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

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Abstract: 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 modulating receptor kinase complex assembly. A similar scaffolding mechanism may underlie RALF function in other signalling pathways.

One Sentence Summary: A broadly accessible receptor perceives antagonistic endogenous peptides to regulate the formation of plant immune receptor complexes.

Main Text: Plant immune pattern recognition receptors (PRRs) are often receptor kinases (*1*). The *Arabidopsis thaliana* (hereafter *Arabidopsis*) receptor kinases FLS2 and 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 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 based on restoration of this response (fig. S1A). To map the *mob6* locus, we sequenced bulked F2 segregants from a backcross between *bak1-5 mob6* and *bak1-5* combined with phenotypic and genetic analysis of an F2 population from a cross between *bak1-5 mob6* and Col-0 (Supplement). We found a homozygous missense mutation (P612S) in At5g19660 encoding the subtilase S1P/SBT6.1 (fig. S1B,C). Allelism tests and trans-complementation assays confirmed that the *mob6* phenotype is caused by a mutation in *S1P* (fig. S1D,E). We thus renamed *mob6* as *s1p-6*.

We characterised 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. S1D,E, fig. S2A, 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 *COR*$^-$ (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 (*4*). The endogenous peptide RAPID ALKALINISATION 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. S4A,B), in a S1P-dependent manner (fig. S4C).

RALF23 treatment led to a dose-dependent inhibition of elf18 ROS (IC_{50} ~ 200 nM; fig. 1A, fig. S5) and induced-resistance to *Pto* DC3000 (fig. 1B). Furthermore, *RALF23* over-expression (*7*) inhibited elf18-triggered ROS and increased susceptibility to *Pto* DC3000 *COR*$^-$ (fig. 1C,D) and to the fungus *Plectosphaerella cucumerina* (*7*). Conversely, loss of *RALF23* (fig. S6A) led to increased elf18-triggered ROS (fig. 1E) and resistance to *Pto* DC3000 *COR*$^-$ (fig. 1F). RALF23 similarly inhibits flg22-triggered ROS (fig. S7). Furthermore, treatment with RALF23 suppressed the heightened elf18-induced ROS in *s1p-6* mutants (fig. S8), suggesting that the negative regulatory function of S1P is executed by the processing of RALF23 or related peptides. For example, the closely related RALF33 (At4g15800) peptide (fig. S9A, S10A) (*8*, *9*) could also inhibit elf18-induced ROS (fig. S6B, S9B,C; S10B). We conclude that RALF23 (and RALF33) negatively regulate 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). Co-treatment with RALF34 (containing a predicted S1P cleavage site) inhibited elf18-induced ROS burst to the same extent as RALF23 and RALF33, while 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) similar to RALF1 (*9*). However, RALF32 does not affect elf18-triggered ROS (fig. S10B). All RALF peptides previously tested induced alkalinisation (*8*, *10*), suggesting that immune inhibition is not

a general property of RALFs and is independent of the alkalinisation activity; consistent with our bioassays involving RALF peptides being performed under buffered conditions (Supplement).

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 (fig. 2A; fig. S12A), which was complemented in a *fer-4*/FER-GFP line (fig. 2A). Furthermore, *RALF23* overexpression in *fer-2* had no effect on ROS (fig. S12B). 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 in insect cells bound to biotin-RALF23 but not biotin-elf24 (fig. 2C), with Kd values ~300 nM and ~600-900 nM as revealed by microscale thermophoresis and isothermal titration calorimetry, respectively (fig. 2D, fig. S13), consistent with values reported for other ligand-receptor kinase pairs (*11*, *12*) and the IC_{50} for RALF23-mediated inhibition of elf18-triggered ROS (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 (fig. 2A, fig. 3A, fig. S12, fig. 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). Co-treatment with RALF23 reduced ligand-induced FLS2/EFR-BAK1 complex formation (fig. 3E,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. 3D-F, 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 pre-formed 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 pro-peptide region (fig. S10A). Treatment with one of these, RALF17, induced ROS production and acted additively to elf18 (fig. S16A,B). RALF17 pre-treatment 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 (*eg.* flg22, elf18) used in our laboratory.

PRORALF23 is processed by S1P within minutes of elicitor perception (fig. S4), suggesting a negative feedback mechanism to inhibit the scaffolding function of FER and 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*), suggesting 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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Figure legends:

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 co-treatment (all in 2 mM MES-KOH pH 5.8). Values are means of total photon counts over 30 min +/- SE, n=16. Letters indicate significance in one-way ANOVA (a-b, $p<0.001$; a-c, $p<0.001$; b-c, $p<0.05$). Kinetics are in fig. S18A. (**B**) Colony forming units (cfu) of *Pto* DC3000 after syringe-inoculation in leaves pre-treated with mock, 1 μM elf18, 1 μM RALF23, or co-treatment (all in 2 mM MES-KOH pH 5.8) for 24 h, determined 2 days after inoculation. Values are means +/- SD, n=4 (one-way ANOVA; $p<0.05$). (**C** and **E**) ROS 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-b, $p<0.001$; a-c, $p<0.001$; b-c, $p<0.05$). Kinetics are in fig. S18B,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 3 independent experiments.

Fig. 2. RALF23-mediated inhibition of immunity is FER-dependent.
(A) ROS after treatment with 100 nM elf18 alone or co-treated 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-b, $p<0.001$; a-c, $p<0.05$; b-c, $p<0.01$). Kinetics are shown in fig. S18D. (**B**) *In vitro* binding assay with MBP-ectoFER or MBP-ectoEFR purified from *E. coli*. Pull-down was done with neutravidin beads; western blots were probed with α-MBP. (**C**) *In vitro* binding assay with ectoFER-V5-6xHis produced in insect cells. Pull-down was performed with Streptavidin beads; western blots were probed with α-V5. (**D**) Quantitative binding analysis using synthetic RALF23 peptide and ectoFER-V5-6xHis produced from baculovirus-infected insect cells using microscale thermophoresis. Similar results were obtained

in 3 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 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-b, $p<0.05$; b-c, $p<0.05$; b-d, $p<0.05$; a-c $p<0.001$; a-d, $p<0.001$). Kinetics are 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-F**) Co-immunoprecipitation of FER-GFP from *fer-4*/FER-GFP (C), FLS2 from Col-0, *fer-4* or *fer-4*/FER-GFP (D), FLS2-GFP from Col-0/FLS2-GFP (E), or EFR-GFP from *efr*/EFR-GFP (F) 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 α-GFP, α-BAK1 and α-FLS2. CBB, Coomassie brilliant blue. Similar results were obtained in 3 independent experiments.

Supplementary Materials:

Materials and Methods

Figures S1-S18

Tables S1-S2

References (*21-30*)