REPORT

PLANT SIGNALING

The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling

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In plants, perception of invading pathogens involves cell-surface immune receptor kinases. Here, we report that the *Arabidopsis* SITE-1 PROTEASE (S1P) cleaves endogenous RAPID ALKALINIZATION FACTOR (RALF) propeptides to inhibit plant immunity. This inhibition is mediated by the malectin-like receptor kinase FERONIA (FER), which otherwise facilitates the ligand-induced complex formation of the immune receptor kinases EF-TU RECEPTOR (EFR) and FLAGELLIN-SENSING 2 (FLS2) with their co-receptor BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED KINASE 1 (BAK1) to initiate immune signaling. We show that FER acts as a RALF-regulated scaffold that modulates receptor kinase complex assembly. A similar scaffolding mechanism may underlie FER function in other signaling pathways.

Plant immune pattern recognition receptors (PRRs) are often receptor kinases (*1*). The *Arabidopsis thaliana* (hereafter, *Arabidopsis*) receptor kinases FLAGELLIN-SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR) bind bacterial flagellin (or the epitope flg22) and EF-Tu (or the epitopes elf18/elf24), respectively, and form ligand-induced complexes with their co-receptor BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED KINASE 1 (BAK1) (*1*).

To decipher the negative regulation of plant PRR-mediated immune signaling, we screened for *modifier of bak1-5* (*mob*) mutants that regain immune responses in the immunodeficient mutant background *bak1-5* (*2*). Here, we report the characterization of MOB6.

bak1-5 is impaired in the production of reactive oxygen species (ROS) upon flg22 or elf18 treatment (*3*). We identified the recessive *bak1-5 mob6* mutant on the basis of restoration of this response (fig. S1A). To map the *mob6* locus, we sequenced bulked F_2 segregants from a backcross between *bak1-5 mob6* and *bak1-5* combined with phenotypic and genetic analysis of an F_2 population from a cross between *bak1-5 mob6* and Col-0 [see the supplementary materials (SM)]. We found a homozygous missense mutation [where Ser replaces Pro^{612} (P612S)] in At5g19660 encoding the subtilase SITE-1 PROTEASE (S1P)/SBT6.1 (fig. S1, B and C). Allelism tests and transcomplementation assays confirmed that the *mob6* phenotype is caused by a mutation in *S1P* (fig. S1, D and E). We thus renamed *mob6* as *s1p-6*.

We characterized the effect of mutant *S1P* alleles on immune signaling. *s1p-3* and *s1p-6* single mutants produced more ROS in response to elf18, flg22, and chitin (fig. S1, D and E; fig. S2A; and fig. S3) and exhibited increased expression of the immune marker genes *FRK1* and *PHI1* upon elf18 treatment (fig. S2B). Also, *s1p-3* and *s1p-6* mutants were more resistant to the hypovirulent bacterium *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 coronatine-minus (*COR*$^-$) strain (fig. S2C). Thus, S1P is a negative regulator of immunity.

Similar to its human ortholog, the *Arabidopsis* subtilase S1P processes substrates with the canonical cleavage site RxxL/RxLx (where R is arginine, L is leucine, and x is any amino acid) (*4*). The endogenous peptide RAPID ALKALINIZATION FACTOR 23 (RALF23; At3g16570)—an established S1P substrate in *Arabidopsis* (*5*)—is a major hub in a flg22-regulated transcriptional network (*6*). Consistent with a potential role of RALF23 in immunity, elf18 treatment or inoculation with the type III–deficient strain *Pto* DC3000 *hrcC*$^-$ rapidly increased the processing of the propeptide PRORALF23 (fig. S4, A and B), in an S1P-dependent manner (fig. S4C).

RALF23 treatment led to a dose-dependent inhibition of elf18-induced ROS production [median inhibitory concentration (IC_{50}) ~ 200 nM] (Fig. 1A and fig. S5) and resistance to *Pto* DC3000 (Fig. 1B). Furthermore, *RALF23* overexpression (*7*) inhibited elf18-triggered ROS production and increased susceptibility to *Pto* DC3000 *COR*$^-$ (Fig. 1, C and D) and to the fungus *Plectosphaerella cucumerina* (*7*). Conversely, loss of *RALF23* (fig. S6A) led to increased elf18-triggered ROS production (Fig. 1E) and resistance to *Pto* DC3000 *COR*$^-$ (Fig. 1F). RALF23 similarly inhibited flg22-triggered ROS production (fig. S7). Furthermore, treatment with RALF23 suppressed the heightened elf18-induced ROS production in *s1p-6* mutants (fig. S8), which suggests that the negative regulatory function of S1P is executed by the processing of

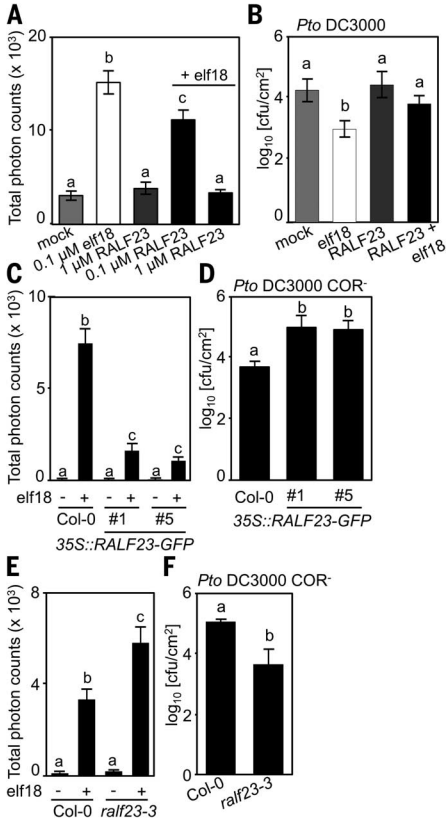

Fig. 1. The endogenous peptide RALF23 negatively regulates immunity. (**A**) ROS production in Col-0 leaf discs treated with 100 nM elf18, 1 µM RALF23, or cotreatment [all in 2 mM 2-(*N*-morpholino)ethanesulfonic acid–potassium hydroxide (MES-KOH), pH 5.8]. Values are means of total photon counts over 30 min ± SE, $n = 16$. Letters indicate significance in one-way analysis of variance (ANOVA) (a and b, $P < 0.001$; a to c, $P < 0.001$; b and c, $P < 0.05$). Kinetics are shown in fig. S18A. (**B**) Colony-forming units (cfu) of *Pto* DC3000 after syringe inoculation in leaves pretreated with mock treatment, 1 µM elf18, 1 µM RALF23, or cotreatment (all in 2 mM MES-KOH, pH 5.8) for 24 hours, determined 2 days after inoculation. Values are means ± SD, $n = 4$ (one-way ANOVA; $P < 0.05$). (**C** and **E**) ROS production after elicitation with 100 nM elf18 or water. Values are means of total photon counts over 30 min ± SE. Letters indicate significance in one-way ANOVA (a and b, $P < 0.001$; a to c, $P < 0.001$; b and c, $P < 0.05$). Kinetics are shown in fig. S18, B and C. (**D** and **F**) Number of *Pto* DC3000 *COR*$^-$ bacteria determined 3 days after surface inoculation. Values are means ± SD, $n = 4$ (one-way ANOVA; $P < 0.05$). Similar results were obtained in three independent experiments.

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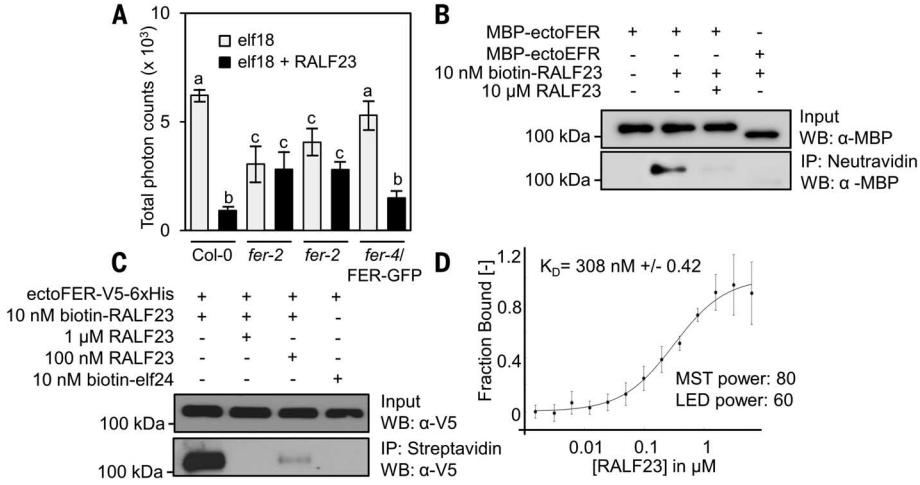
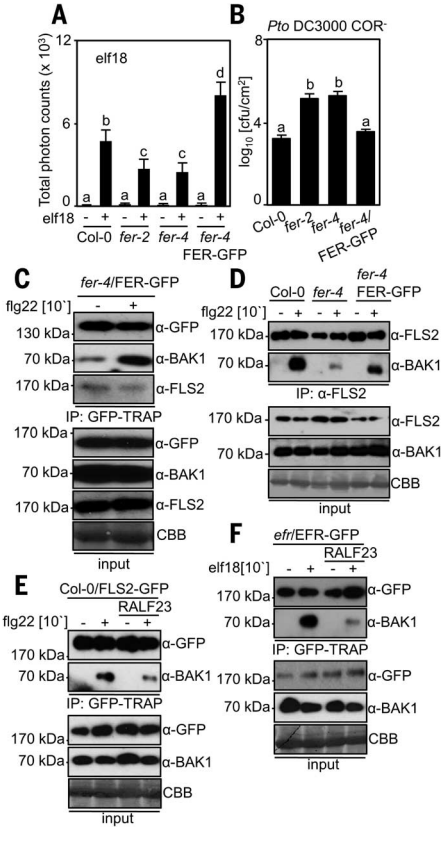


Fig. 2. RALF23-mediated inhibition of immunity is FER dependent. (**A**) ROS production after treatment with 100 nM elf18 alone or cotreated with 1 µM RALF23 (all in 2 mM MES-KOH, pH 5.8). Values are means of total photon counts over 40 min ± SE, n = 16. Letters indicate significance in one-way ANOVA (a and b, $P < 0.001$; a to c, $P < 0.05$; b and c, $P < 0.01$). Kinetics are shown in fig. S18D. (**B**) In vitro binding assay with maltose-binding protein (MBP)–ectoFER or MBP-ectoEFR purified from *E. coli*. Pull-down assay for immunoprecipitation (IP) was done with neutravidin beads; Western blots (WB) were probed with antibody against MBP (α-MBP). (**C**) In vitro binding assay with ectoFER–V5-6xHis (ectoFER tagged with V5 antibody epitope and hexahistidine) produced in insect cells. Pull-down was performed with streptavidin beads; Western blots were probed with antibody against V5 (α-V5). (**D**) Quantitative binding analysis using synthetic RALF23 peptide and ectoFER–V5-6xHis using microscale thermophoresis. Similar results were obtained in three independent experiments, except for (C), where the assays were performed twice with identical results.

Fig. 3. FER is a RALF-regulated scaffold for immune receptor complexes. (**A**) ROS production after elicitation with 100 nM elf18 or water. Values are means of total photon counts over 30 min ± SE, n = 8. Letters indicate significance in one-way ANOVA (a and b, $P < 0.05$; b and c, $P < 0.05$; b to d, $P < 0.05$; a to c, $P < 0.001$; a to d, $P < 0.001$). Kinetics are shown in fig. S18E. (**B**) Number of *Pto* DC3000 *COR*⁻ bacteria 3 days after surface inoculation. Values are means ± SD, n = 4 (one-way ANOVA; $P < 0.05$). (**C** to **F**) Coimmunoprecipitation of (C) FER-GFP from *fer-4*/FER-GFP; (D) FLS2 from Col-0, *fer-4*, or *fer-4*/FER-GFP; (E) FLS2-GFP from Col-0/FLS2-GFP; or (F) EFR-GFP from *efr*/EFR-GFP seedlings treated with or without the indicated peptides (100 nM flg22, 100 nM efl18, 1 µM RALF23, or water) for 10 min. Western blots were probed with antibodies α-GFP, α-BAK1, and α-FLS2. CBB, Coomassie brilliant blue. Similar results were obtained in three independent experiments.



RALF23 or related peptides. For example, the closely related RALF33 (At4g15800) peptide (figs. S9A and S10A) (*8*, *9*) could also inhibit elf18-induced ROS production (figs. S6B; S9, B and C; and S10B). We conclude that RALF23, as well as RALF33, negatively regulates immunity.

The *Arabidopsis* genome encodes about 35 RALF peptides (*8*, *9*). Only 11 RALFs (including RALF23 and RALF33) display a S1P cleavage site (fig. S10A). Cotreatment with RALF34 (containing a predicted S1P cleavage site) inhibited elf18-induced ROS burst to the same extent as RALF23 and RALF33, whereas RALF32 (lacking a predicted S1P cleavage site) did not (fig. S10B). This suggests that S1P-cleaved RALFs inhibit immunity. RALF23, RALF33, and the more divergent RALF32 triggered seedling growth inhibition (fig. S10C) in a way similar to RALF1 (*9*). However, RALF32 does not affect elf18-triggered ROS production (fig. S10B). All RALF peptides previously tested induced alkalinization (*8*, *10*), suggesting that immune inhibition is not a general property of RALFs and is independent of the alkalinization activity, which is consistent with our bioassays involving RALF peptides being performed under buffered conditions (SM).

The *Arabidopsis* malectin-like receptor kinase FERONIA (FER; At3g51550) was recently identified as a receptor for RALF1 (*9*). RALF1, RALF23, and RALF33 are closely related (fig. S9A and S10A) and have overlapping gene expression patterns with *FER* (fig. S11) (*8*, *9*). *fer-2* and *fer-4* mutants were insensitive to the inhibitory effect of RALF23 or RALF33 peptide on elf18-induced ROS production (Fig. 2A and fig. S12A), which was complemented in a *fer-4*/FER–GREEN FLUORESCENT PROTEIN (*fer-4*/FER-GFP) line (Fig. 2A). Furthermore, *RALF23* overexpression in *fer-2* had no effect on elf18-induced ROS production (fig. S12B). The genetic dependence of *FER* in RALF23-, RALF33-, and RALF32-triggered growth inhibition (fig. S10C) suggested that FER may bind additional RALF peptides.

Biotinylated RALF23 bound to recombinant FER ectodomain (ectoFER) but not the unrelated EFR receptor ectodomain (ectoEFR) (Fig. 2B). Similarly, ectoFER expressed and purified from insect cells bound to biotin-RALF23 but not biotin-elf24 (Fig. 2C), with dissociation constant (K_d) values ~300 nM and ~600 to 900 nM, as revealed by microscale thermophoresis and isothermal titration calorimetry, respectively (Fig. 2D and fig. S13), which is consistent with values reported for other ligand-receptor kinase pairs (*11*, *12*) and the IC_{50} for RALF23-mediated inhibition of elf18-triggered ROS production (fig. S5). Thus, in addition to RALF1 (*9*), FER is also a receptor for RALF23.

FER is enriched in detergent-resistant membranes after flg22 treatment (*13*). The *fer-2* and *fer-4* mutants were hyposensitive to elf18, flg22, and chitin (Figs. 2A and 3A and figs. S12 and S14) and were more susceptible to *Pto* DC3000 *COR*⁻ (Fig. 3B), indicating that FER can also positively regulate immunity. FER weakly associates with both FLS2 and BAK1, with the latter being strongly enhanced upon flg22 treatment (Fig. 3C). Flg22-induced FLS2-BAK1 complex formation was reduced in *fer-4* and restored in *fer-4*/FER-GFP (Fig. 3D). Cotreatment with RALF23 reduced ligand-induced FLS2/EFR-BAK1 complex formation (Fig. 3, E and F). *RALF23* overexpression had a similar effect on flg22-induced

FLS2-BAK1 complex formation (fig. S15). RALF23 or the loss of *FER* did not affect accumulation of FLS2, EFR, or BAK1 (Fig. 3, D to F, and fig. S15). Thus, ligand-induced complex formation between FLS2/EFR and their co-receptor BAK1 is promoted by FER and inhibited by RALF23.

Our data suggest that FER acts as a scaffold to regulate immune receptor–complex formation. FER may reside in plasma membrane microdomains as part of preformed signaling "platforms," together with receptors and co-receptors. Whether FER-mediated regulation intersects with other regulators of FLS2/EFR-BAK1 complex formation, such as BIR2 and IOS1 (*14*, *15*), will be an interesting topic for future investigation. Two-thirds of RALF proteins lack a predicted S1P cleavage site and are devoid of a propeptide region (fig. S10A). Treatment with one of these, RALF17, induced ROS production and acted additively to elf18 (fig. S16, A and B). RALF17 pretreatment was also sufficient to induce resistance to *Pto* DC3000 (fig. S16C). As RALF17-induced ROS production is dependent on *FER* (fig. S16B), the activity is not caused by a possible contamination with synthetic peptides (e.g., flg22 or elf18) used in our laboratory.

PRORALF23 is processed by S1P within minutes of elicitor perception (fig. S4), which suggests a negative-feedback mechanism to inhibit the scaffolding function of FER and to dampen immune signaling. Fungal pathogens secrete peptides with homology to RALF23 (*16*, *17*); these fungal RALF orthologs may suppress immunity by inhibiting the formation of active receptor complexes.

FER has emerged as a regulator of many biological processes, ranging from fertilization to inhibition of cell elongation and growth (*18*). Many of these processes involve receptor kinases and co-receptors (*19*). This suggests that FER may have a similar scaffolding function within other receptor kinase complexes. Different, but overlapping, expression patterns of *FER* and *RALF* genes (fig. S11) (*9*, *20*) suggest that a variety of FER-RALF modules may regulate diverse receptor kinase complexes during growth, development, or environmental sensing.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/355/6322/287/suppl/DC1
Materials and Methods
Figs. S1 to S18
Tables S1 and S2
References (*21*–*30*)

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Small peptides allow rapid responses

RALFs (rapid alkalinization factors), a family of small peptides in plants, are produced in response to rapidly changing conditions. Stegmann *et al.* studied the agility and diversity built into this signaling network. Some RALFs, such as RALF23 and its relative RALF33, are activated by proteolytic cleavage. Others, such as RALF32, are not. RALF23 and RALF33 are called into play after a pathogen triggers immune responses. RALF32, on the other hand, regulates seedling growth. All three of these RALFs use the same receptor kinase, which can interact with other signaling components. Thus, plant responses can be fine-tuned by rapid release of peptides.

Science, this issue p. 287