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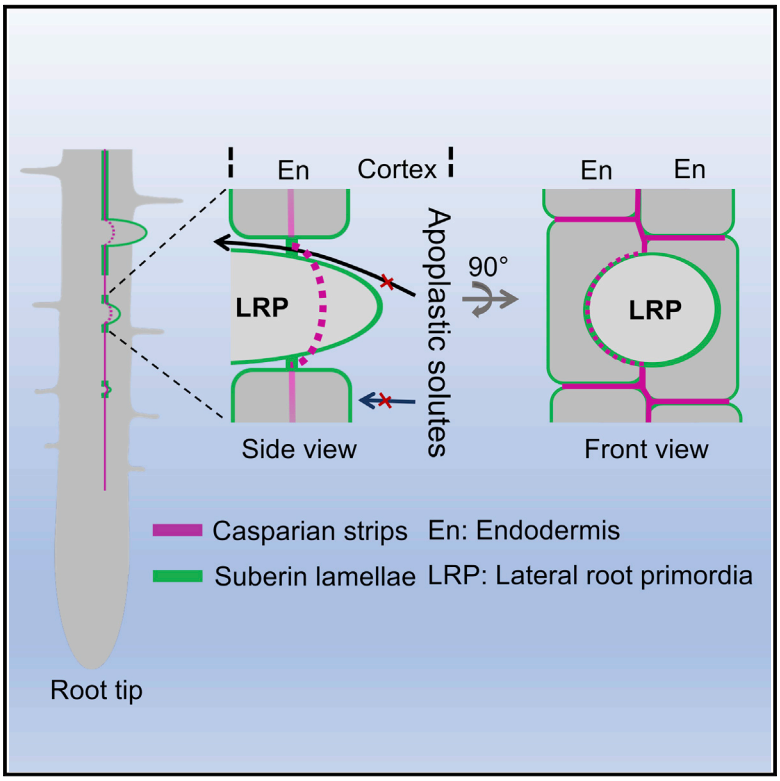
Role of *LOTR1* in Nutrient Transport through Organization of Spatial Distribution of Root Endodermal Barriers

Graphical Abstract



Authors

Baohai Li, Takehiro Kamiya, Lothar Kalmbach, ..., David E. Salt, Niko Geldner, Toru Fujiwara

Correspondence

atorufu@mail.ecc.u-tokyo.ac.jp

In Brief

Li, Kamiya, et al. identify a novel gene required for ion transport by organizing Casparian strip formation and suberin deposition. Further, through the analysis of the Casparian strip mutants, they show that suberin lamella functions as an apoplastic barrier at the site of lateral root emergence where Casparian strips are disrupted.

Highlights

- *lcs2* has a defect in Casparian strip formation and accumulates more suberin
- The causal gene of *lcs2* is required for the correct localization of CASP1
- Ectopic suberin accumulation inhibits the calcium transport from roots to shoots
- Suberin functions as an apoplastic diffusion barrier at lateral root emergence sites

Li et al., 2017, Current Biology *27*, 758–765
March 6, 2017
http://dx.doi.org/10.1016/j.cub.2017.01.030

CellPress

Current Biology
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Role of *LOTR1* in Nutrient Transport through Organization of Spatial Distribution of Root Endodermal Barriers

Baohai Li,[1,7,8] Takehiro Kamiya,[1,7] Lothar Kalmbach,[2] Mutsumi Yamagami,[3] Katsushi Yamaguchi,[4] Shuji Shigenobu,[4] Shinichiro Sawa,[5] John M.C. Danku,[6,9,10] David E. Salt,[6,9] Niko Geldner,[2] and Toru Fujiwara[1,11,*]

[1]Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan
[2]Department of Plant Molecular Biology, University of Lausanne–Sorge, 1015 Lausanne, Switzerland
[3]Department of Radioecology, Institute for Environmental Sciences, Aomori 039-3212, Japan
[4]NIBB Core Research Facilities, National Institute for Basic Biology, Aichi 444-8585, Japan
[5]Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan
[6]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK
[7]Co-first author
[8]Present address: Gregor Mendel Institute, Austrian Academy of Sciences, Vienna Biocenter, 1030 Vienna, Austria
[9]Present address: Centre for Plant Integrative Biology, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK
[10]We dedicate this article to Dr. John M.C. Danku, who passed away on December 13, 2016
[11]Lead Contact
*Correspondence: atorufu@mail.ecc.u-tokyo.ac.jp
http://dx.doi.org/10.1016/j.cub.2017.01.030

SUMMARY

The formation of Casparian strips and suberin lamellae at the endodermis limits 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.

RESULTS

The *low Ca sensitive 2* 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 to 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

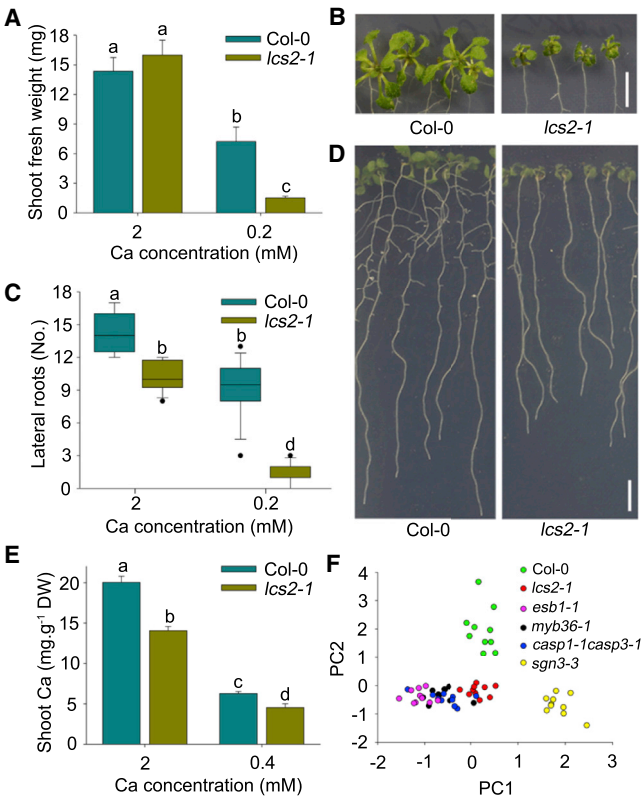

Figure 1. Growth Phenotype and Ionome Pattern of the *lcs2-1* Mutant

(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 roots grown in agar medium for 9 days and shown with boxplots (n > 12). The middle line, boxes, and whiskers represent the median, first quartile to third quartile, and 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). DW, dry weight.

(F) Principal-component analysis of *lcs2-1* and known Casparian-strip-defective mutants based on 20 element concentrations (as shown in Table S1) in shoots grown on agar medium with normal Ca for 14 days (n = 10).

Error bars represent mean ± SD in (A) and (E). Different letters indicate the significant difference (Tukey's honestly significant difference [HSD] tests, $p < 0.05$). Scale bars, 5 mm in (B) and (D). See also Figure S1.

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 ethyl methanesulfonate (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; Figures 1A–1D and S1A). Detailed analysis revealed that the LRP was normally formed, while lateral root emergence was delayed in *lcs2-1*, under low Ca treatment (Figures 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 those of *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 that of 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 mutant [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 Experimental Procedures) in the wild-type and at around the 34th endodermal cell after the onset of elongation in *lcs2-1* and *esb1-1* mutants (Figures 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 (Figures 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 patterns in the wild-type, large gaps in the Casparian strips were observed in *lcs2-1* (Figures 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 (Figures 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 (Figures 2D and 2E). This suberin deposition pattern of *lcs2-1* was similar to that of *esb1-1* (Figures 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 (Figures 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 (Figures 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 (Figures 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 *Ler*, 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 transfer DNA (T-DNA) insertion in the fourth exon of *At5g50150*, displayed a phenotype similar to that of *lcs2-1* in that it exhibited low Ca sensitivity, Casparian strip, and suberin deposition (Figures 2J and S3A–S3D) and the F_1 between *lcs2-1* and SALK_051707 did not recover these low Ca-sensitive phenotypes (Figures 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*, which 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 the 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 those of 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 (Figures S1B and S1C), this result suggests that enhanced suberin

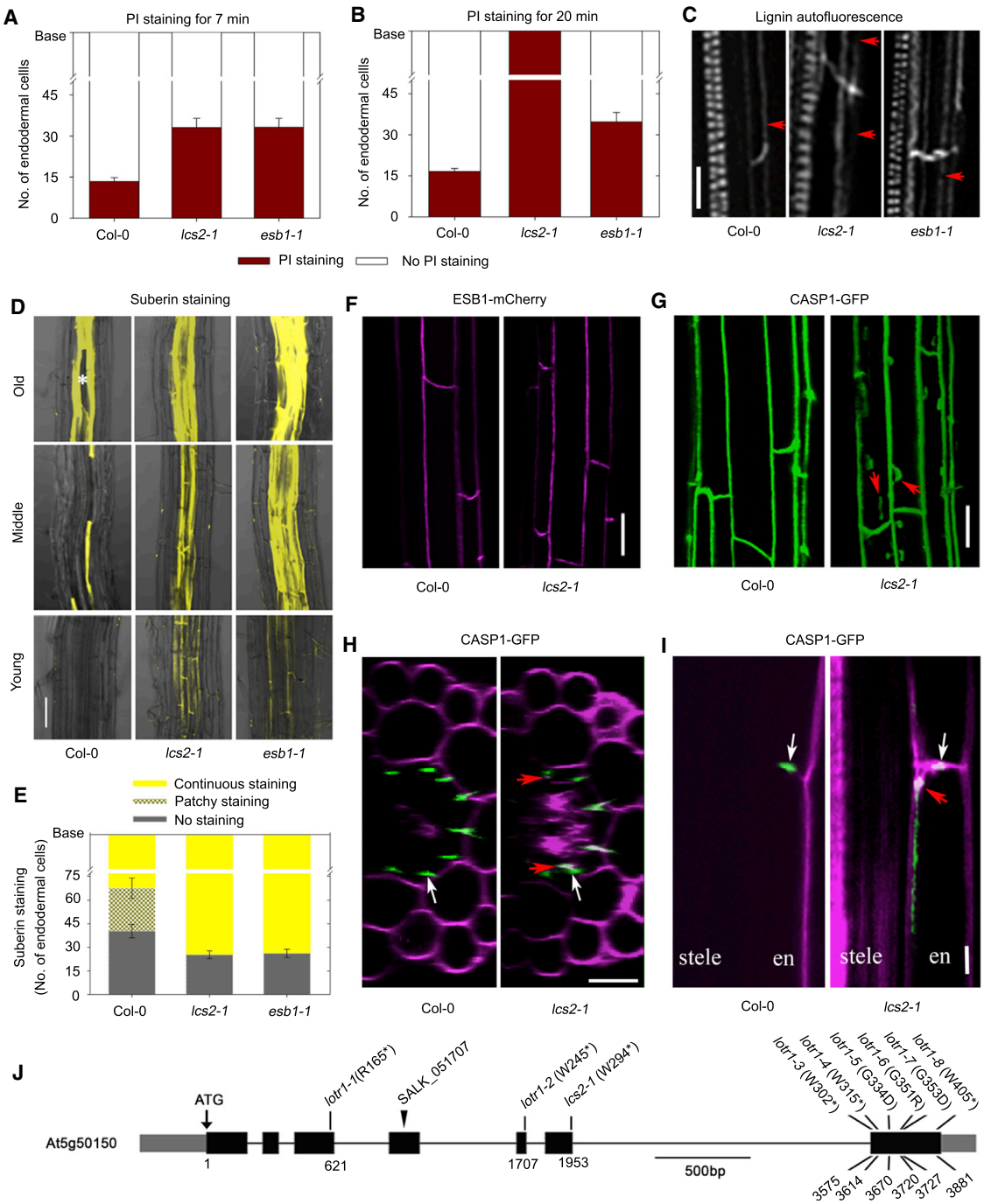

Figure 2. *lcs2-1* Is a Novel Mutant Showing Defects in Casparian Strips

(A and 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 is 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, and 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.

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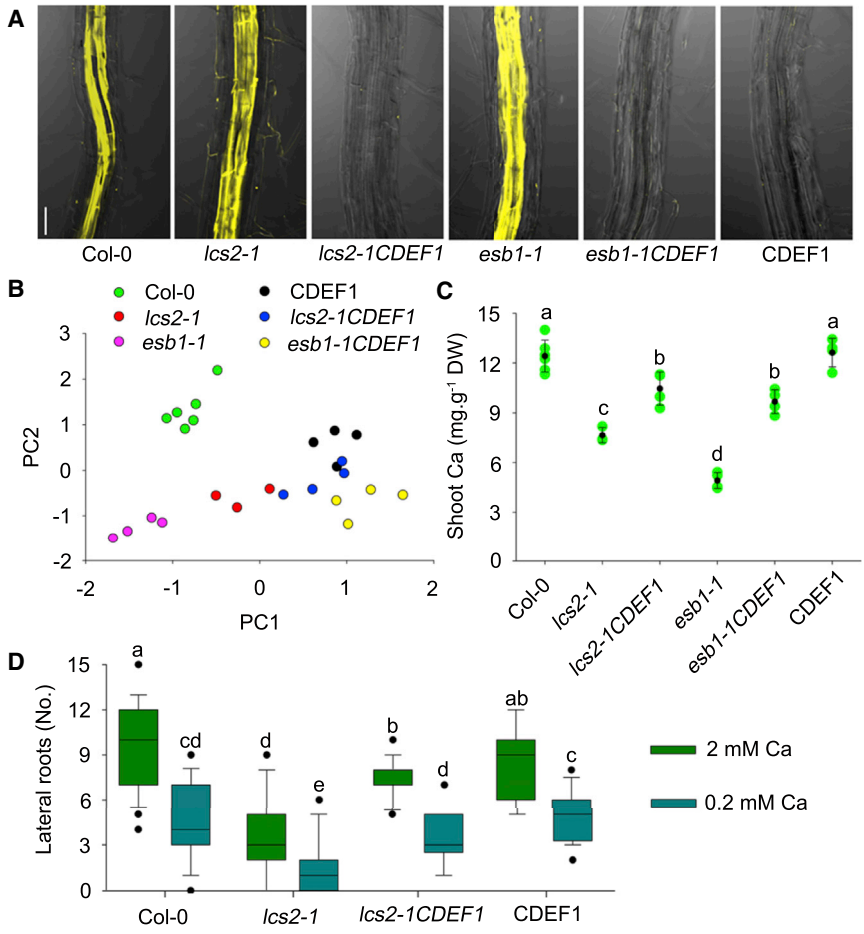

Figure 3. Ectopic Suberin Accumulation Reduces Calcium Delivery to Shoot and Lateral Root Formation in *lcs2-1* Mutant

(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 and C) Principal-component analysis based on the concentrations of ten elements (Li, B, Na, Mg, P, K, Ca, Mn, Cu, and 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) and (C) were grown in agar medium under normal conditions for 14 days. Scatterplots and mean ± SD are shown. DW, dry weight.

(D) The visible lateral root number was partially recovered in *lcs2-1* by expressing *CDEF1* and shown with boxplots, 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.

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, rather, was 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 in 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].

(G–I) CASP1-GFP localization was altered in *lcs2-1* mutant. (G) The z stack image was constructed with 30 images obtained by confocal microscopy. CASP1-GFP was localized into both stele (red arrow) and the central portion of endodermal cells (en) (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 in (I).

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

All seedlings were grown for 5 days. Error bars represent the mean ± SD in (A), (B), and (E). Scale bars, 10 μm in (C), 50 μm in (D), 20 μm in (F)–(H), and 5 μm in (I). See also Figures S1–S3.

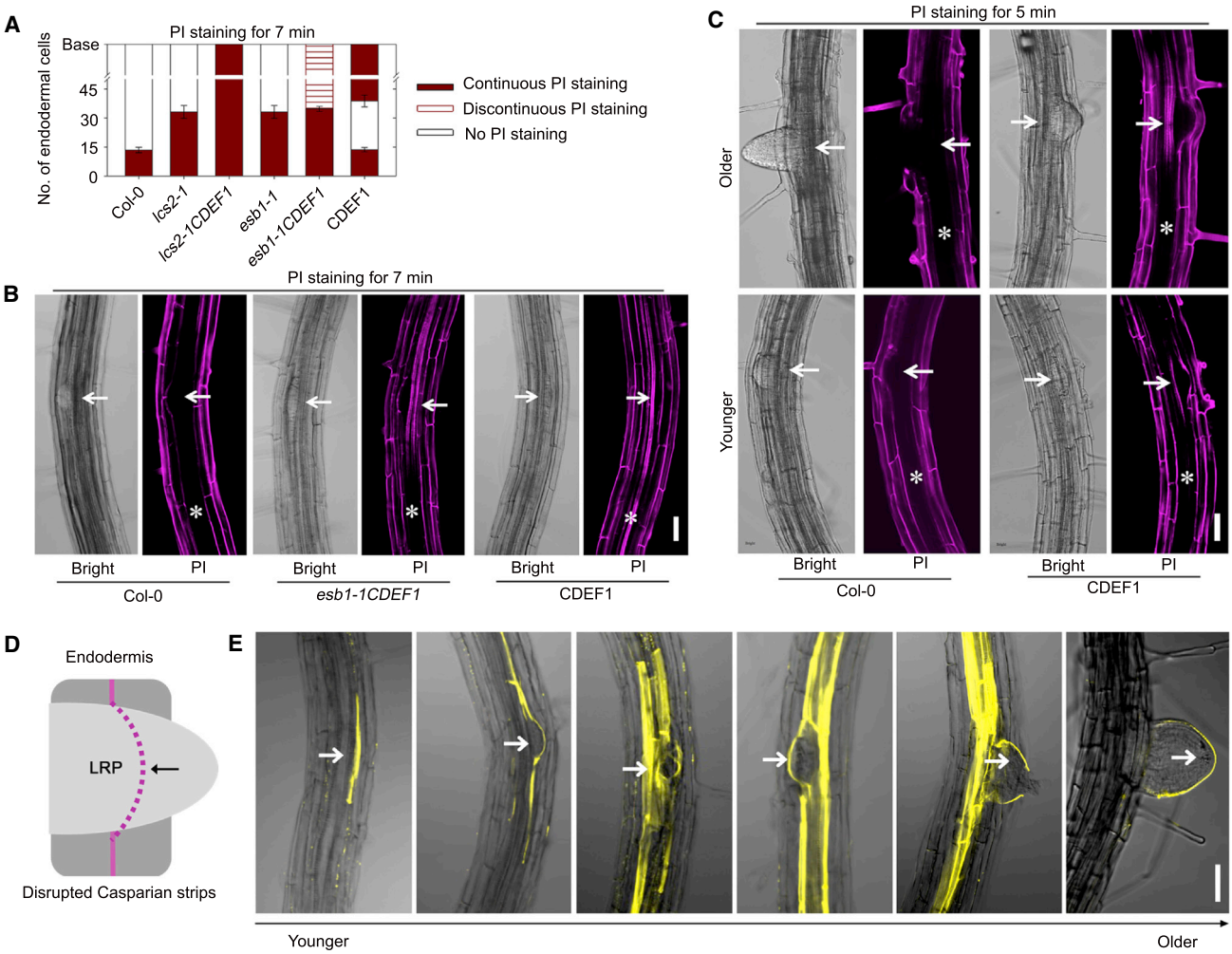

Figure 4. Suberin Lamellae Function as Apoplastic Diffusion Barrier at Lateral Root Emergence Sites

(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). Error 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 bars, 50 μm in (B), (C), and (E). See also Figure S4.

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* (Figures 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 emergence 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 (Figures 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 first deposited on the surface of LRPs 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 (Figures 2G–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 that the domain of unknown function 239 (DUF239) in LOTR1 is denoted as a glucoamylase domain (The Arabidopsis Information Resource, 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 the 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 (Figures S1A and S1C), the seedlings of *lotr1* mutants can grow as 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 leakage of Ca from the stele through the disrupted Casparian strips.

Suberin is a 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 transmission electron microscopy (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 affects lateral root emergence in *lcs2-1* mutant (Figures 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 a 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 proposes 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 are described in detail in the Supplemental Information.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.01.030.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

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.), 15H01224, and 25221202 (to T.F.) and by a JSPS postdoctoral fellowship for overseas researchers (grant number P11394 to B.L.).

Received: August 28, 2016
Revised: December 2, 2016
Accepted: January 17, 2017
Published: February 23, 2017

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