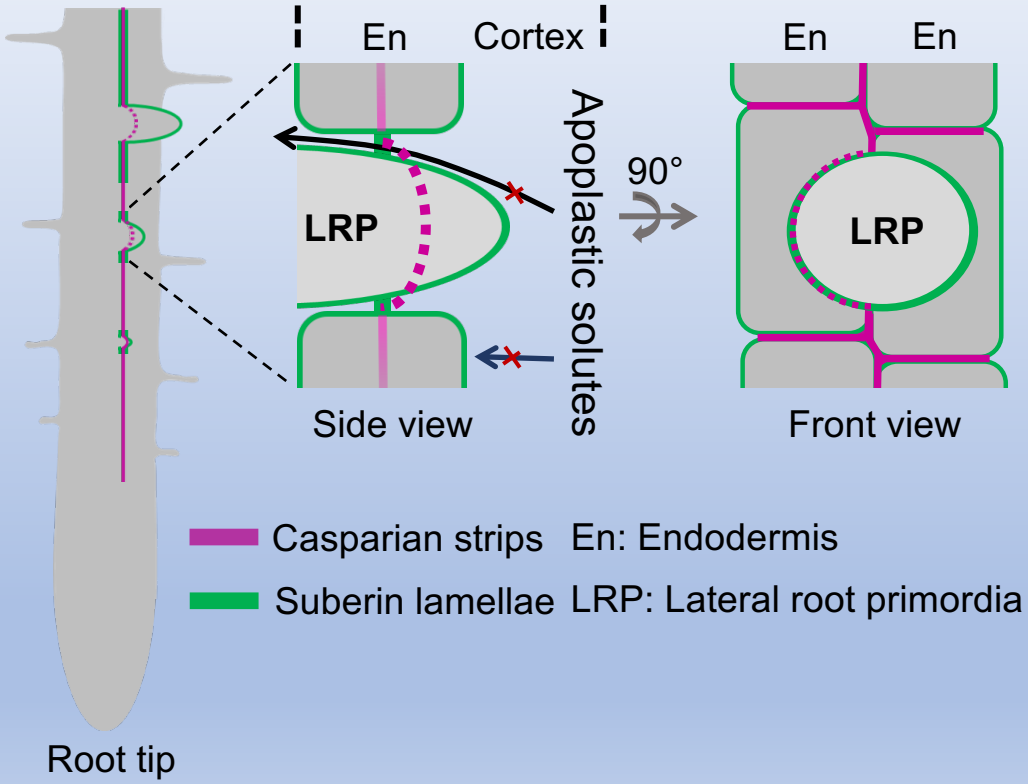

Title

Role of *LOTR1* in nutrient transport through organization of spatial distribution of root endodermal barriers

Authors

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Additional Title Page Footnote

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[10]We dedicate this article to Dr. John M. C. Danku, who passed away on 13th December, 2016.

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SUMMARY

The formation of Casparian strips and suberin lamellae at the endodermis limit the free diffusion of nutrients and harmful substances via the apoplastic space between the soil solution and the stele in roots [1-3]. Casparian strips are ring-like lignin polymers deposited in the middle of anticlinal cell walls between endodermal cells and fill the gap between them [4-6]. Suberin lamellae are glycerolipid polymers covering the endodermal cells and likely function as a barrier to limit transmembrane movement of apoplastic solutes into the endodermal cells [7-8]. However, the current knowledge on the formation of these two distinct endodermal barriers and their regulatory role in nutrient transport is still limited. Here, we identify an uncharacterized gene, *LOTR1*, essential for Casparian strip formation in *Arabidopsis thaliana*. The *lotr1* mutants display altered localization of CASP1, an essential protein for Casparian strip formation [9], disrupted Casparian strips, ectopic suberization of endodermal cells, and low accumulation of shoot calcium (Ca). Degradation by expression of a suberin-degrading enzyme in the mutants revealed that the ectopic suberization at the endodermal cells limits Ca transport through the transmembrane pathway, thereby causing reduced Ca delivery to the shoot. Moreover, analysis of the mutants showed that suberin lamellae function as an apoplastic diffusion barrier to the stele at sites of lateral root emergence where Casparian strips are disrupted. Our findings suggest that the transmembrane pathway through unsuberized endodermal cells rather than the sites of lateral root emergence mediates the transport of apoplastic substances such as Ca into the xylem.

Keywords

Casparian strip, suberin, apoplast, transmembrane pathway, lateral root, cell wall

RESULTS

The *low Ca sensitive 2* (*lcs2*) mutant exhibits reduced Ca accumulation in shoots.

In roots, the endodermis bearing the Casparian strips and suberin lamellae is considered to block the radial delivery of nutrients derived from soils to the stele via the apoplastic pathway [1-3]. Two pathways are proposed for apoplastic nutrients such as calcium (Ca) to pass through the endodermis [10, 11]. The first is the transmembrane pathway mediated by the unsuberized endodermal cells, including passage cells, where the Casparian strips are formed [10, 12]. This pathway is supported by recent findings of the *enhanced suberin 1* (*esb1*) mutant [13]. In *esb1*, suberin is ectopically deposited on normally unsuberized endodermis and *esb1* shows alteration of ionome patterns in shoots, including low Ca accumulation [13]. However, the Casparian strip formation is also defective in *esb1* [6], which raises another possibility that the alteration of ionome patterns might be due the defect of Casparian strip formation. Therefore, the contribution of suberin in plant nutrient transport needs to be evaluated separately from that of the Casparian strips. The other pathway to pass through the endodermis is an apoplastic bypass at the root tip where Casparian strips are not formed, as well as at the site of lateral root emergence where Casparian strips are disrupted by lateral root primordia (LRPs) emerging from the pericycle, a cell layer inside of the endodermis [11, 14]. The site of lateral root emergence is widely regarded as an important pathway for apoplastic substances such as minerals and for bacterial entry into plants [15-18]. However, other reports show that there is no correlation between bypass flow and the number of lateral roots [19-21]. Therefore, the presence of a barrier at the lateral root emergence site remains controversial. Furthermore, the substances that are involved in the possible blockage at this site are unclear.

To identify a regulator(s) involved in the transport of Ca, a typical nutrient transported via the apoplastic pathway in plants [10, 11], we isolated *low calcium sensitive 2-1* (*lcs2-1*) from an EMS-mutagenized M_2 population of *A. thaliana* Col-0. The mutant was isolated based on the shoot growth phenotype grown on agar medium with low Ca. Both shoot biomass and visible lateral root number but not the primary root length of *lcs2-1* were much more reduced than Col-0 in response to the low Ca condition (0.2 mM, Figure 1A-1D, S1A). Detailed analysis revealed that the LRP was normally formed, while lateral root emergence was delayed in *lcs2-1* under low Ca

treatment (Figure S1B and S1C). The Ca concentration in shoot decreased by 30% in *lcs2-1* under both the normal (2 mM Ca) and low Ca conditions in agar medium compared with wild type (Figure 1E). The leaf Ca concentration also decreased to 40% in *lcs2-1* when grown in soil (Table S1). In addition to Ca and its congener, strontium (Sr), we also found that the accumulation of 11 other elements in leaves was significantly altered in the *lcs2-1* mutant (Table S1).

In our previous study, we found that several Casparian strip defective mutants exhibit changes in concentrations of multiple elements including Ca [6, 13, 22, 23]. Therefore, we compared the ionomic profiles of *lcs2-1* and known Casparian strip defective mutants: *esb1-1*, *casp1-1casp3-1, myb36-1*, and *sgn3-3*. The principal component analysis of elemental concentrations in the leaves showed that the ionomic profile of *lcs2-1* was similar to *esb1-1, casp1-1casp3-1*, and *myb36-1* mutants (Figure 1F), which are defective in Casparian strip formation and show enhanced suberin accumulation [6, 22]. The leaf ionome profile of *sgn3-3,* which possesses defective Casparian strips and normal suberin accumulation [23], was distinct from the other mutants (Figure 1F). These results suggest that *lcs2-1* presents defects in both the Casparian strips and the suberin lamellae.

Formation of an apoplastic diffusion barrier is delayed in *lcs2* endodermis.

To examine the function of endodermal apoplastic barrier in *lcs2-1* roots, we analyzed the permeability of the apoplastic tracer, propidium iodide (PI), in the stele. Because the endodermal barrier is similarly disrupted among *esb1-1*, *casp1-1casp3-1*, and *myb36-1* mutants [6, 22], we took *esb1-1* as a representative of these mutants in this study. We also included *sgn3-3,* which is a different type of Casparian strip mutants [24] for comparison. After incubation in PI solution for 7 min, PI penetrated into the stele from the root tip to the 14th endodermal cell after the onset of elongation (See the Method) in the wild type and at around the 34th endodermal cell after the onset of elongation in *lcs2-1* and *esb1-1* mutants (Figure 2A and S1D). In *sgn3-3*, PI penetration was observed in the entire root, as reported previously [24] (Figure S1E). On incubation for 20 min, penetration occurred throughout the stele along with the whole root of *lcs2-1*, but not in the wild type and *esb1-1* mutant (Figure 2A and S1F). These results indicate that the apoplastic barrier function in *lcs2-1* is weaker than that of *esb1-1*, but stronger than that of *sgn3-3* mutant. Thus, we concluded that *lcs2-1* represents a novel type of mutant in terms of diffusion barrier formation.

lcs2 shows disrupted Casparian strips and enhanced suberin accumulation in endodermis.

To confirm alteration in Casparian strips formation in *lcs2-1* roots, we observed the lignin autofluorescence of the Casparian strips. Unlike the linear and continuous pattern in the wild type, large gaps in the Casparian strips were observed in *lcs2-1* (Figure 2C and S1G). These characteristics also differed from *esb1-1*, which showed small gaps in Casparian strips, along with ectopic lignin deposition at the corners of endodermal cells between both the cortex and the pericycle [5, 22] (Figure 2C). These differences in Casparian strips may explain the increased permeability of PI in *lcs2-1* compared with *esb1-1* (Figure 2C). The lignin autofluorescence signal was stronger in *lcs2-1* compared with Col-0, which might be due to a feedback response of lignin deposition. Next, we observed the suberin accumulation using fluorol yellow 088 staining, a fluorescence suberin dye, in *lcs2-1* roots as suberin ectopic deposition is observed in other Casparian strip mutants such as *esb1*, *casp1-1casp3-1*, and *myb36* [6, 22]. In Col-0 roots, suberin lamellae were first observed around the 39th endodermal cell from the onset of elongation and both suberized and unsuberized cells were observed, giving it a 'patchy' appearance. Above the 67th cell, all the endodermal cells were suberized, presenting a continuous suberin-staining pattern (Figure 2D and 2E). In *lcs2-1*, unlike Col-0, suberin lamellae were continuously deposited starting from an earlier phase of the mature endodermis (from the 25th endodermal cell) to the junction connecting with the hypocotyls (Figure 2D and 2E). This suberin deposition pattern of *lcs2-1* was similar to that of *esb1-1* (Figure 2D and 2E). Suberin ectopic accumulation is also observed in *casp1-1casp3-1* and *myb36* [6, 22]. Therefore, suberin accumulation could be a secondary effect of the disruption of the Casparian strip. It has been reported that the kinase-like receptor SGN3 is required for the ectopic suberin accumulation in *esb1-1* and *casp1-1casp3-1* [23], though the underlying mechanism is still unclear. Similarly to these mutants (Figure 2D and 2E), the ectopic suberin deposition may also be mediated by SGN3 in *lcs2-1*.

Subcellular localization of CASP1 but not ESB1 is altered in _lcs2_.

The altered Casparian strips suggest a possibility that the machinery of Casparian strip formation is mislocalized in *lcs2-1*. To test this, we observed ESB1 and CASP1 localization by introducing ESB1 (*ProESB1:ESB1-mCherry*) and CASP1 (*ProCASP1*:*CASP1-GFP*) into the *lcs2-1* mutant. We found that localization of

CASP1-GFP, but not ESB1-mCherry, in *lcs2-1* was different from that in the wild type (Figure 2F and 2G), suggesting that ESB1 localization mechanism is different from that of CASP1. The CASP1-GFP fluorescence was observed in isolated islands outside of its normal position, in addition to a CASP1-GFP signal at the normal position (Figure 2G). Furthermore, an optical cross-section revealed that the CASP1-GFP was localized on the stele-side of the endodermis in conjunction with its presence in the middle of endodermal cells where CASP1-GFP usually accumulates in the wild type (Figure 2H and 2I). This pattern of CASP1-GFP in *lcs2-1* suggests that the causal gene is required for the localization of CASP1-GFP to the Casparian strip domain.

The causal gene of *lcs2-1* is *At5g50150*.

Using a selfed F_2 population from F_1 derived from the crosses between *lcs2-1* and L*er*, we found a candidate gene (*At5g50150*) with a nonsense mutation in the 294th amino acid residue tryptophan (Figure 2J) through genetic mapping and next-generation sequencing. In an independent forward genetic screen for players controlling the making of the ring-like CASP1-GFP domain, a complementation group of eight alleles was identified that displayed a mislocalization of CASP1-GFP similar to that of *lcs2-1* (Figure S2). The gene responsible was identified by genome sequencing of a mutant pool from a segregating, backcrossed population (Figure S2) and was named *LORD OF THE RINGS 1* (*LOTR1*), after the novel by J.R.R. Tolkien, based on the CASP1-GFP mislocalization phenotype. Non-complementation of a cross in F_1 demonstrated allelism of *LOTR1* with *lcs2-1*. In addition, SALK_051707 with a T-DNA insertion in the 4th exon of *At5g50150* displayed a similar phenotype to *lcs2-1*, in that it exhibited low Ca sensitivity, Casparian strip, and suberin deposition (Figure 2J and S3A-S3D) and the F_1 between *lcs2-1* and SALK_051707 did not recover these low Ca sensitive phenotypes (Figure S3A and S3B). Moreover, the localization of CASP1-GFP was recovered by introducing a wild-type genomic DNA fragment corresponding to *At5g50150* into the *lcs2-1* background (Figure S3E). Taken together, these results established that *At5g50150* is the causal gene of *lcs2-1* mutant. Hereafter, we refer to *At5g50150* as *LOTR1*. The LOTR1 protein contains domains of unknown function 4409 (DUF4409) and 239 (DUF239) (Figure S2A) and is predicted to localize in the extracellular space (http://suba.plantenergy.uwa.edu.au). Phylogenetic analyses of proteins carrying these two domains identified 86 genes present in 26

species (Figure S3F), suggesting that the family of these genes exists widely among plant species.

Enhanced suberin accumulation reduces Ca delivery to shoots in *lcs2*.

To dissect the roles of suberin lamellae and Casparian strips on nutrient transport in *lcs2-1* and *esb1-1* mutants, we introduced the gene *CDEF1* that encodes a suberin degrading enzyme into *lcs2-1* and *esb1-1* mutants, under the control of the endodermis-specific *CASP1* promoter (*ProCASP1:CDEF1*) [4]. No suberin accumulation was detected in endodermal cells of transgenic mutant plants (*lcs2-1* and *esb1-1)* carrying *ProCASP1:CDEF1* (Figure 3A). We confirmed that Casparian strip formation was not affected by introduction of the construct (Figure S4A), which is in agreement with previous reports [4, 24]. Using these lines, we first measured the shoot ionomic profiles. The principal component analysis of which showed that ionome profiles of *lcs2-1* and *esb1-1* expressing *ProCASP1:CDEF1* were different from their parental mutants (Figure 3B). This supports the notion that suberin lamellae play an important role in the regulation of nutrient delivery into the xylem and subsequently to the shoot [1, 10, 13]. With reference to the Ca concentration in shoots (Figure 3C), the *ProCASP1:CDEF1* significantly increased the Ca accumulation in *lcs2-1* and *esb1-1* shoots, but not in the wild type. This result proved that the enhanced suberin in root endodermis causes the decreased Ca accumulation observed in both *lcs2-1* and *esb1-1* shoots.

Subsequently, we examined the shoot growth and lateral root formation in *lcs2-1* carrying *ProCASP: CDEF1*. The shoot biomass of *lcs2-1* was not salvaged by the expression of *ProCASP1:CDEF1* (Figure S4B). However, the number of visible lateral roots was partially recovered in *lcs2-1* mutant by the introduction of *ProCASP1:CDEF1* (Figure 3D). Taken together with the results that low Ca inhibits the emergence of lateral root (Figure S1B and S1C), this result suggests that enhanced suberin could be partially responsible for the delay of lateral root emergence in *lcs2-1*. To test whether the reduced lateral root formation is the reason for the low Ca accumulation in shoots, we examined the Ca accumulation in shoots of these mutants without lateral roots. As shown in Figure S4C, none of the mutants showed reduced Ca accumulation in shoots as compared to the wild type. These results suggest that low accumulation of Ca in shoots in *lotr1/lcs2-1* mutants could not have been due to the decrease in lateral root formation caused by the ectopic suberin (Figure 3D), but as a

result of the limited Ca transmembrane transport into the endodermal cells by the ectopic suberin accumulation as suggested by previous studies [10, 13].

Degradation of suberin enhances the defect in the apoplastic diffusion barrier of *lcs2*.

It has been shown that suberin functions as an apoplastic diffusion barrier in roots. In the root of suberin defective triple mutant (*abcg2 abcg6 abcg20*) and the wild type expressing the suberin-degrading enzyme CDEF1, PI penetration into stele is observed in the older portion of roots [24]. Therefore, it is possible that suberin accumulation above the 35th endodermal cell from the onset of elongation in *lcs2-1* and *esb1-1* contributes to the formation of the apoplastic diffusion barrier (Figure 2A). To test this hypothesis, we performed the PI permeability assay in *lcs2-1* and *esb1-1* mutants carrying *ProCASP1:CDEF1* [4]. We found that introduction of *ProCASP1:CDEF1* increased PI permeability in the zone above the 35th cell of the endodermis in *lcs2-1* and *esb1-1* mutants (Figure 4A), indicating that the enhanced suberin indeed functions as an apoplastic diffusion barrier above the 35th endodermal cell of these mutants. In contrast to the *lcs2-1* carrying *ProCASP1:CDEF1*, PI staining in stele was observed in a discontinuous manner (indicated as horizontal lines on the bar of Figure 4A) in the roots of *esb1-1* expressing *CDEF1* (Figure 4A), indicating that the apoplastic diffusion barrier above the 35th endodermal cell of Casparian strip mutants (Figure 2A) is partly formed by suberin in *esb1-1*. The remained apoplastic diffusion barrier in *esb1-1* expressing *CDEF1* might be due to two additional types of lignin deposition in the cell corner of *esb1-1* [6, 22].

Suberin lamellae function as an apoplastic diffusion barrier at lateral root emergence sites.

To elucidate where and how suberin functions as an apoplastic barrier, we observed in detail the PI staining site in the discontinuous staining region (after 35th endodermal cell) in *esb1-1* expressing *ProCASP1:CDEF1*. Along with the bright field image, we found that PI staining was observed in the stele only at lateral root emergence sites, when the roots were incubated in PI solution for 7 min (Figure 4B). Such PI staining near the lateral root emergence sites was not observed in Col-0 and *esb1-1* without *ProCASP1:CDEF1* (Figure 4A and 4B). After incubation with PI for 7 min, the PI staining was observed at both the lateral root emergence sites and neighboring regions in Col-0 expressing *ProCASP1:CDEF1* (Figure 4B). This outcome raises the

possibility that PI penetration into the stele initially occurs around the lateral root emerging sites and then diffuses into the neighboring regions, or through both the primordia and other parts of the endodermis. To differentiate these two possibilities, we performed PI penetration assays in the root of Col-0 expressing *ProCASP1:CDEF1* with shorter incubation periods of 3 and 5 min, which do not allow the diffusion of PI. Under both conditions, we observed PI penetration in the stele at lateral root emergence sites, but not in the other regions of *ProCASP1:CDEF1* line (Figure 4C and S4D). In 3 min, PI uniformly stained the root stele of *sgn3-3* whose apoplastic barrier is completely broken in whole roots [23] (Figure S4D), suggesting that this duration is sufficient for apoplastic diffusion of PI into the stele. Taken together, these results indicate that suberin deposition functions as an apoplastic diffusion barrier at lateral root emergence sites, where Casparian strips are disrupted [14] (Figure 4D).

In agreement with the function of suberin at lateral root emergence sites, we found that suberin lamellae were firstly deposited on the surface of lateral root primordia (LRP) at younger stages and then spread to their surrounding endodermal cells as the LRP grew, but not in other endodermal cells in the wild type (Figure 4E) in the initial phase of development.

DISCUSSION

The proper localization of CASPs at the site on the plasma membrane where Casparian strips are to be deposited is a critical step for directing to this domain the lignin polymerizing machinery in order to form Casparian strips [9, 25]. CASP1, at first, is randomly distributed on the plasma membrane and then laterally diffuses into the Casparian strip domain [9]. ESB1 and SGN3 are required for the fusion of CASP patches at the Casparian strip membrane domain [6, 23]. Unlike these proteins, LOTR1 is required to prevent the ectopic localization of CASP protein to the outside of the normal position of Casparian strip deposition (Figure 2G, 2H, 2I, S2, and S3E). Considering that CASP1 displays very low endocytosis and lateral diffusion once it has become localized [9], LOTR1 could be involved in its lateral diffusion from the initial random distribution to the restricted localization at the final deposition site. Lateral diffusion of plasma membrane proteins can be constrained by the cell wall

[26]. Considering that LOTR1 is predicted to be an apoplastic protein (see Results) and the domain of unknown function 239 (DUF239) in LOTR1 is denoted as a glucoamylase domain (TAIR: http://www.arabidopsis.org), we speculate that LOTR1 is involved in cell wall modifications that influence the CASP1 lateral diffusion into the Casparian strip membrane domain. Based on HanaDB-AT microarray gene expression database (http://evolver.psc.riken.jp/seiken), *LOTR1* is widely expressed in all the tissues examined and is enriched in the root, stem, and young silique. This gene expression pattern suggests that the function of *LORT1* is not specific to Casparian strip deposition. However, except for the short primary root and less lateral roots (Figure S1A and 1C), the seedlings of *lotr1* mutants can grow normally as the wild-type under our normal condition.

Apoplastic Ca movement through the endodermis has been suggested to be mediated via the unsuberized endodermal cells and/or an apoplastic bypass at the lateral root emergence sites [10, 11]. However, in the present study, we found a suberin-based apoplastic barrier at the sites of lateral root emergence in the wild type (Figure 4). This finding, together with the observation that the enhanced suberization of endodermal cells causes low Ca accumulation in shoots (Figure 3C), suggests that the transmembrane pathway through unsuberized endodermal cells, but not an apoplastic bypass at lateral root emergence sites, mediates apoplast Ca transport through the endodermis bearing the Casparian strip. The ectopic suberin observed in Casparian strip mutants may inhibit the Ca transmembrane transport mediated by Ca transporters/channels in the normally unsuberized endodermal cells as proposed in previous studies [27]. This idea is also supported by the observance that salt stress induces suberin deposition in roots in wild-type Arabidopsis [28], while reducing Ca accumulation in the shoot [29].

The shoot Ca concentration in Casparian strip mutants (*lcs2-1* and *esb1-1*) expressing *ProCASP1:CDEF1* is higher than that of the mutants alone, but is not fully restored to the levels observed in the wild type (Figure 2C). This result suggests that Casparian strips are also important for Ca delivery to shoots. *sgn3* has a disrupted apoplastic barrier in the entire root and normal suberin [23]. In *sgn3-3*, Ca concentration in shoots is lower than that of the wild type (Figure S4E), indicating that the Casparian strip is required to maintain higher Ca concentrations in the stele than in the medium. The partial recovery observed in *lcs2-1* and *esb1-1* may be

explained by the reverse Ca leakage of Ca from the stele through the disrupted Casparian strips.

Suberin is hydrophobic polymer deposited between the plasma membrane and cell wall, covering the endodermal cells [7]. Since suberin deposition between adjacent endodermal cells is separated by the cell wall [30], suberin cannot function as an apoplastic barrier [6] (Figure 2A). However, we observed that suberin is required to maintain an apoplastic barrier between LRP and its surrounding endodermal cells (Figure 4). At the lateral root emergence site, the cell wall of the endodermal cell overlaying the LRP is degraded during the process of lateral root emergence [31]. This elimination of the cell wall may make it possible for suberin to fill the gap between adjacent plasma membranes of the LRP and the surrounding endodermal cells. A layer of suberin lamellae between LRP and surrounding endodermal cells has been observed in Arabidopsis with TEM analysis [5], which supports our findings. Alternatively, suberin might be deposited as a primary cell wall modification at these sites. In addition to apoplastic transport, we also found that the ectopic deposition of suberin affect lateral root emergence in *lcs2-1* mutant (Figure S1C and 3C). The enhanced suberin deposition on LRPs and their surrounding endodermal cells might disturb water flow into LRPs, which is required for the emergence of lateral roots [32], or increase the physical properties of the LRP-overlaying endodermal cells, resulting in the delay of lateral root emergence as described in the previous study [33].

In summary, we identified a novel gene *LOTR1* from an uncharacterized protein family essential for correct Casparian strip formation and suberin lamellae deposition. Through the analysis of Casparian strip mutants, we revealed the novel function of suberin in nutrient transport regulation in roots. The finding of suberin lamellae acting as an apoplastic diffusion barrier at the lateral root emergence site propose that the transmembrane pathway through unsuberized endodermal cells is a major pathway to mediate the transport of apoplastic substances such as Ca into the xylem.

EXPERIMENTAL PROCEDURES

The materials, experimental procedures, and statistical methods were described in detail in supplemental information.

AUTHOR CONTRIBUTIONS

B.L., T.K., D.E.S., N.G. & T.F. designed research; B.L., T.K., L.K., M.Y., K. Y., S.S., S.S. and J.M.C.D. performed research; B.L., T.K., L.K., D.E.S., N.G., & T.F., analyzed data and discussion; and B.L., T.K. & T.F. wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Emiko Yokota for technical assistance; and Hidehiro Fukaki (Kobe University) for providing *slr-1* and *arf7-1arf19-1* seeds. This research is supported by JSPS KAKENHI Grant Numbers 26712008 (to T.K.) and 15H01224 and 25221202 (to T.F.) and JSPS postdoctoral fellowship for overseas researchers (B.L.).

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FIGURE LEGENDS

Figure 1. Growth phenotype and ionome pattern of the *lcs2-1* mutant.

(A) Shoot fresh weight of the wild type (Col-0) and *lcs2-1* mutant grown for 14 days on agar medium with normal Ca (2 mM) or low Ca (0.2 mM) (n = 3).
(B) Representative images of Col-0 and *lcs2-1* shoots grown under 0.2 mM Ca conditions for 14 days.
(C) The number of visible lateral root grown in agar medium for 9 days and shown with Box plots (n > 12). The middle line, boxes, and whiskers represent the median, the first and third quartiles, maximum and minimum respectively.
(D) Representative images of Col-0 and *lcs2-1* grown under 0.2 mM Ca conditions for 9 days.
(E) Ca concentrations in shoots of Col-0 and *lcs2-1* grown on agar medium for 14 days (n = 3).
(F) Principal component analysis of *lcs2-1* and known Casparian strip defective mutants based on 20 elements concentrations (as shown in Table S1) in shoots grown on agar medium with normal Ca for 14 days (n = 10).
Bars represent mean ± SD in (A, E). Different letters indicate the significant difference (Tukey's HSD tests, $p < 0.05$). Scale bar: 5 mm in (B, D). See also Figure S1.

Figure 2. *lcs2-1* is a novel mutant showing defects in Casparian strips.

(A, B) Disruption of apoplastic diffusion barrier in *lcs2-1* was quantified by propidium iodide (PI) (n = 6). The roots were incubated with PI for 7 min (a) and 20 min (b), respectively.
(C) Z-stack confocal image of lignin-based Casparian strip autofluorescence in roots. Spiral-like signal in the left is from the xylem vessel. Red arrows indicate the normal Casparian strips in the wild-type and the absence of Casparian strips in *lcs2-1* and *esb1-1*.
(D) Suberin lamellae in root endodermis were identified with fluorol yellow 088 staining. Merged images between fluorescent signal from fluorol yellow 088 (yellow) and bright field (gray) were shown. Passage cell was denoted with an asterisk. Pattern of suberin deposition is grouped into three types in the wild-type roots: continuous

suberization in the old parts, patchy suberization in the middle section, no suberization in young parts [23].

(E) Quantitative analysis of suberin accumulation. The endodermal cell with suberin was counted from the onset of elongation to the junction (base) between root and hypocotyl (n = 6).

(F) The accumulation pattern of ESB1-mCherry (Magenta) was similar to the wild type in *lcs2-1* mutant.

(G, H, I) CASP1-GFP localization was altered in *lcs2-1* mutant. Z-stack image was constructed with 30 images obtained by confocal microscopy (G). CASP1-GFP was localized into both stele- (red arrow) and the central portion of endodermal cells (white arrow) (H, radial section; I, longitudinal section). Cell profile was stained with PI (Magenta). Red arrows indicate ectopic localization of CASP1-GFP in *lcs2-1* mutant (I).

(J) Schematic representation of *LOTR1* alleles. *LOTR1* (*At5g50150*) is the causal gene of *lcs2-1*.

All seedlings were grown for 5 days. Bars represent the mean ± SD in (A, B, E). Scale Bar: 10 μm in (C), 50 μm in (D), 20 μm in (F-H), 5 μm in (I). See also Figure S1, S2, and S3.

Figure 3. Ectopic suberin accumulation reduces calcium delivery to shoot and lateral root formation in *lcs2-1* mutant.

(A) Suberin accumulation in roots. *lcs2-1CDEF1* and *esb1-1CDEF1* are *lcs2-1* and *esb1-1* expressing CDEF1 (encoding the suberin degrading enzyme) by endodermis specific gene (*CASP1*) promoter. Suberin was detected with fluorol yellow 088 staining. Merged image of fluorescent signal from fluorol yellow 088 (yellow) and bright field (gray) are shown. Scale Bar: 50 μm.

(B, C) Principal component analysis based on the concentrations of 10 elements (Li, B, Na, Mg, P, K, Ca, Mn, Cu, Mo) in shoots (B). Ca accumulation in *lcs2-1* and *esb1-1* shoots was recovered by expressing *CDEF1* (n = 3-5) (C). The seedlings in (B, C) were grown in agar medium under normal condition for 14 days. Scatter plots and mean ± SD were shown.

(D) The visible lateral root number was partially recovered in *lcs2-1* by expressing *CDEF1* and shown with box plots as defined in the legend of Figure 1C. The seedlings were grown for 9 days (n >15).

Different letters indicate the significant difference (Tukey's HSD tests, $p < 0.05$). See also Figure S4.

Figure 4. Suberin lamellae function as apoplastic diffusion barrier at lateral root emergence sites.

(A) PI permeability in root endodermis was increased in *lcs2-1* expressing *CDEF1* (*lcs2-1CDEF1*) and *esb1-1* expressing *CDEF1* (*esb1-1CDEF1*) as compared to the parental mutants (n = 6). Bars represent means ± SD.

(B) PI penetration into the stele was blocked at the lateral root emergence sites (arrow) as well as other regions (asterisk) in Col-0, but not in *esb1-1CDEF1* and *CDEF1*.

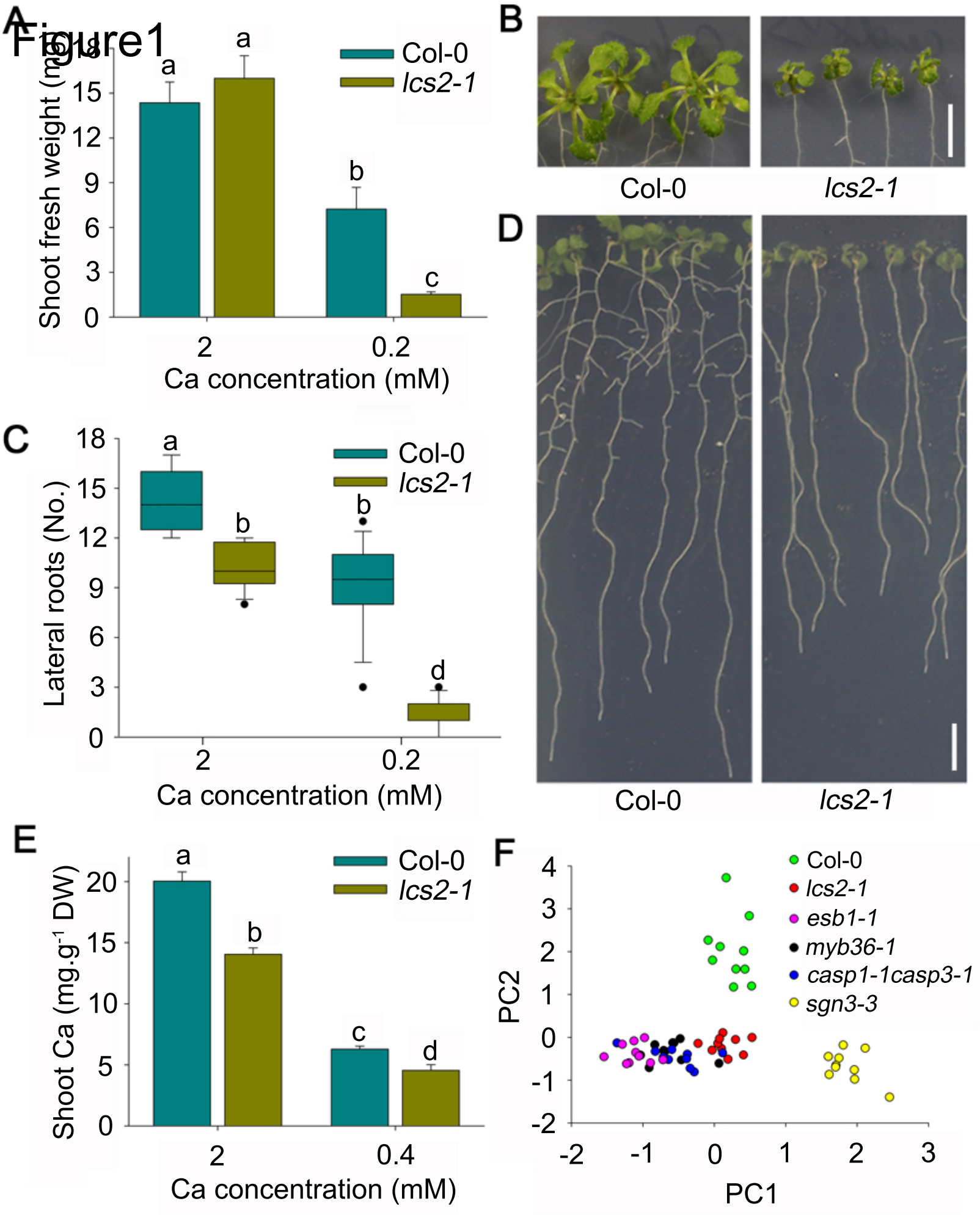
(C) PI penetrated the stele at the lateral root emergence sites (arrow) but not in the other regions (asterisk) of *CDEF1*, within 5 min of staining.

(D) Schematic diagram of Casparian strips disrupted (arrow) by lateral root emergence [14].

(E) Suberin lamellae (fluorol yellow 088 staining) deposit on the surface of lateral root primordium (LRP) and the surrounding endodermal cells in Col-0.

Scale Bar: 50 μm in (B, C, E). See also Figure S4.

Figure1


A
Shoot fresh weight (mg)
Col-0
lcs2-1
a
a
b
c
2
0.2
Ca concentration (mM)
B
Col-0
lcs2-1
C
Lateral roots (No.)
Col-0
lcs2-1
a
b
b
d
2
0.2
Ca concentration (mM)
D
Col-0
lcs2-1
E
Shoot Ca (mg.g-1 DW)
Col-0
lcs2-1
a
b
c
d
2
0.4
Ca concentration (mM)
F
PC2
PC1
Col-0
lcs2-1
esb1-1
myb36-1
casp1-1casp3-1
sgn3-3

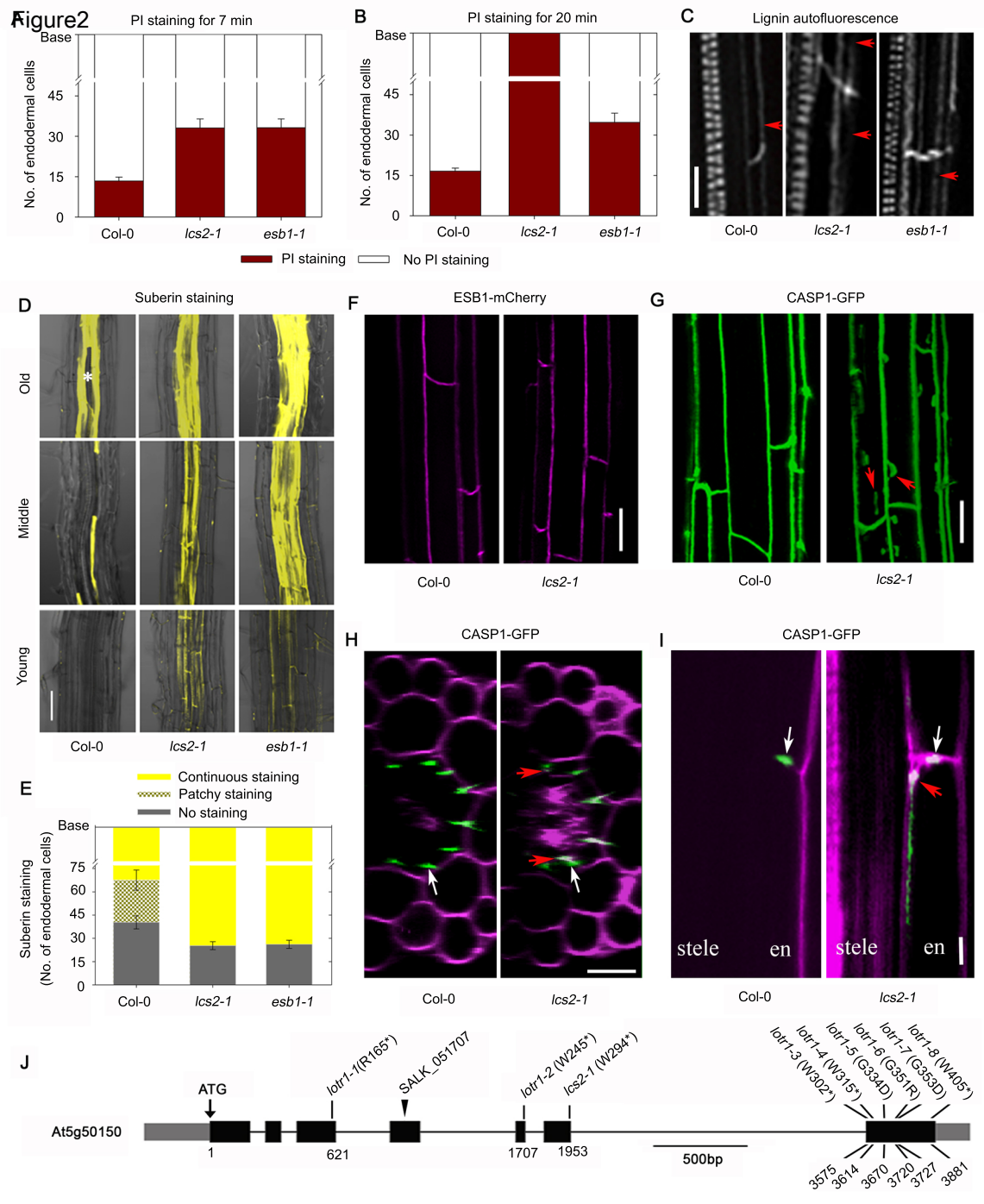


Figure2
A
PI staining for 7 min
Base
No. of endodermal cells
45
30
15
0
Col-0
lcs2-1
esb1-1
B
PI staining for 20 min
C
Lignin autofluorescence
PI staining
No PI staining
D
Suberin staining
Old
Middle
Young
E
Continuous staining
Patchy staining
No staining
Suberin staining
(No. of endodermal cells)
75
60
F
ESB1-mCherry
G
CASP1-GFP
H
I
stele
en
J
ATG
lotr1-1(R165*)
SALK_051707
lotr1-2 (W245*)
lcs2-1 (W294*)
lotr1-3 (W302*)
lotr1-4 (W315*)
lotr1-5 (G334D)
lotr1-6 (G351R)
lotr1-7 (G353D)
lotr1-8 (W405*)
At5g50150
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500bp
3575
3614
3670
3720
3727
3881

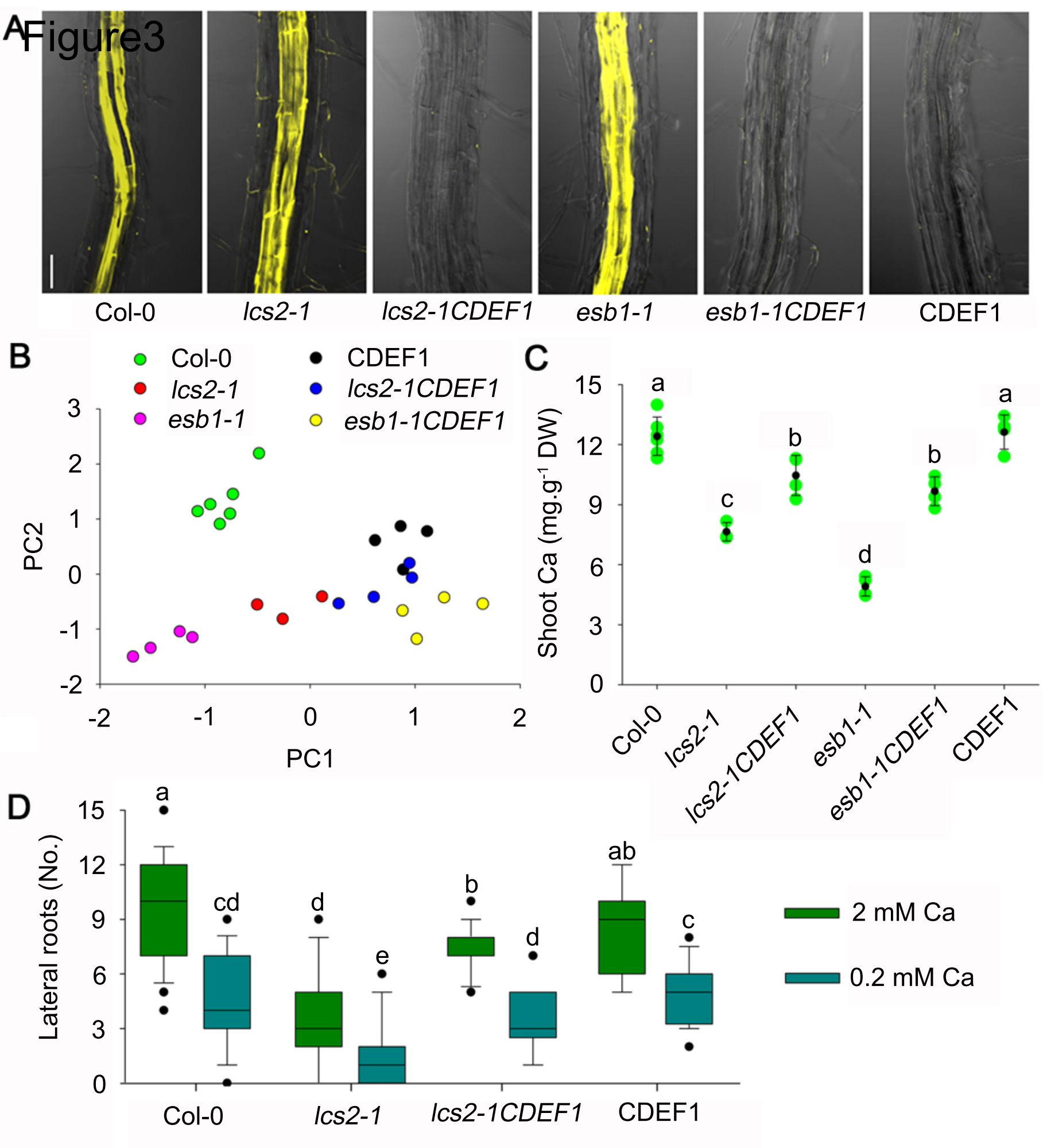
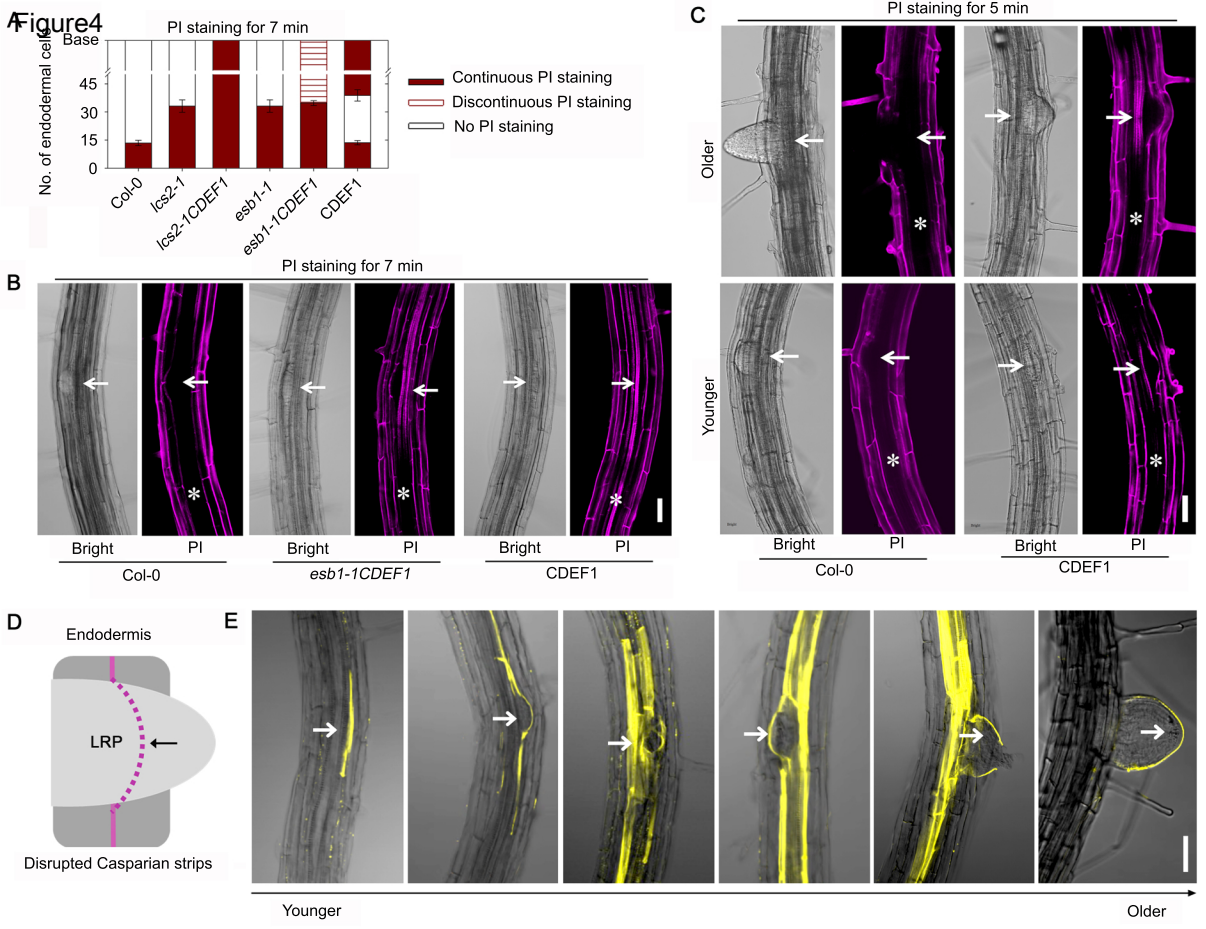


Figure3
A
Col-0
lcs2-1
lcs2-1CDEF1
esb1-1
esb1-1CDEF1
CDEF1
B
Col-0
lcs2-1
esb1-1
CDEF1
lcs2-1CDEF1
esb1-1CDEF1
PC2
PC1
C
Shoot Ca (mg.g-1 DW)
a
c
b
d
b
a
Col-0
lcs2-1
lcs2-1CDEF1
esb1-1
esb1-1CDEF1
CDEF1
D
Lateral roots (No.)
a
cd
d
e
b
d
ab
c
2 mM Ca
0.2 mM Ca
Col-0
lcs2-1
lcs2-1CDEF1
CDEF1

Figure4


A
PI staining for 7 min
No. of endodermal cells
Base
45
30
15
0
Col-0
lcs2-1
lcs2-1CDEF1
esb1-1
esb1-1CDEF1
CDEF1
Continuous PI staining
Discontinuous PI staining
No PI staining
B
PI staining for 7 min
Bright
PI
Col-0
esb1-1CDEF1
CDEF1
C
PI staining for 5 min
Older
Younger
Bright
PI
Col-0
CDEF1
D
Endodermis
LRP
Disrupted Casparian strips
E
Younger
Older

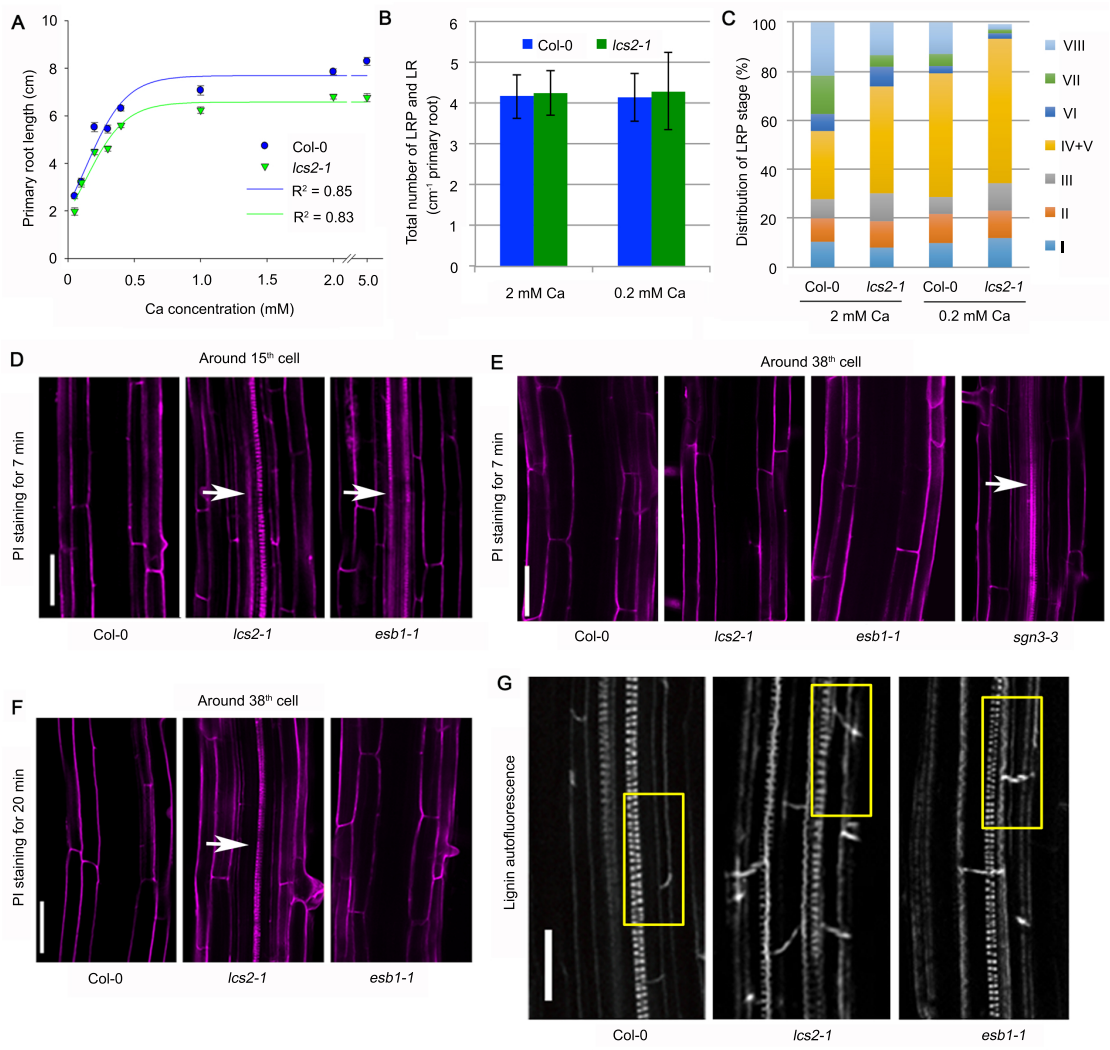


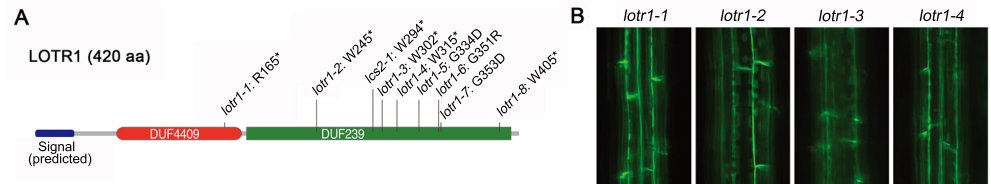
Figure S1. The primary root length, lateral root primordium (LRPs) development, the apoplastic barrier function and Casparian strip deposition in *lcs2-1*(Related to Figure 1 and 2)

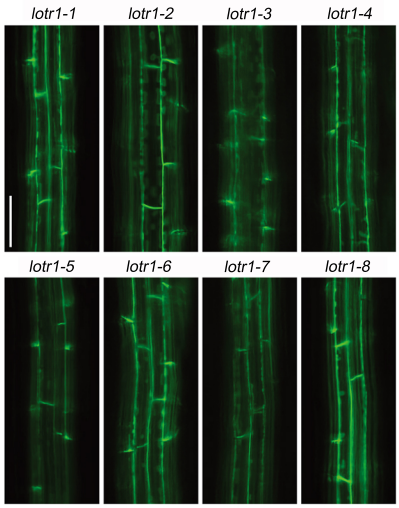
(A) The *lcs2-1* primary root was shorter than Col-0 in regardless of Ca supply. Seedlings were germinated and grown in the medium with different Ca concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 1, 2, and 5 mM) for 10 days. Each treatment contained three independent plates. Col-0 and *lcs2-1* seedlings were grown alongside in each plate. Values are mean ± SE (n = 20-24), shown in scatter plots and fitted with Nonlinear Regression - Dynamic Fitting Equation: Sigmoidal, Sigmoid, 3 Parameter ('S' curve).

(B) The total number of lateral root primodium (LPR) initiated including both LRPs and visible lateral roots (LR) is similar between *lcs2-1* carrying *proPIN1:PIN1-GFP* and the wild-type carrying *proPIN1:PIN1-GFP*. The seedlings were germinated and grown in 2 mM Ca and 0.2 mM Ca for 10 days. The number of LRPs was counted using confocal microscopy based on the signal of PIN1-GFP. Values are mean ± SD (n = 8 seedlings in the wild-type, n=10 seedlings in *lcs2-1* mutant). (C) Distribution of LRP stage was changed in *lcs2-1* carrying *proPIN1:PIN1-GFP*. The developmental stages of the same seedlings used in (B) were classified based on the criteria described in [S1]. The number of LRP at the stage IV and V was combined because it is difficult to distinguish between them in our condition. At the stage IV, the LRP has penetrated the parent endodermal layer [S1].

(D-F) The apoplastic barrier function in *lcs2-1* is lower than that in *esb1-1* but higher than that in *sgn3-3* mutant. The representative images show similar delay of blockage for PI diffusion into the stele around 15th (D) and 38th (E and F) endodermal cell. PI staining for 7 min (D and E) and 20 min (F). Scale bar: 50 μm. White arrows indicate PI staining in the stele.

(G) Large gaps in the Casparian strip were observed in *lcs2-1*. Disrupted Casparian strip was observed in the whole root. The representative image was shown in the earlier phase of mature endodermis. The insets (yellow rectangles) are enlarged in Figure 2C. Scale bar: 25 μm.





C

Allele	Code change	Amino acid change	Protein domain
lcs2-1	UGG -> UGA	W294*	DUF239
lotr1-1	CGA -> UGA	R165*	DUF4409
lotr1-2	UGG -> UGA	W245*	DUF239
lotr1-3	UGG -> UGA	W302*	DUF239
lotr1-4	UGG -> UGA	W315*	DUF239
lotr1-5	GGC -> GAC	G334D	DUF239
lotr1-6	GGA -> AGA	G351R	DUF239
lotr1-7	GGC -> GAC	G353D	DUF239
lotr1-8	UGG -> UGA	W405*	DUF239

Allele	T-DNA line	Insertion site
lotr1-9	SALK_051707	Exon 4

Figure S2. *lotr1* mutants contain polymorphisms in *At5g50150* (*LOTR1*) (Related to Figure 2)

(A) Schematic representation of the LOTR1 protein, its predicted domains based on information accessible through Uniprot (identifier: Q9FG96; www.uniprot.org) and the location of the isolated mutations.

(B) Epifluorescence images of CASP1-GFP in eight identified *lotr1* alleles display a correctly localized fluorescence signal with occasional large gaps and frequent ectopic CASP1-GFP-labeled structures. Scale bar: 50 µm.

(C) All known mutant alleles of *LOTR1*.

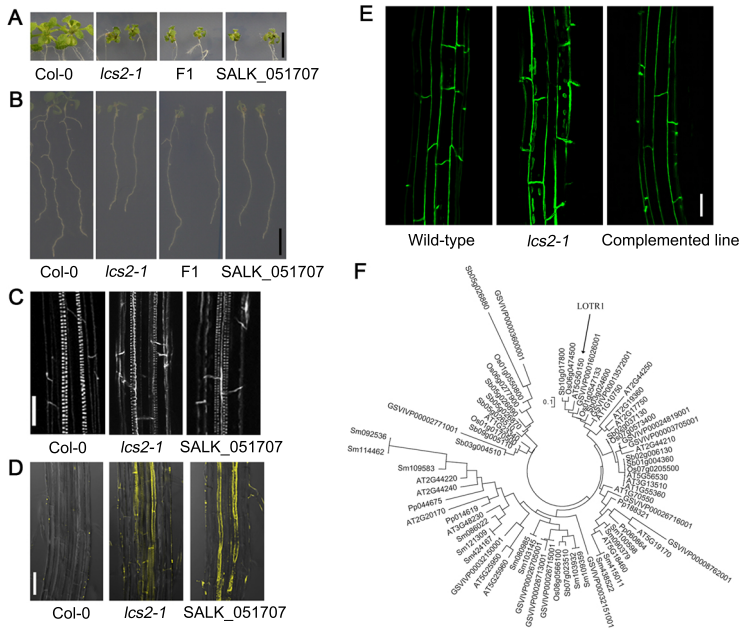


Figure S3. The phenotypes of *LOTR1* (*At5g50150*) T-DNA alleles, complemented test, and phylogenetic analysis of *LOTR1* homologs in plants (Related to Figure 2)

(A and B) The T-DNA line of *At5g50150* (SALK_051707) showed similar leaf growth and visible lateral root number with *lcs2-1* and their F1 crosses under 0.2 mM Ca condition. The plants were grown for 14 days (A) and 9 days (B), respectively. Scale bar: 5 mm.

(C) Z-stack confocal image of lignin-based Casparian strip autofluorescence in roots. Spiral-like signal in the center is from the xylem vessel.

(D) Suberin lamellae were detected with fluorol yellow 088 staining in the young section (around 30 cells after the onset of endodermal cell elongation) of roots.

(E) The localization of CASP1-GFP was recovered in *lcs2-1* carrying wild-type *At5g50150* genomic DNA (Complemented line). The *At5g50150* genomic DNA including the native promoter was introduced into the *lcs2-1* expressing *ProCASP1:CASP1-GFP* lines. Z-stack confocal images of CASP1-GFP are shown. Scale bar: 25 μm.

(F) Phylogenetic tree of *LOTR1* homologs in plants. Phylogenetic tree of 89 genes in the family containing both the domain of unknown function 4409 (DUF4409) and the domain of unknown function 239 (DUF239) in 26 plant species was shown. *LOTR1* (*At5g50150*) belongs to the gene family ORTHO03D000423 which includes 89 genes in 26 species in PLAZA3.0 (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots).

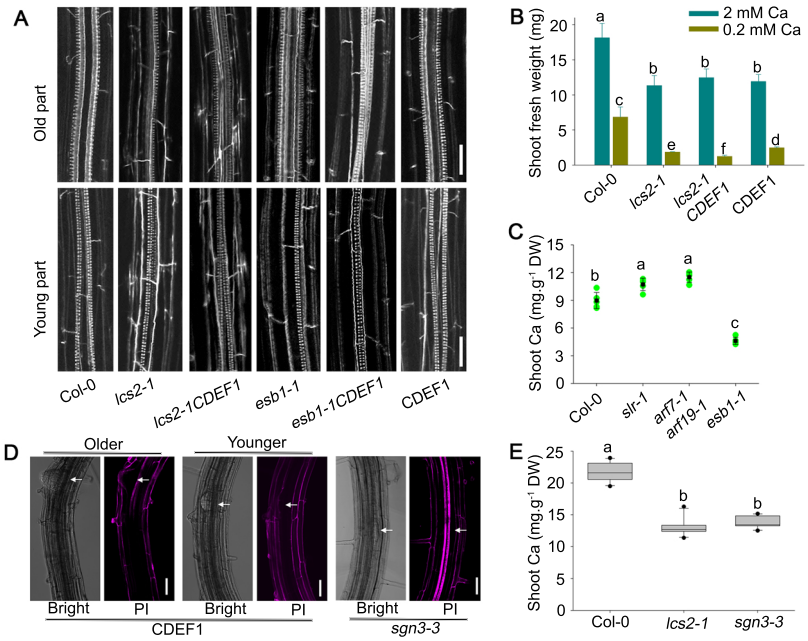


Figure S4. Effects of *ProCASP1:CDEF1* (CDEF1) on Casparian strip formation, shoot growth, PI penetration at lateral root emergence site and shoot Ca accumulation in no lateral root mutants and *sgn3*(Related to Figure 3 and 4)

(A) The expression of *ProCASP1:CDEF1* (CDEF1) did not affect the disrupted Casparian strips in *lcs2-1* and *esb1-1* roots. Z-stack confocal images of lignin-based Casparian strip autofluorescence were presented in the earlier phase of mature endodermis (young part) and the more mature region developed with lateral root primordium (old part). Spiral-like signal in the center is from xylem vessel. Scale bar: 25 μm.

(B) The expression of *ProCASP1:CDEF1* (CDEF1) did not salvage the shoot growth of *lcs2-1* mutant. The seedlings were grown for 16 days. The seedlings from each plate were collected as a pool for shoot fresh weight measurement. The shoot fresh weight per plant was calculated by dividing the weight of the pool by the number of plants. The bars represent the mean ± SD (n = 4). Different letters indicate significant difference by Tukey's HSD tests ($p < 0.05$).

(C) Shoot calcium accumulation in no lateral root mutants: *slr-1* [S2] and *arf7-1arf19-1* [S3]. The plants were grown under normal conditions for 14 days. Ca accumulation of *slr-1* and *arf7-1arf19-1* slightly increased against Col-0. Scatter plots and mean ± SD are shown (n = 5). Different letters indicate significant difference (Tukey's HSD tests, $p < 0.05$).

(D) PI penetrated initially into the stele around the mature lateral root primordium (LRP) region in *ProCASP1:CDEF1* (CDEF1) roots but uniformly in *sng3-3* roots with PI staining for 3 min. White arrow indicates the LRP. Magenta color indicates the PI staining. The stele was in the center of roots. Scale bar: 50 μm.

(E) The Ca concentration in shoots is reduced in *sgn3-3* mutant. Box plots showed the Ca concentration in shoots (n = 10). The seedlings were grown in agar medium with normal Ca for 14 days. Different letters indicate significant difference (Tukey's HSD tests, $p < 0.05$).

Table S1. **Altered leaf ionomic profile in *lcs2-1* mutant grown in soil for 5 weeks (Related to Figure 1)**

Elements	Col-0 (ppm)		*lcs2-1* (ppm)		Difference	Corrected
	Mean	± SD	mean	± SD	(%)	*p*-value
Li	21.3	3.7	22.6	4.3	6.47	0.509
B	230	68	496	224	116	0.006
Na	544	130	744	234	36.7	0.029
Mg	17900	1270	16700	1070	-6.7	0.057
P	8980	1330	9860	1230	9.71	0.071
S	10300	1770	12500	2080	20.9	0.012
K	60500	14700	71100	13800	17.5	0.049
Ca	53600	4140	31800	2490	-40.6	1.46E-08
Mn	247	30.8	186	22.7	-24.6	2.00E-04
Fe	121	18.7	102	16.4	-15.2	0.05
Co	2.67	0.29	3.17	0.52	18.8	0.035
Ni	1.1	0.15	1.17	0.33	6.55	0.512
Cu	5.02	1.02	6.32	0.75	26.0	0.003
Zn	78.3	5.3	74.1	9.0	-5.33	0.148
As	3.07	1.68	6.41	2.04	109	0.002
Se	53.9	24.2	54.8	12.8	1.63	0.908
Rb	116	29.9	150	32.6	29.0	0.011
Sr	138	12.6	87.1	6.6	-37.1	1.10E-07
Mo	1.17	0.4	1.72	0.55	46.5	0.01
Cd	1.22	0.21	1.92	0.25	57.1	2.00E-04

Note: The data was statistically analyzed with two-tailed Student's *t*-test (n = 12). *P-value* was corrected with Benjamini and Hochberg's FDR correction for multiple comparisons and shown. Raw data are available from the tray 2017 in the iHUB (http://www.ionomicshub.org/home/PiiMS). *lcs2-1* was named as Ca-f in the iHUB. The ambient growth conditions were as follows: day length, 12 h; temperature, 22°C; humidity, 40.0%; growth media, Bul 1A; light intensity, 110.0 μE; fertilization, 0.25 Hoagland;s + 1 ml/L Fe HBED.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *lcs2-1* mutant was isolated from EMS-mutagenized Columbia (Col-0) ecotype of *Arabidopsis thaliana*. PCR primers (5'-TGGAAAAGCAGACTGATGCATACC-3' and 5'-AATGGAAGGAAATGAAGACTGACCAT-3') and the BccI enzyme were used to identify the point-mutated *LOTR1* in *lcs2-1* mutant. SALK_051707 was obtained from ABRC and homozygous lines were established with the PCR primers (5'-TACCGTCTCATTTACAACCCG-3' and 5'-TCAAACACAAAACGAATGTGG-3'). Casparian strip defective mutants *esb1-1* [S4], *sgn3-3* [S5], *myb36-1* [S6] and *casp1-1casp3-1* [S7] were used in this study. The transgenic lines *ProCASP1:CASP1-GFP* [S7], *ProESB1:ESB1-mCherry* [S8], *ProCASP1:CDEF1* [S9] and *ProPIN1:PIN1-GFP*[S10] were introduced in *lcs2-1* by crossing or *Agrobacterium*-mediated floral dip method. All the materials were in Col-0 background. The seeds were surface-sterilized and sown on Yamagami medium solidified with the 1.5% purified agar (catalog no. 01056-15, Nacalai Tesque, Inc., Japan) [S11]. After incubation for 2 days at 4°C, the plates were placed vertically in a growth chamber at 22°C under 16 h light/8 h dark.

Phenotype and ionome analysis

The seedlings were grown in agar medium. After 9 days, the plants were photographed and the number of visible lateral roots was counted. After 14 days, the shoots were photographed and harvested for fresh weight measurement. The LRPs were observed based on the signal of proPIN1:PIN1-GFP, which produces a very clear GFP signal in each stage of LRPs particularly in these LRPs at the early stages[S10]. The stage of LRPs is classified as based on the criteria described in the paper [S1]. For the ionomics analysis, the shoots of several 14-day-old seedlings were harvested as one sample, washed with deionized water, and dried at 60°C for 3 days. After measuring the dry weight, the samples were digested with 61% HNO_3 and dissolved with 0.08M HNO_3 containing 2 ppb indium as an internal standard. The concentrations of elements were determined by inductively coupled plasma (ICP)-MS (NexION, PerkinElmer, U.S.A in Figure 1F and Table S1; SPQ9700, Hitachi High Technologies, Japan). Due to the difference of the sensitivity of the instruments, the concentrations of 10 elements (Li, B, Na, Mg, P, K, Ca, Mn, Cu, Mo) were measured and used for PC analysis in Figure 3B. The size of shoot under 0.2 mM Ca condition is too small to obtain enough amount of samples for ICP-MS analysis, the plants grown under 0.4 mM Ca condition were subjected to the analysis for low Ca condition.

Plasmid constructs and transformation in *Arabidopsis*

The 6537-bp genomic DNA of *At5g50150* (including 2637-bp sequences upstream of ATG) from wild-type Col-0 was amplified with PCR primers (5'-CACCCACAGAGGTGCGTGGTTGTATGA-3' and 5'-AAAAGGACACCTTGGGTTCCGACCAG-3'). The DNA fragment was introduced into pENTR™/D-TOPO vector (Life Technologies) and then transferred into the Gateway plant destination vector pGWB559 [S12] by using LR clonase (Life Technologies). The construct was transformed into *lcs2-1* carried with *ProCASP1:CASP1-GFP* by the *Agrobacterium*-mediated flower dip method [S13].

Confocal laser scanning microscopy observation

The penetration of PI into the stele was used to assess the function of apoplastic diffusion barriers in roots [S14]. The seedlings were incubated in a fresh solution of 15 μM (10 μg mL^{-1}) PI in the dark for the period indicated in the figure legend (3, 5, 7, and 20 min), and rinsed twice with water. Fluorol yellow 088 staining was used to detect suberin accumulation in roots [S15]. For quantification of the number of endodermal cells, the onset of elongation was defined as the endodermal cell whose median optical section was more than twice in width [S14]. Lignin autofluorescence of cleared roots was used to observe the Casparian strip deposition [S14]. The 5- or 6-day-old seedlings (as stated in figure legends) were used to observe Casparian strip autofluorescence, suberin lamella stained with fluorol yellow 088, PI permeability, GFP and mCherry fluorescence. The fluorescence was observed using confocal microscopy FV1000 (Olympus). The excitation and emission wavelengths were set as follows: 488 nm and 485–545 nm for Casparian strip autofluorescence, fluorol yellow 088 and GFP; 559 nm and 570–670 nm for PI and mCherry. The z-stack and radial optical section images were constructed with a successive series of 1-μm step-size images with Fiji (http://fiji.sc/Fiji). The signal of PIN1-GFP is observed with Confocal microscopy. All the experiments were reproduced in different trials.

Statistical analysis

The significant difference was performed with one-way ANOVA with Tukey's HSD tests using SPSS 13.0 software, except for the data in Supplementary Table 1 that were analyzed with two-tailed Student's *t*-test followed by Benjamini and Hochberg's FDR correction. The bar graphs, box plots, principal component analysis plots, and scatter plots were produced in SigmaPlot10.0 and Microsoft Excel15.24 software.

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