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A Peptide Hormone and Its Receptor Protein Kinase Regulate Plant Cell Expansion

Miyoshi Haruta[1,2], **Grzegorz Sabat**[1], **Kelly Stecker**[1,2], **Benjamin B. Minkoff**[1,2], and **Michael R. Sussman**[1,2,*]

[1]Biotechnology Center, University of Wisconsin, Madison, WI 53706, USA

[2]Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA

Abstract

Plant cells are immobile; thus, plant growth and development depend on cell expansion rather than cell migration. The molecular mechanism by which the plasma membrane initiates changes in the cell expansion rate remains elusive. We found that a secreted peptide, RALF (rapid alkalinization factor), suppresses cell elongation of the primary root by activating the cell surface receptor FERONIA in *Arabidopsis thaliana*. A direct peptide-receptor interaction is supported by specific binding of RALF to FERONIA and reduced binding and insensitivity to RALF-induced growth inhibition in *feronia* mutants. Phosphoproteome measurements demonstrate that the RALF-FERONIA interaction causes phosphorylation of plasma membrane H^+–adenosine triphosphatase 2 at Ser^{899}, mediating the inhibition of proton transport. The results reveal a molecular mechanism for RALF-induced extracellular alkalinization and a signaling pathway that regulates cell expansion.

Cell expansion is a fundamental cellular process driving plant growth, and regulation of its rate and direction is a highly dynamic process that changes during development, as well as during adaptation to variations in the external environment. To maintain appropriate cell expansion rates, plant cells fine-tune signal transduction pathways involving protein phosphorylation cascades catalyzed by protein kinases and protein phosphatases. For example, light and the growth-stimulating hormone auxin increase cell expansion rates and decrease apoplastic pH via phosphorylation of the penultimate Thr^{947} residue (1, 2) in a C-terminal regulatory domain of plasma membrane H^+–adenosine triphosphatase (H^+-ATPase).

RALF (rapid alkalinization factor), a 5-kD secreted peptide first discovered with assays searching for endogenous plant peptides with the ability to increase the pH of the media of cultured cells (3, 4), also induces a rapid increase in cytoplasmic calcium (5). Among more

*Corresponding author. msussman@wisc.edu.

Supplementary Materials

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than 30 RALF-like genes in the *Arabidopsis* genome (fig. S1), RALF is most highly expressed in roots and was suggested to be involved in root development (6, 7) (figs. S2 and S3).We report here on signaling events at the plasma membrane that involve a peptide-receptor interaction leading to the suppression of root cell elongation. RALF mobilizes calcium in a manner resembling the signal transduction pathways of animal peptide growth factors, wherein the factor binds to a cell surface receptor that then autophosphorylates (8). We demonstrate that FERONIA receptor-like kinase binds to RALF and initiates a downstream phosphorylation signaling cascade that inhibits plasma membrane H^+-ATPase activity, increases apoplastic pH, and reduces cell elongation. We identify RALF and FERONIA as a ligand-receptor pair and provide a molecular mechanism for a FERONIA-mediated growth-inhibitory signaling pathway that may also intersect with the innate immune response.

To obtain material for biochemical and genetic studies, we expressed RALF as an N-terminal 6×His fusion peptide in *Escherichia coli* that was purified to homogeneity using an affinity column and reversed-phase high-performance liquid chromatography (fig. S4, A to D). His-tagged RALF was biologically active in cytoplasmic calcium mobilization assays (fig. S4C). As a negative control, we also produced and purified an inactive RALF analog, RALF(Δ2–8), which lacks seven amino acids at its N terminus and is a biologically inactive analog (figs. S4E and S5) (9). To examine RALF-induced rapid changes in phosphorylation of plasma membrane proteins, we performed mass spectrometry–based quantitative phosphoproteomic profiling using an ^{15}N metabolic labeling technique (10, 11) (fig. S6A). Seedlings were treated with 1 μM RALF peptide or water as a control for 5 min, homogenized, and plasma membranes purified. For a biological duplicate, the samples were reciprocally labeled. Mass spectrometric analyses of phosphopeptides allowed the quantification of ~550 $^{14}N/^{15}N$ phosphopeptide pairs, with median linear ratio changes of 1.14 and 1.17 for samples A(^{14}N RALF/^{15}N control) and B (^{14}N control/^{15}N RALF), respectively (fig. S6, B and C; fig. S7, A and B). Among the phosphopeptides quantified, five proteins displayed a change in abundance, observed in a reciprocal manner, by a factor of at least 2.5 (table S1). Four of these proteins increased in abundance—FERONIA receptor kinase (pS871 and pS874, factor of 8.3 to 13.4, Fig. 1A); plasma membrane H^+-ATPase 2 (AHA2; factor of 2.6 to 4.3, fig. S8); calcium-dependent protein kinase 9 (CPK9; factor of 4.0 to 12.1, fig. S9); and PEN3/ABCG36 transporter (factor of 4.17 to 4.52)—and one decreased: a second FERONIA-related receptor-like kinase we term ERULUS (ERU; factor of 0.22, fig. S10A).

We also observed the increased abundance of a FERONIA phosphopeptide containing a third serine phosphorylation site, pS858. Analysis was hampered by an unrelated peptide coeluting during the liquid chromatography–mass spectrometry analysis, but we manually reconstructed the chromatogram for the FERONIA phosphopeptide, SSDVYEGNVTDsR (Fig. 1B). The pS858 phosphopeptide increased by a factor of ~20 in the RALF-treated tissues. Using selective reaction monitoring with a heavy isotope–labeled synthetic phosphopeptide containing pS858 (fig. S7, C and D), we determined that pS858 is increased by a factor of 6.4 to 11.5 in RALF-treated seedlings. By analogy with the phospho-regulated mammalian epidermal growth factor receptor (12), we hypothesized that FERONIA was the

receptor for RALF and that phosphorylation at the C terminus activated the kinase and initiated a RALF-induced signaling cascade (Fig. 1C).

The FERONIA receptor-like kinase was first described with *Arabidopsis* mutants defective in pollen tube elongation arrest (13). During normal fertilization, the elongating pollen tube stops and releases its sperm nuclei at the egg, whereas in the *fer* mutant, the pollen tube overgrows and fails to release sperm nuclei, resulting in reduced fertility. FERONIA is among the most widely expressed members of the malectin receptor kinase family (14) (fig. S10B). In addition to changes in FERONIA, we detected another member of the malectin receptor family that responded to RALF, in this case with a decrease in abundance of the phosphopeptide containing pS497 located at the juxtamembrane domain (fig. S10C). Because FERONIA was named after the Etruscan fertility goddess, we have named this protein ERULUS, after a son of FERONIA in Etruscan mythology. To test the in planta function of these two receptor kinases (FERONIA and ERULUS) that show RALF-dependent changes in phosphorylation, we compared the growth of transferred DNA (T-DNA) knockdown (*fer5*) and knockout (*fer4*) mutants with that of the wild type in the presence and absence of RALF. At 1 μM RALF, wild-type growth was inhibited, whereas the *fer4* null mutant was insensitive to RALF and the *fer5* knockdown mutant was moderately inhibited (Fig.2Aand fig. S11A). In contrast, T-DNA mutants of ERULUS showed shorter root hair phenotypes (fig. S10, D to G), but there were no differences in sensitivity to RALF relative to the wild type (Fig. 2B), despite changes in its phosphorylation in response to RALF. Inhibition of wild-type root elongation after RALF treatment was due to reduced cell elongation (fig. S11, B to D).

To characterize RALF-induced early cellular responses occurring within a few seconds after RALF treatment, we examined changes in cytoplasmic calcium of the *fer4* mutant using an aequorin-expressing *Arabidopsis* seedling as cytoplasmic calcium reporter. The RALF-induced calcium increase observed in the wild type was absent in the *fer4* mutant (Fig. 2C), although a control experiment with ATP treatment showed that the *fer4* mutant fully responds to this calcium-mobilizing agonist and is thus not generally defective in the calcium response machinery (fig. S12). These results indicate that the *fer4* mutant is specifically deficient in the RALF-dependent calcium signaling system.

RALF and FERONIA are both highly expressed in the mature zone of the root during the seedling stage (fig. S13), and their mRNA expression patterns and RALF-induced root growth arrest suggest that the two proteins act by restricting cell elongation in the post-elongation zone. Consistent with this idea, transcriptome analyses after RALF treatment for 30 min revealed that the families of genes encoding proteins known to be involved in cell expansion—SAUR63 [small auxin up RNA (15)], expansin (16), and the rate-liming enzymes for biosynthesis of plant growth regulators, brassinosteroid-6-oxidase 2 (BR6OX2) and gibberellin-3-oxidase 1—were all down-regulated (fig. S14) (17, 18). Genes associated with calcium and ethylene signaling, including calmodulin and ethylene response factors (19), were up-regulated by RALF treatment. The RALF-induced change of BR6OX2 expression was absent in the *fer4* mutant (Fig. 2D).

A peptide derived from pathogenic bacterial flagellin, flg22, is sensed by the FLS2 receptor and also induces a phosphorylation change of AHA2 at Ser^{899} that coincides with apoplastic alkalinization (20, 21). An AHA2 S899D ($Ser^{899}\rightarrow$Asp) mutant expressed in yeast demonstrated that this phosphomimetic mutation reduced growth relative to wild-type AHA2; thus, an increase in AHA2 phosphorylation at Ser^{899} after RALF treatment is predicted to down-regulate H^+-ATPase function, providing a molecular explanation for the observed RALF-induced apoplastic alkalinization (22). As a check on specificity of the RALF-FERONIA interaction, we examined the flg22 sensitivity of the *fer* mutant as well as the RALF sensitivity of the *fls2* mutant. The *fer4* mutant was insensitive to RALF but not to flg22 peptide, and the *fls2* mutant was insensitive to flg22 but not to RALF (fig. S15, A and B).We selected 17 other lines containing knockout mutations for receptor-like kinases and cell surface receptor–like proteins that are implicated in RALF signaling because they are co-regulated with RALF or are induced by RALF after 30 min of treatment (table S2). None of these other lines showed differences in RALF sensitivity of root elongation relative to the wild type (Fig. 2B), and thus we conclude that the *fer4* response to RALF is specific.

The above observations suggest that FERONIA mediates RALF's inhibitory effect on root elongation by inhibiting the activity of AHA2, which secretes protons into the apoplast (23). Our model predicts that the H^+-ATPase activity is constitutively up-regulated in the *fer4* mutant; consistent with this prediction, experimental measurements showed that *fer4* mutants acidify the bathing media faster than the wild type (Fig. 3A). Moreover, in a root elongation assay in the presence of the inhibitory cation lithium, *fer4* mutant roots were hypersensitive and growth was poorer than in wild-type roots (Fig. 3B). This response is a typical phenotype of mutant plants containing increased H^+-ATPase activity and a hyperpolarized plasma membrane, which causes increased uptake of the inhibitory cation into the cytoplasm (24). The effect of increased H^+-ATPase activity on root growth was examined by growing seedlings under blue light, which promotes root elongation (25). The roots of both *fer4* and *ralf* loss-of-function mutants were longer than wild-type roots (Fig. 3C and fig. S16, A to D).

To test whether RALF binds to FERONIA, we performed coimmunoprecipitation of HisRALF or its inactive analog HisRALF(Δ2–8) with hemagglutinin (HA)–tagged FERONIA (FER-HA) expressed in *Nicotiana benthamiana*. FER-HA protein, with an apparent molecular mass of ~140 kD, bound HisRALF (~8 kD; Fig. 4, A and B). His-RALF binding was greater than with the inactive analog by a factor of 4.5 to 6. The lack of effect of HisRALF(Δ2–8) when added together with HisRALF in root inhibition assays indicates that HisRALF(Δ2–8) lacks the ability to interact with the RALF receptor, and this was borne out by binding measurements (Fig. 4, A and C, and fig. S17A). In a separate experiment with 25 nM RALF labeled with ^{125}I, we measured the binding of RALF peptide to plasma membranes isolated from wild-type or *fer4* plants in the presence or absence of an excess amount (25 μM) of nonradioactive RALF (Fig. 4D). Approximately half of the iodinated RALF bound to the wild-type plasma membrane was reduced by an excess amount of nonradioactive RALF, indicating that this portion of the binding is not only specific but also saturable. In the *fer4* mutant, the saturable binding was reduced by ~40% but not completely eliminated. The possibility that there are additional sites for RALF binding other than

FERONIA in the plasma membrane of wild-type plants is supported by our observation that the *fer4* mutant is not completely insensitive to RALF at a higher concentration (5 μM) in both the root growth inhibition test and cytoplasmic calcium assay (fig. S15, D and E). Direct and specific binding of RALF to FERONIA was further confirmed with in vitro binding assays using the ectodomain of FERONIA (ectoFER) expressed as fusion proteins with either glutathione *S*-transferase (GST) or maltose binding protein (MBP) produced in *E. coli* (fig. S18, B to F). RALF specifically bound to GST-ectoFER as well as HisMBP-ectoFER; the inactive RALF analog showed little or no binding in pull-down assays with the ectoFER proteins (Fig. 4, E and F).

Our results show that FERONIA is a receptor for RALF and that this receptor kinase mediates RALF's inhibitory effects on the cell elongation rate in *Arabidopsis* primary roots. Our demonstration that RALF is a naturally occurring ligand for FERONIA and that this kinase plays an important role in negatively regulating cell expansion provides an impetus for future work in evaluating the role that each RALF-like peptide plays in plant growth and development. The results also support the hypothesis that RALF, or another member of the 34 RALF-like peptides, is the ligand functioning in pollen tube arrest prior to fertilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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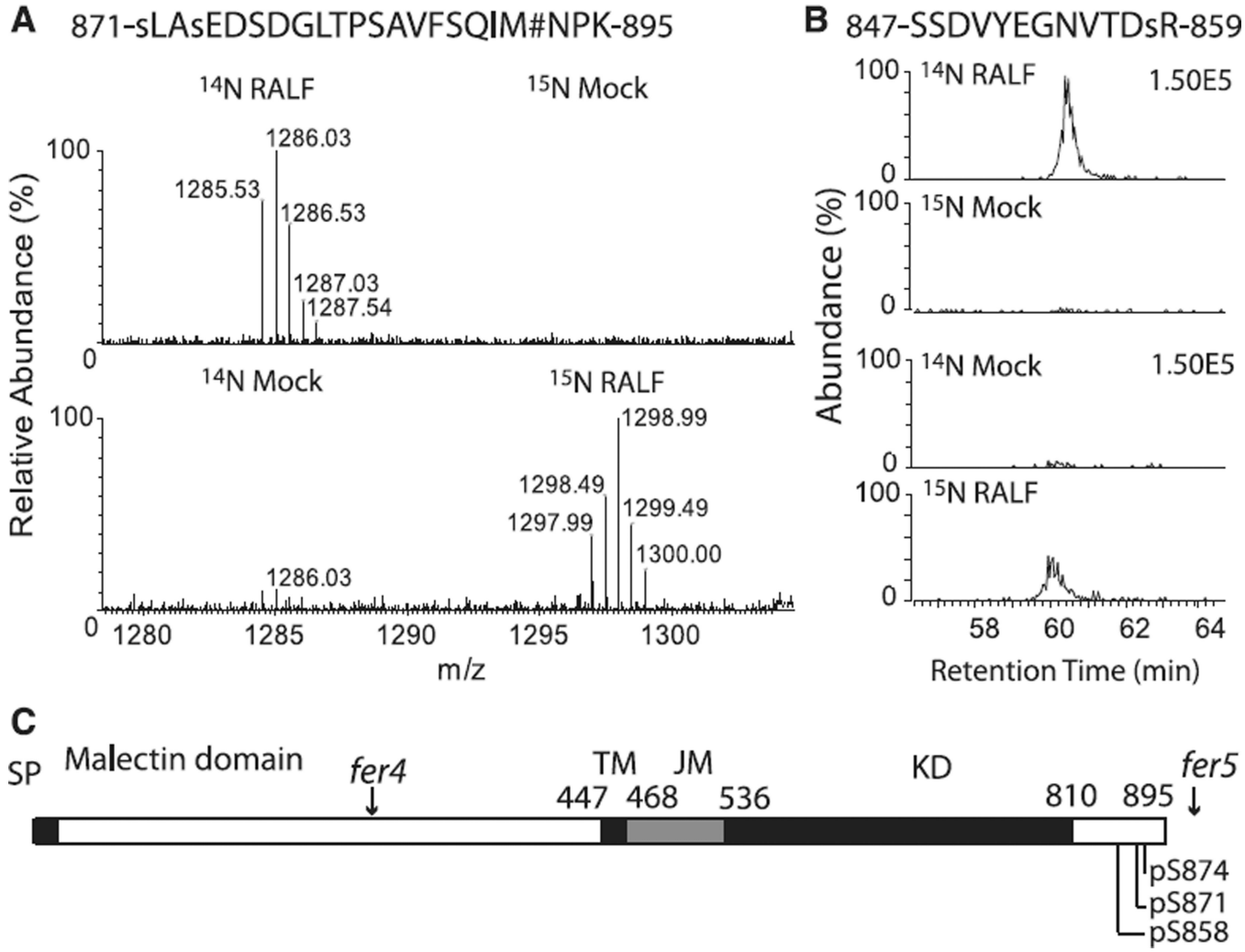

Fig. 1. RALF induces increased phosphorylation of FERONIA receptor kinase

(**A**) Mass spectrometric spectra showing an increased abundance of a FERONIA phosphopeptide containing pS871 and pS874 after RALF treatment. (**B**) Extracted ion chromatogram of a FERONIA phosphopeptide showing RALF-induced increase of phosphorylation at Ser^{858}. (**C**) Structure of FERONIA receptor kinase. SP, signal peptide; TM, transmembrane domain; JM, juxtamembrane domain; KD, kinase domain. Arrows indicate the positions of T-DNA insertions in *fer* mutants. Abbreviations for amino acids: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M#, oxidized Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr; s, phosphorylated serine.

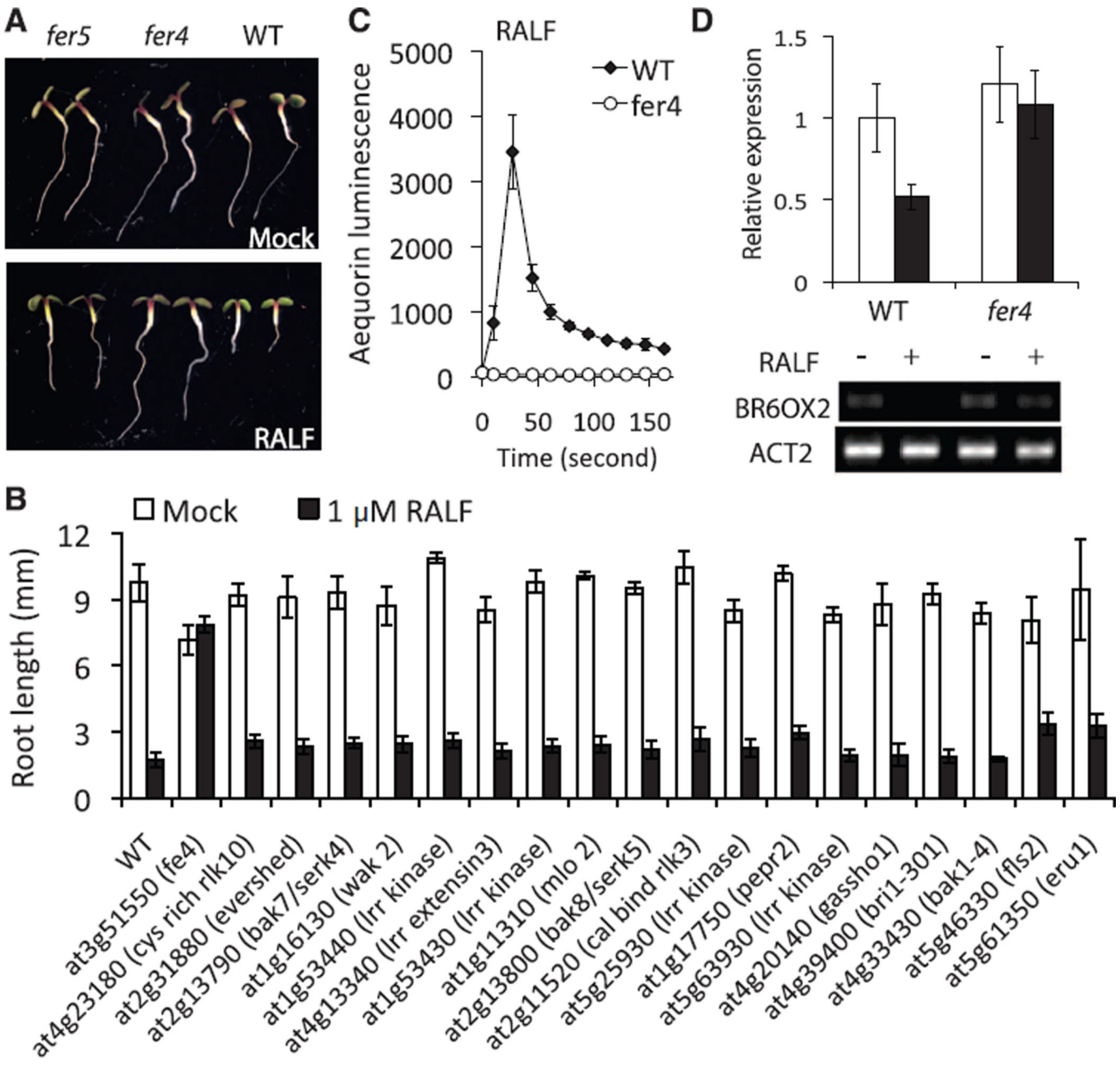

Fig. 2. Loss-of-function *fer* mutants are specifically insensitive to RALF

(**A**) The *fer4* and *fer5* plants show reduced sensitivity to RALF-induced root growth inhibition. Seedlings were treated with 1 μM RALF. (**B**) Insensitivity of *fer* mutants to RALF is specific. Mutants of 18 other receptor-like proteins respond to RALF normally. (**C**) Normal FERONIA function is required for RALF-induced cytoplasmic calcium increase. Aequorin-expressing seedlings were treated with 500 nM RALF, *n*=6. (**D**) RALF treatment causes a decrease of BR6OX expression in the wild type but not in the *fer4* mutant. ACT2 denotes actin. Error bars in (B), (C), and (D) denote SEM.

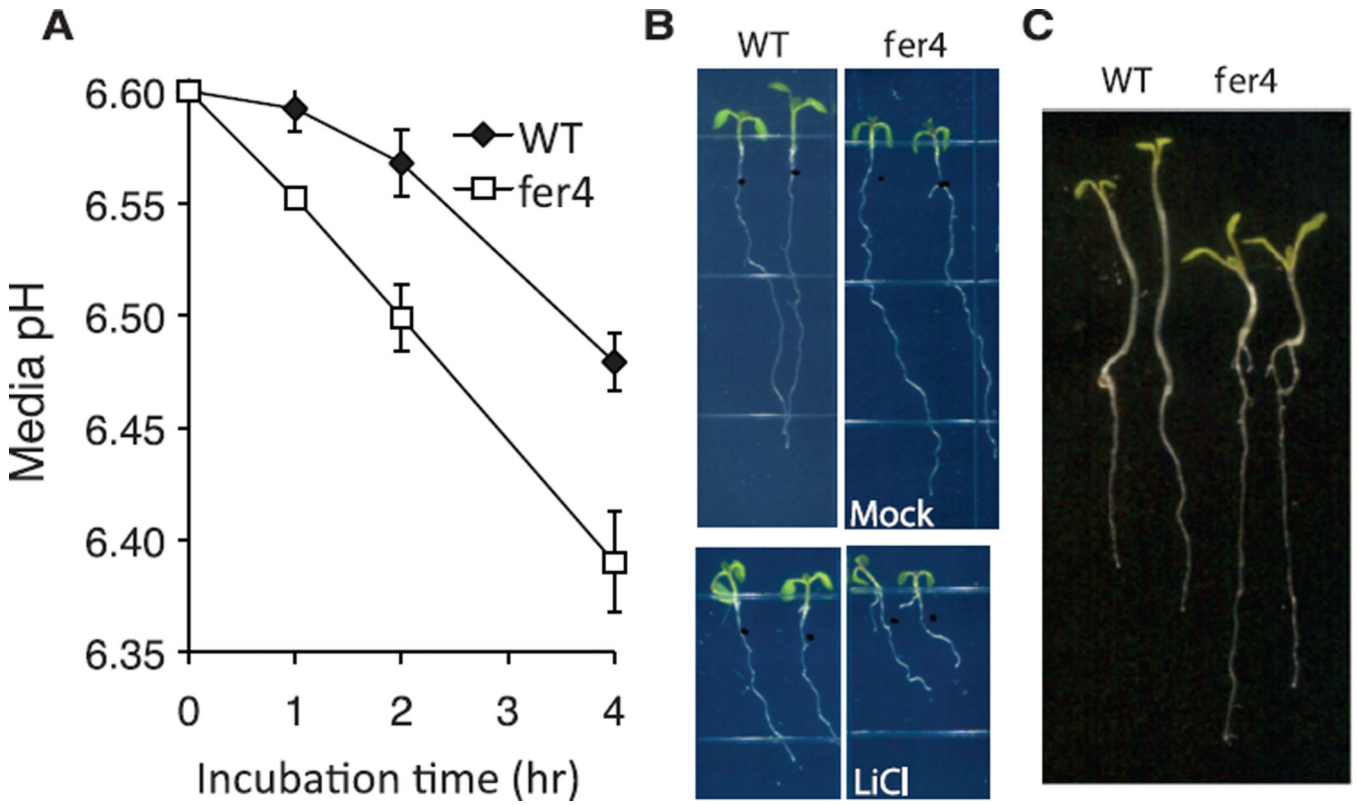

Fig. 3. Loss-of-function *fer* mutant exhibits the phenotype typical of plants with a higher plasma membrane H^+-ATPase activity

(**A**) Seedlings of *fer4* mutant acidify bathing media faster than the wild type (n = 7 each). Error bars denote SEM. (**B**) The *fer4* root growth is hypersensitive to lithium ion stress, consistent with a hyperactive pump and a deeper membrane potential. (**C**) The *fer4* mutant shows longer root length. Seedlings were grown under blue light for 7 days.

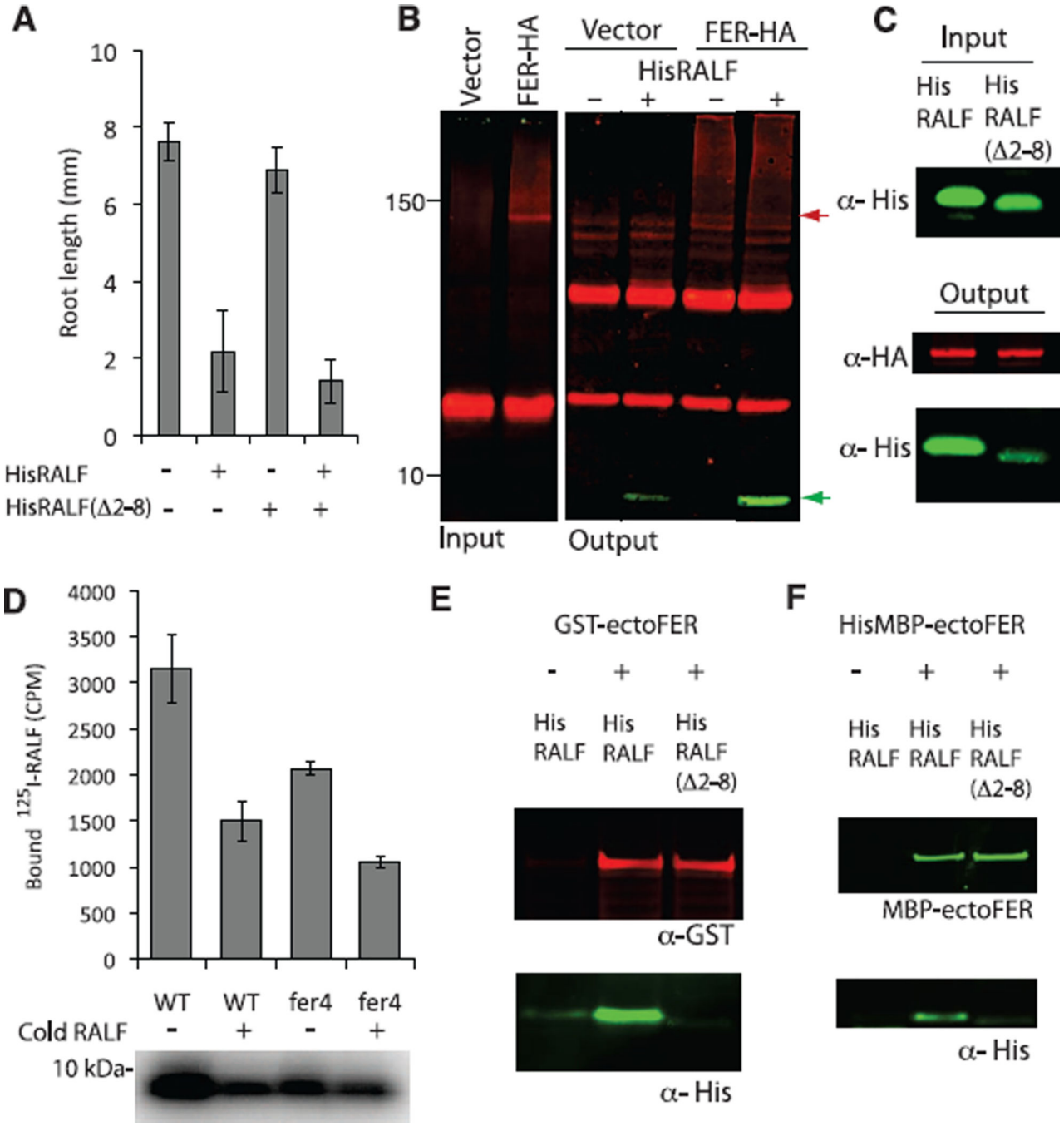

Fig. 4. RALF binds to FERONIA

(**A**) HisRALF(Δ2–8) is an inactive analog of RALF and does not compete with RALF in root growth inhibition assay. Data are means ± SD (n = 6). (**B**) RALF binds to the FERONIA protein expressed in tobacco. Red arrow indicates FER-HA; green arrow indicates HisRALF. (**C**) Binding of RALF to FERONIA is sequence-specific, and binding of the inactive analog HisRALF(Δ2–8) to FERONIA is similar to background binding. Binding was immunodetected with antibodies to His tag (α-His) or HA (α-HA). (**D**) The *fer4* mutant plasma membrane shows reduced saturable binding of ^{125}I-RALF relative to the

wild type. Data are means ± SE. Bound and released ^{125}I-RALF detected in protein gel shows reduced binding in the *fer4* mutant. (**E**) HisRALF, but not HisRALF (Δ2–8), binds to GST-tagged ectodomain of FERONIA receptor (GST-ectoFER). Data are representative of three experiments. (**F**) HisRALF, but not HisRALF(Δ2–8), binds to HisMBP-tagged ectodomain of FERONIA (HisMBP-ectoFER).