A peptide hormone required for Casparian strip diffusion-barrier formation in *Arabidopsis* roots

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Abstract

Plants achieve mineral ion homeostasis by restricting lateral diffusion of ions between the root vascular bundles and the soil by a hydrophobic barrier on endodermal cells called the Casparian strip. We identified a family of sulfated peptides required for contiguous Casparian strip formation in *Arabidopsis* roots. These peptide hormones, which we named Casparian strip integrity factor 1 (CIF1) and CIF2, are expressed in the root stele and specifically bind endodermis-expressed leucine-rich-repeat receptor kinase, GASSHO1 (GSO1)/SCHENGEN3, and its homolog, GSO2. A mutant devoid of CIF peptides is defective in ion homeostasis in the xylem. *CIF* genes are environmentally responsive. Casparian strip regulation is not just a passive process driven by root developmental cues but also serves as an active strategy to cope with adverse soil conditions.

One-Sentence Summary

We identified a peptide hormone required for formation of the Casparian strip in plants, a physical barrier preventing passive diffusion of mineral ions and water between the root vascular bundles and the soil.

Main text

Nutrient uptake by roots is a fundamental process for plant growth and development. Mineral ions enter into the cytoplasm of epidermal cells and the cortex or outer face of endodermal cells primarily via transporter-mediated pathways. These ions then move across the endodermis by symplastic transport through plasmodesmata and are ultimately secreted into the xylem for transfer to the shoot. As nutrients often accumulate against a concentration gradient in the xylem vessels, vascular plants have evolved a ring-like physical barrier that prevents passive apoplastic diffusion of ions and water across the endodermal cells that surround the vascular bundles. This hydrophobic barrier embedded within the walls of endodermal cells is called the Casparian strip (*1*).

The Casparian strip consists primarily of a hydrophobic, water-impermeable lignin polymer that seals the extracellular spaces between neighboring endodermal cells. Molecular and genetic screening studies have identified several key factors required for Casparian strip formation (*2*-*6*), including Casparian strip membrane domain protein (CASP) family acting as scaffolds (*7*) and GASSHO1 (GSO1)/SCHENGEN3 (SGN3) leucine-rich-repeat receptor kinase (LRR-RK) (*8*). Of these proteins, the LRR-RK GSO1/SGN3 expressed in root endodermal cells is of particular interest, as accumulating evidence suggests that LRR-RKs of subfamily XI act as receptors for small peptide hormones (*9*). Loss-of-function mutations in GSO1/SGN3 result in incorrect patch-like localization of the CASPs, leading to the formation of a repeatedly interrupted, discontinuous Casparian strip. This finding led to the hypothesis that peptide ligand-mediated GSO1/SGN3 receptor activation is crucial for contiguous Casparian strip formation in roots.

In most cases, genes encoding the precursors of small peptide hormones exist as multiple paralogous copies in the genome (*9*). Individual precursor polypeptides share a family-specific conserved domain close to the C-terminus, from which mature functional peptide hormones are generated through posttranslational modification followed by proteolytic processing. In the course of screening novel peptide hormone candidates in *Arabidopsis* based on these empirical rules (*10*), we identified two closely related paralogous genes, *At2g16385* and *At4g34600*, each of which encodes an ≈80-amino acid polypeptide characterized by a conserved domain at the C-terminus (Fig. S1A). Phylogenetic analyses indicate that possible orthologues of these

polypeptides are widely distributed among land plants (Fig. S1B). Nano-LC MS/MS analyses of the peptides secreted from *Arabidopsis* plants overexpressing At2g16385 determined that the mature form is a 21-amino acid tyrosine-sulfated peptide derived from the conserved domain (Fig. 1A-B, Fig. S1C-D).

To identify receptors that directly interact with members of this peptide family, we chemically synthesized a cross-linkable derivative of At2g16385 in which photoactivatable 4-azidosalicylic acid (ASA) is incorporated into the 11th residue from the N-terminus, a residue not conserved among paralogs (Fig. S2A). After radioiodination, we performed an exhaustive binding assay by photoaffinity labeling against an *Arabidopsis* receptor kinase expression library (*11*) and found that two related LRR-RKs, GSO1/SGN3 and GSO2, in subfamily XI directly interact with the [^{125}I]ASA-At2g16385 peptide (Fig. 1C, Fig. S2B). This binding was competitively inhibited by excess unlabeled At4g34600 as well as At2g16385 but not by an unrelated peptide, RGF1 (*12*), indicating that the At2g16385 and At4g34600 peptides act as specific ligands for GSO1/SGN3 and GSO2 receptor kinases (Fig. 1D).

β-Glucuronidase (GUS) reporter-aided histochemical analyses revealed At2g16385 promoter activity in the stele, especially at the phloem pole, of the mature region of the primary roots (Fig. 2A). No signal was detected in shoots. At4g34600 promoter activity was also confined to the root stele in the elongation and differentiation zones of both primary and lateral roots (Fig. 2B). GUS activity disappeared at the sites of lateral root emergence (Fig. 2B). In the root tip, the GUS signal became visible at and above approximately 10 cells after the onset of elongation (Fig. 2C).

To determine whether loss of the At2g16385 and At4g34600 peptides phenocopies the *gso1/sgn3* single or *gso1/sgn3 gso2* double mutants in roots, we identified T-DNA insertion lines that carry mutations in the 5'-untranslated region of the *At2g16385* gene and in the intron of the *At4g34600* gene (Fig. 3A, Fig. S3A). Although the At2g16385 At4g34600 ligand double mutant germinated normally on agar plates without ectopic adhesion of cotyledons caused by embryonic cuticle defects reported in *gso1/sgn3 gso2* double mutants (*13*), (Fig. 3B, Fig. S3B-C), we observed that the mutant displayed an obvious defect in endodermal barrier formation in the roots, as visualized by penetration of the hydrophilic dye, propidium iodide (PI), into the vasculature (Fig. 3C). This phenotype was comparable to that observed in the roots of the

gso1/sgn3 gso2 receptor double mutant. The root defect in the ligand double mutant was restored when the mutant was treated with synthetic At2g16385 peptide. In contrast, the *gso1/sgn3 gso2* receptor double mutant was completely insensitive to the peptide.

We next directly observed the Casparian strip by lignin autofluorescence and confirmed that loss of the ligands as well as receptors resulted in the formation of a repeatedly interrupted, discontinuous Casparian strip (Fig. 3D-E). Peptide treatment restored the defective Casparian strip in the ligand double mutant but not in the receptor double mutant. Dose-dependence experiments demonstrated that both the At2g16385 and At4g34600 peptides were effective at concentrations as low as 1 nM (Fig. 3F). These results unambiguously indicate that the At2g16385 and At4g34600 peptides are critical for integrity of the Casparian strip, and we therefore named the peptides Casparian strip integrity factor (CIF) 1 and 2, respectively.

A *cif1-1* single mutant showed no detectable phenotype, but a *cif2-1* mutant was defective in endodermal barrier formation (Fig. S3D-F). We confirmed that defects in the Casparian strip and rosette leaf size in the *cif1-1 cif2-1* double mutant were fully complemented by a 3.2-kb DNA fragment containing the *CIF2* gene (Fig. S3G-H). Additionally, we found that the *gso1/sgn3* receptor single mutant that is defective in Casparian strip formation was still sensitive to the CIF1 peptide (Fig. S3I-J). Because the *GSO2* transcript has been detected in roots (*14*), we concluded that, in addition to GSO1/SGN3, the GSO2 receptor kinase plays a certain—albeit minor—role in perception of CIF peptides in roots. The *cif1-1 cif2-1* ligand double mutant, however, differs from the *gso1/sgn3 gso2* receptor double mutant in that the former displays no cuticle defects in cotyledons, suggesting that additional ligands other than CIF family peptides may exist for GSO1/SGN3 and GSO2.

The Casparian strip defects in the *cif1-1 cif2-1* double mutant were further characterized by live-cell imaging using a CASP1-GFP that has been used to visualize the Casparian strip domain (*7*). We observed that CASP1-GFP accumulated in discontinuous patches in the *cif1-1 cif2-1* mutant (Fig. 3G), in a pattern similar to that reported for the *gso1/sgn3* receptor mutant (*8*). Endogenous expression of the *CASP1* and *CASP2* genes was also downregulated in the *cif1-1 cif2-1* mutant (Fig. S4A). When treated with CIF1 peptide, the discontinuous CASP1-GFP patches fused into contiguous bands comparable to those in wild-type plants, accompanied by upregulation of *CASP1* and *CASP2* expression (Fig. 3H-I, Fig. S4B). This fusing response was

detected after 5 h of peptide treatment and completed by 6-9 h (Fig. 3H-I). We also observed that when the *cif1-1 cif2-1* double mutant that had been treated with CIF1 peptide for 24 h was transferred to new medium devoid of the peptide, small breaks occasionally formed in the CASP1-GFP bands 24 h after transfer, accompanied by reduced expression of *CASP1* and *CASP2* (Fig. 3J, Fig. S4C-D). These results suggest that the CIF peptides are required for formation and maintenance of the contiguous Casparian strip.

The Casparian strip is thought to play roles in environmental adaptation by acting as a physical barrier that prevents unfavorable inward and outward leakage of ions between the xylem and the soil (*15*). Under nutrient-limiting conditions, for example, the *gso1/sgn3* receptor mutant exhibits symptoms of potassium deficiency (*8*). In the course of testing the response of the *cif1-1 cif2-1* double mutant to excess essential minerals, we found that this mutant is highly sensitive to excess iron (Fig. 4A). Although wild-type *Arabidopsis* plants tolerated excess iron at concentrations as high as 500 μM, the *cif1-1 cif2-1* double mutant showed a noticeable growth defect at 300 μM and exhibited severely stunted growth accompanied by bronzing of the leaves at 500 μM after 7 days of treatment. Excessive intake of iron by plants has been reported to induce the production and accumulation of toxic reactive oxygen species, causing damage to the plants (*16*). These growth defects were fully restored by supplementation of the medium with CIF1 peptide (Fig. 4A). These results indicate that mutants exhibiting defects of the Casparian strip cannot adapt to fluctuating iron levels.

To confirm whether excess iron intake into the xylem occurred in the mutants, we collected xylem sap from the hypocotyl of decapitated *Arabidopsis* plants and analyzed the mineral ion concentrations by synchrotron radiation X-ray fluorescence spectrometry. At the normal iron concentration (75 μM), the iron content in the xylem sap of the *cif1-1 cif2-1* mutant was virtually the same as that of the wild type (Fig. 4B). In contrast, the iron level in the xylem sap of the *cif1-1 cif2-1* mutant cultured under excess iron conditions was considerably higher than that of the wild type, probably due to inward leakage. Notably, we found that expression of both *CIF1* and *CIF2* was upregulated by excess iron, and further synergistically regulated by lowering the medium pH (Fig. 4C-D, Fig. S5A). Because iron is more soluble in acidic soils than neutral soils, this response may reflect an adaptation to ensure the growth and survival of the plants under unfavorable mineral conditions. In addition, we found that the *cif1-1 cif2-1* mutant

exhibited retarded growth under low potassium conditions, and the potassium level in the xylem sap of the mutant was lower than that of the wild type, probably due to concentration-dependent outward leakage (Fig. S5B-C). These findings suggest that Casparian strip mutants are defective in ion homeostasis in the xylem associated with inward or outward leakage of ions, depending on the ionic concentration gradient across the endodermis cell layer, leading to pleiotropic phenotypes in unfavorable mineral environments.

As sessile organisms, plants have evolved sophisticated machinery to acquire nutrients without interference from a complex array of fluctuating environmental conditions. The Casparian strip, which forms an essential diffusion barrier between the roots and soil environment, is one such example. Our biochemical and physiological analyses revealed that the stele-expressed peptide hormones CIF1 and CIF2 mediate the formation and maintenance of a functional, contiguous Casparian strip by activating endodermis-expressed GSO1/SGN3 and GSO2 receptor kinases. Given that CIF peptides are environmentally responsive, Casparian strip regulation is likely not just a passive process driven by root developmental cues but also serves as an active strategy to cope with adverse soil conditions.

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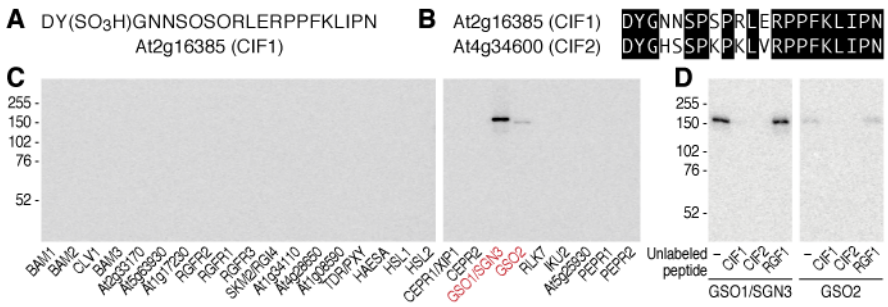

Fig. 1. Structures and receptors of the mature At2g16385 and At4g34600 peptides. (**A**) Structure of mature At2g16385 peptide (later named CIF1). O denotes hydroxyproline. (**B**) Sequence alignment of the mature peptide domains of At2g16385 and At4g34600. The residues conserved in both peptides are shaded in black. (**C**) Exhaustive photoaffinity labeling using [^{125}I]ASA-At2g16385 against membrane fractions derived from individual receptor kinase expression lines. (**D**) Competitive displacement of [^{125}I]ASA-At2g16385 binding by 300-fold excess unlabeled peptide.

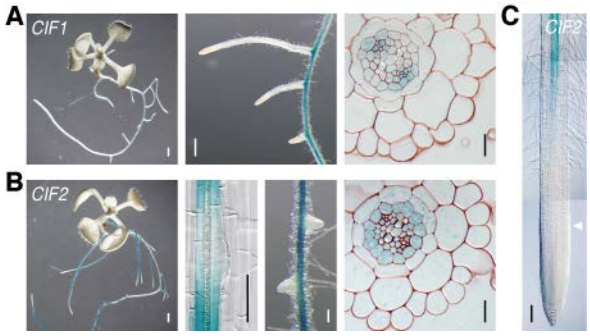

Fig. 2. Promoter activities of the *At2g16385* and *At4g34600* genes. (**A**) Promoter activity of the *At2g16385* gene. Whole view of a plant (left), a close view of the mature region of the primary roots (middle), and root cross-section (right). (**B**) Promoter activity of the *At4g34600* gene. Whole view (left), at the sites of lateral root initiation (middle left) and emergence (middle right), and root cross-section (right). (**C**) Promoter activity of the *At4g34600* gene in the root tip. Arrowhead marks the first elongating cortical cell. Scale bar: (A left, middle, B left) 1 mm; (B middle left, middle right, C) 100 μm; (A right, B right) 20 μm.

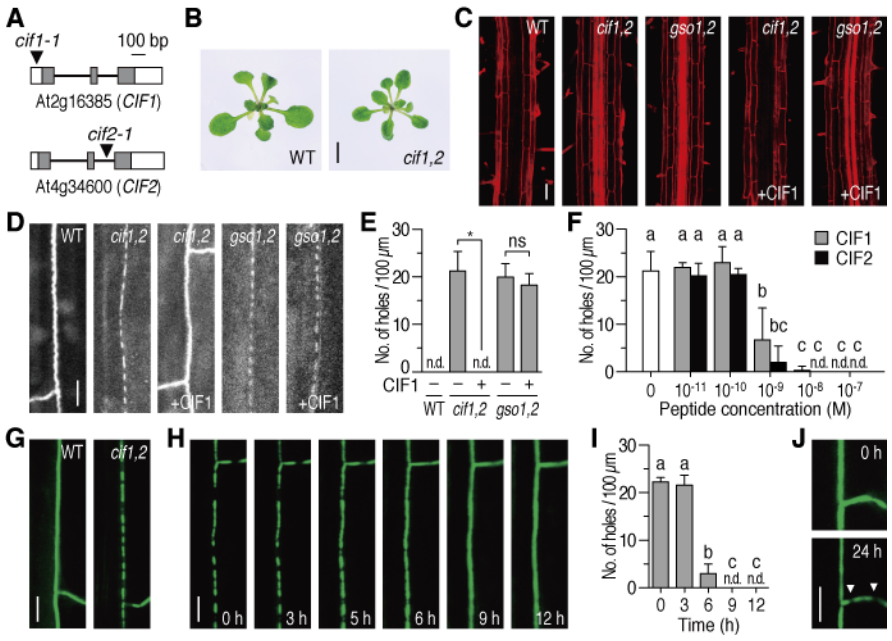

Fig. 3. Phenotypes of T-DNA insertion mutants of the *At2g16385* (*CIF1*) and *At4g34600* (*CIF2*) genes. (**A**) Schematic representation of T-DNA insertion sites in the mutants. (**B**) Two-week-old wild-type and mutant plants. (**C**) Absence of endodermal diffusion barrier in the ligand and receptor mutants visualized by penetration of propidium iodide into the vasculature. Seedlings were treated with 100 nM At2g16385 (CIF1) peptide for 24 h. (**D**) Casparian strip in the ligand and receptor mutants visualized by autofluorescence. (**E**) Quantitative analysis of the number of holes per 100 µm in the Casparian strips shown in (D). Data represent mean values ± SD (*P < 0.05, Student's *t* test, n = 4-5). (**F**) Dose-response of contiguous Casparian strip formation to peptide treatment for 24 h. Significant differences are indicated by different letters (P < 0.05, one-way ANOVA, n = 3-7). (**G**) CASP1-GFP localization in wild-type and mutant plants. (**H**) Time-lapse imaging of the fusing dynamics of the Casparian strip domain in the *cif1-1 cif2-1* mutant after treatment with CIF1 peptide. (**I**) Quantitative analysis of the number of holes in the Casparian strips after CIF1 treatment (n = 4-6). (**J**) CASP1-GFP localization pattern in *cif1-1 cif2-1* mutant after CIF1 deprivation. Scale bar: (B) 5 mm; (C) 50 µm; (D, G, H, J) 10 µm.

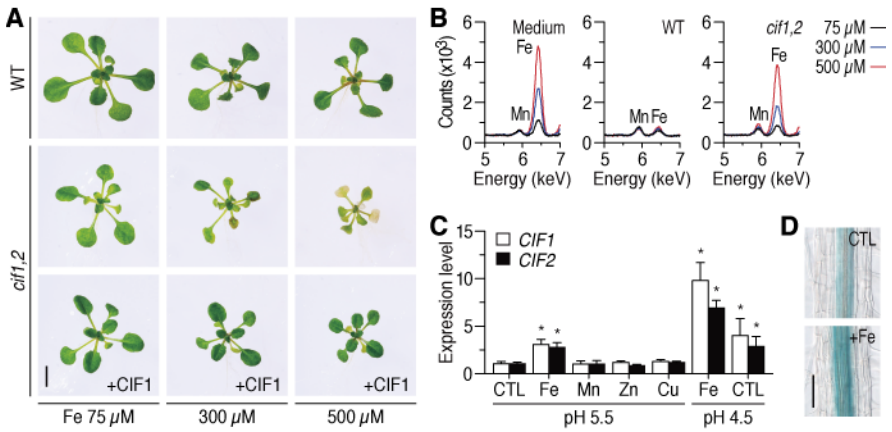

Fig. 4. The *cif1-1 cif2-1* double mutant is hypersensitive to excess iron. (**A**) Growth of wild-type and mutant plants under excess iron conditions for 7 days in the presence or absence of CIF1 peptide. (**B**) Comparison of iron levels between the culture medium, wild-type xylem sap. and mutant xylem sap using synchrotron radiation X-ray fluorescence spectrometry. (**C**) Expression levels of *CIF1* and *CIF2* in wild-type plants treated with excess mineral ions for 24 h at pH 5.5 or 4.5 (n = 3). (**D**) Promoter activity of *CIF2* in roots treated with 75 μM iron at pH 5.5 as a control or 500 μM iron at pH 4.5 for 24 h. Scale bar: (A) 5 mm; (D) 100 μm.

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Supplementary materials

Materials and Methods

Figs. S1 to S5

Table S1

References (*17-24*)