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Supplementary Information for

Leucine-rich Repeat Extensin Proteins Regulate Plant Salt Tolerance in Arabidopsis

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Dataset S1

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Supplemental Information

Materials and Methods

Recombinant Protein Expression

To express LRR3 and mature RALF22 in *Escherichia coli*, the constructs of pGEX-4T-1-LRR3 and pET-28a-mRALF22 were transformed to strain BL21, respectively. A single clone of each transformant was transferred to 3 ml liquid LB medium with corresponding antibiotics for incubation overnight. The bacteria suspensions were transferred to 300 ml LB liquid medium for incubation until the OD_{600} reaching to 0.4-0.6. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (1 μM of final concentration) for 5 h prior to protein extraction. GST-LRR3 was purified by using Glutathione Sepharose 4B (GE Healthcare), and His-RALF22 was purified by using Ni-NTA agarose (Qiagen).

In Vitro Pull-down

Purified GST-LRR3 or GST was mixed with His-RALF22 in 1 ml buffer (20 mM Tris-HCl, pH 7.5, 1% IGEPAL) and incubated at 4°C for 2 h. 30 μL pre-equilibrated glutathione beads (GE Healthcare) was added to mixture and incubated for another 1 h. The mixture was washed with buffer 4-5 times. The proteins were boiled for 5 min after adding 50 μL 1xSDS loading buffer and subjected to SDS-PAGE and immunoblotting using anti-GST and anti-His antibodies.

Split Luciferase Complementation Assay

A. tumefaciens GV3101 strains harboring different constructs were incubated in LB liquid medium containing 25 μg ml^{-1} gentamycin and 50 μg ml^{-1} kanamycin for 24 h. The bacteria were spun down at 4,000 rpm for 5 min and the pellets were resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 100 μM acetosyringone) with a final concentration of OD_{600} = 0.5. The strain suspensions were kept at room temperature for 2 h before infiltration. For co-infiltration, equal volume of two different strains carrying the indicated nLUC and cLUC constructs were mixed prior to infiltration. The bacteria were infiltrated into *N. benthamiana* by using a 1-ml disposable syringe. After 48 h the infiltrated leaves were sprayed by luciferin, and the fluorescence was detected by CCD camera (Princeton instruments).

Synthetic Peptides

The mature RALF22 and RALF23 peptides were synthesized by EZBiolab (United States) with a purity of >95%. The peptides were dissolved in deionized water. The corresponding sequences of RALF22 and RALF23 are listed in *SI Appendix*, Table S2.

RALF22 Cleavage Assay

For the RALF22 cleavage assay, 8-day-old seedlings were transferred to liquid MS medium and incubated overnight. NaCl (final concentration of 150 mM) was added to the medium and seedlings were harvested in liquid nitrogen after a time course incubation. The seedlings were ground into powder and proteins were extracted by using the following extraction buffer: 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% Glycerol, 1 mM DTT, 1 mM PMSF, 10 μM antipain, 10 μM aprotinin, 10 μM leupeptin, and

phosphatase inhibitor cocktail set II. The extracts were mixed well and centrifuged at 14,000 rpm and 4°C for 20 min, and suspensions were transferred to new tubes. The concentration of total proteins was measured by using Quick Start Bradford Dye Reagent (Bio-Rad) and the final concentration of each sample was adjusted to the same level. The proteins were boiled for 5 min after adding SDS loading buffer. For immunoblotting, proteins were separated by SDS-PAGE (10% acrylamide gel) and transferred to a supported nitrocellulose membrane (Bio-Rad) using electro-transfer at 20 V for 30 min. The membrane was blocked in TBST buffer containing 5% skim milk powder and further incubated with primary antibody (anti-GFP) and secondary antibody. Finally, the proteins were detected using chemiluminescent HRP substrate (Millipore).

Co-immunoprecipitation Assay

Proteins were expressed in *N. benthamiana* by infiltrating agrobacteria containing the corresponding constructs. The leaves of *N. benthamiana* were collected 48 h later and ground in liquid nitrogen. Total proteins were isolated by using protein extraction buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 μM antipain, 10 μM aprotinin, and 10 μM leupeptin). After centrifugation at 14,000 rpm for 10 min at 4 °C, 2 ml protein suspension was transferred to a new tube and 3 μL anti-HA antibody was added. The mixture was incubated at 4°C for 4 h and then 40 μL pre-equilibrated protein G agarose beads (50% slurry, Millipore) were added and incubated for additional 2 h to capture immunocomplex. The beads were pelleted and washed 3 times with protein extraction buffer. Finally, the agarose beads were resuspended in 50

μL 1xSDS loading buffer and boiled for 5 min. 20 μL supernatant of each sample was used for immunoblotting analysis.

Fluorescence Assay

To detect the internalization of FER, 6-day-old seedlings of *pFER::FER-GFP* transgenic plants were transferred to 4 ml liquid MS medium, and synthesized RALF22 (1 μM), RALF23 (1 μM), flg22 (1 μM), or NaCl (150 mM) were added to treat seedlings for the indicated time points as described in the figure legends. The fluorescent signal in the elongation zone of roots was detected by using confocal microscopy. Identical parameters, including the same laser strength and the same pinhole, were applied for all samples.

LC-MS/MS Analysis

Two grams of 10-day-old seedlings of *35S::LRX3-YFP-HA*, *35S::LRX4-YFP-HA*, and *35S::LRX5-YFP-HA* transgenic plants were harvested and ground in liquid nitrogen with a mortar and pestle. Total proteins were extracted by using 10 ml of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 % glycerol, 5 mM DTT, 1% (v/v) protease inhibitor cocktail (P9599, Sigma), 1% (v/v) IGEPAL CA-630, 1 mM $Na_2MoO_4 \cdot 2H_2O$, 1 mM NaF, 1.5 mM Na_3VO_4, 1 mM EDTA, 1% (v/v) protein phosphatase inhibitor (Sigma). Samples were centrifuged at 13,000xg for 15 min after incubation at 4°C for 30 min, and the supernatants were transferred to new 15 ml tubes. 5 μL anti-GFP antibodies (Roche) were added and the proteins were incubated at 4°C for 4 h before adding 60 μL of protein G agarose beads (Millipore). After incubation for another 4 h with rotation, the beads were

spun down at 1,000xg for 2 min and washed 4 times with lysis buffer and 1 time with PBS buffer. Immunoprecipitated proteins were digested by trypsin overnight. Digested peptides were desalted using SDB-XC StageTip and analyzed by Easy-nLC coupled with LTQ-Orbitrap Velos Pro mass spectrometer. Peptides were identified by using Proteome Discoverer version 2.2 software.

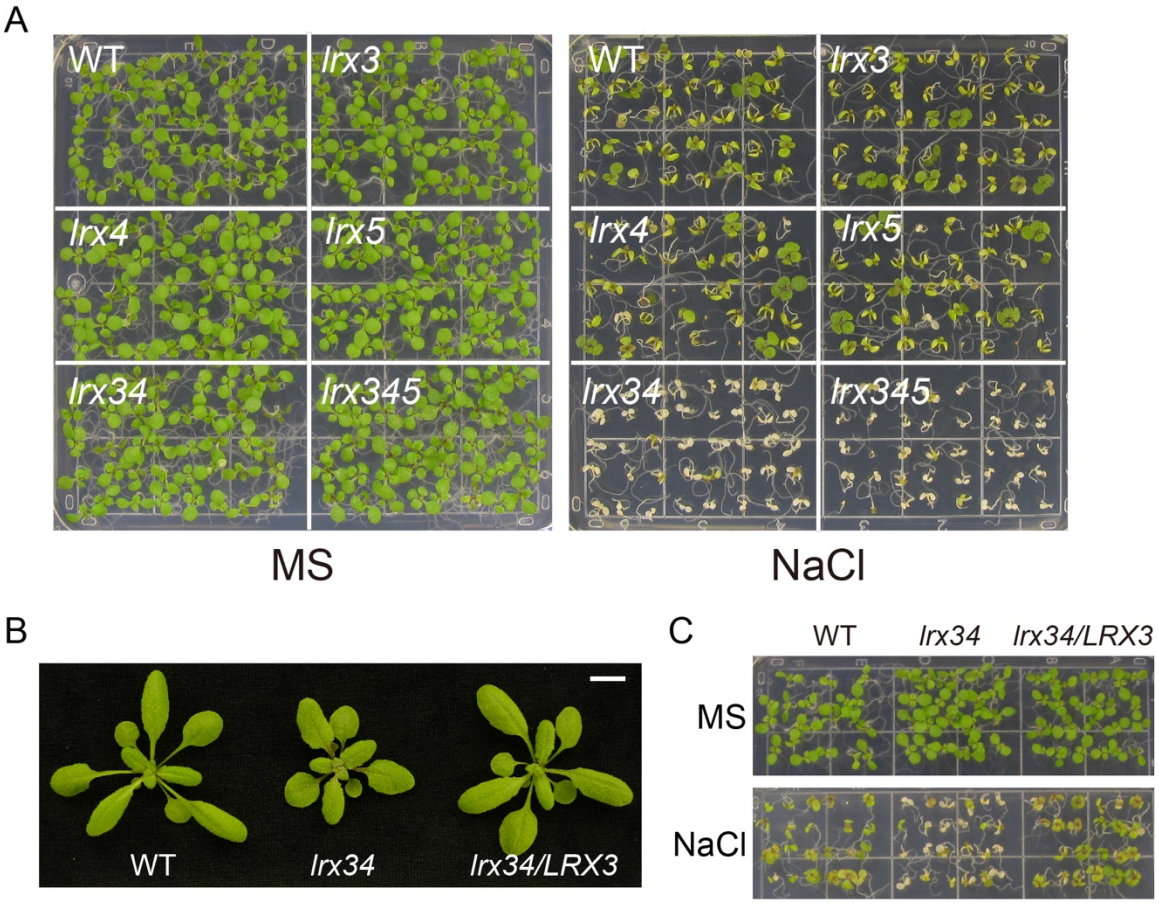

Fig. S1. Phenotypes of *lrx* single, double, and triple mutants.

(*A*) Phenotypes of wild type and *lrx3, lrx4, lrx5, lrx34*, and *lrx345* mutants germinated on MS and MS+NaCl (120 mM) media.

(*B*) *lrx34*/*LRX3* plants were generated by transformation of *35S::LRX3-YFP-HA* into the *lrx34* double mutant. Rosette morphology of wild type, *lrx34*, and *lrx34*/*LRX3* transgenic plants is shown. Scale bar, 1cm.

(*C*) Phenotypes of wild type, *lrx34*, and *lrx34*/*LRX3-YFP-HA* plants grown on MS and MS+NaCl (120 mM) media.

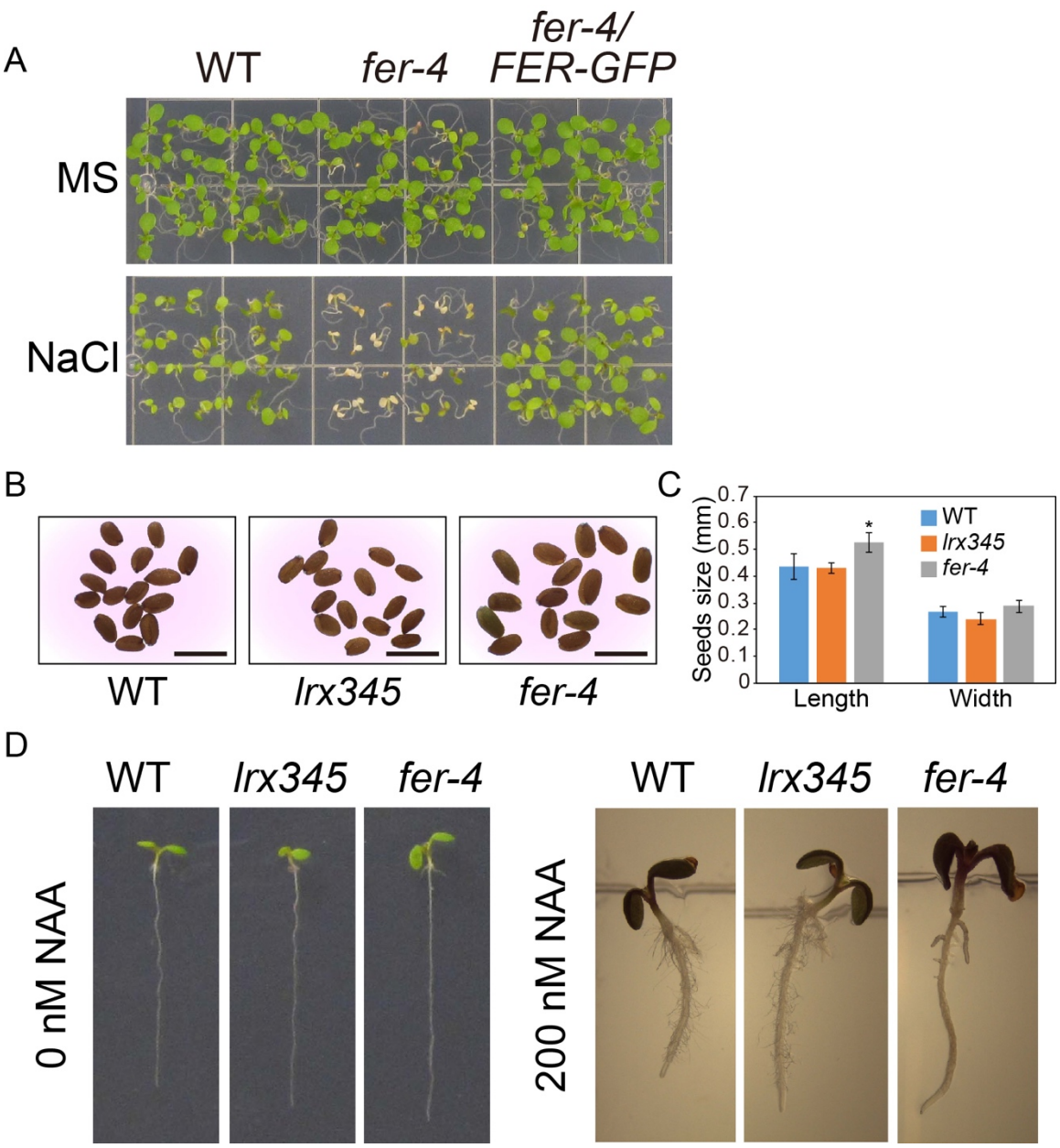

Fig. S2. Some of the phenotypes of the *fer-4* mutant are not found in *lrx345*.

(*A*) Phenotypes of wild type, *fer-4*, and *fer-4/FER-GFP* plants grown on MS and MS+NaCl (120 mM) media.

(*B*) Seeds of wild type and *lrx345* and *fer-4* mutants were photographed under microscopy. Scale bar, 1 mm.

(*C*) Quantification of the length and width of seeds. Values are means ± SD (n=15), $^{*}p < 0.05$ (Student's *t*-test).

(*D*) Root hairs of wild type, *lrx345* and *fer-4* seedlings grown on MS medium (left) or MS medium with 1-naphthaleneacetic acid (NAA) (right).

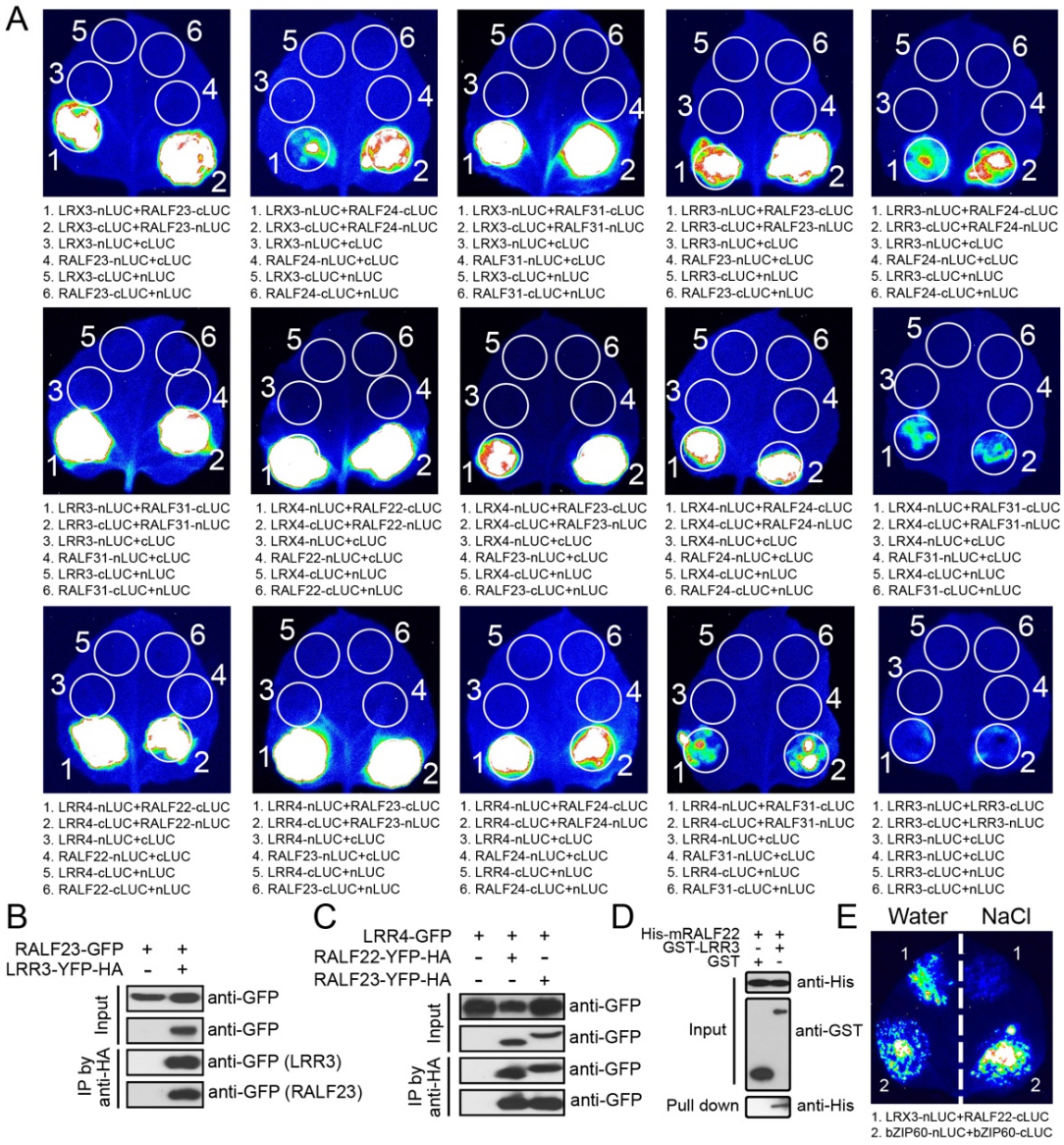

Fig. S3. LRX3 and LRX4 proteins interact with several RALF peptides.

(*A*) Split luciferase complementation assays showing the interactions of RALF peptides with LRX proteins or with the LRR domain of LRX proteins. Construct to express the indicated fusion proteins were co-transformed to *N. benthamiana* leaves through *Agrobacterium* infiltration. Luciferase activity was determined at 48 h after infiltration.

(*B-C*) *Agrobacterium* strains containing constructs to express the indicated fusion proteins were infiltrated into *N. benthamiana*. Immunoprecipitations were performed by using anti-HA antibodies. Immunoblottings were conducted by using anti-GFP antibodies.

(*D*) Interaction between His-mRALF22 (mature RALF22) and GST-LRR3 using in vitro pull-down assay.

(*E*) *Agrobacerium* strains expressing *LRX3-nLUC* and *RALF22-cLUC* were co-infiltrated into the two sides of one *N. benthamiana* leaf. bZIP60, which can form homo-dimers, was used as a control. Half of the leaf was sprayed with water and another half was sprayed with 200 mM NaCl. After 1 h of the treatment, the luciferase activity was examined. The experiment was repeated for 5 times with similar results.

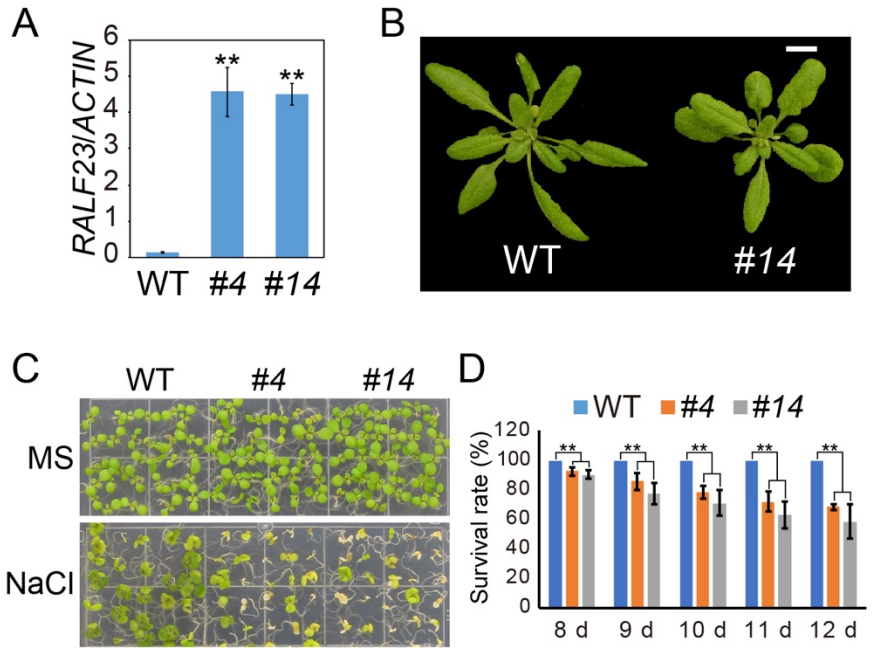

Fig. S4. *RALF23* overexpressing plants phenocopy *lrx345* and *fer-4* mutants.
(*A*) qRT-PCR analysis of the transcript levels of *RALF23* gene in wild type and two independent *RALF23* overexpressing lines (*#4* and *#14*). *ACTIN8* was used as the internal control. Values are means ± SD (n=3), $^{**}p < 0.01$ (Student's *t*-test).
(*B*) Rosette morphology of plants grown in soil for 5 weeks. Scale bar, 1 cm.
(*C*) Phenotypes of seedlings grown on MS and MS+NaCl (120 mM) media.
(*D*) Survival rates of wild type and *RALF23* overexpressing seedlings on MS+NaCl (120 mM) medium. Values are means ± SD (n=3), $^{**}p < 0.01$ (Student's *t*-test).

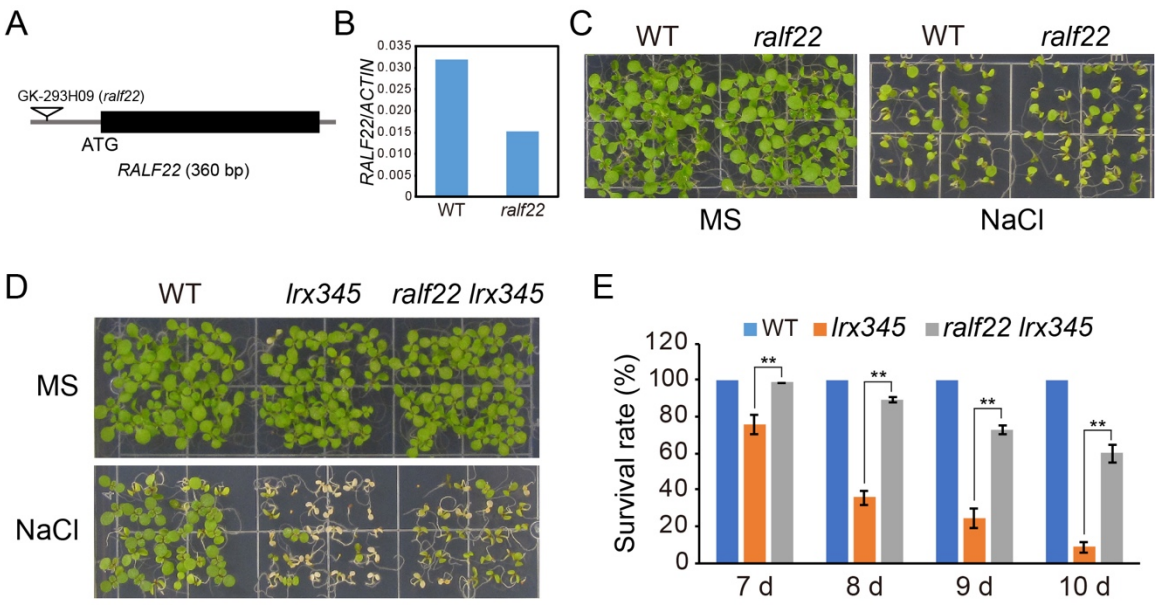

Fig. S5. A *ralf22* mutation partially suppresses the salt-hypersensitive phenotype of *lrx345* mutant.

(*A*) Diagram of the *RALF22* gene. The triangle represents the transfer DNA insertion site.

(*B*) qRT-PCR analysis of the transcript level of *RALF22* in wild type and *ralf22* mutant. *ACTIN8* was used as the internal control.

(*C*) Phenotypes of wild type and *ralf22* grown on MS and MS+NaCl (120 mM) media.

(*D*) Phenotypes of wild type, *lrx345*, and *ralf22 lrx345* grown on MS and MS+NaCl (120 mM) media.

(*E*) Survival rates of wild type, *lrx345*, and *ralf22 lrx345* seedlings on MS+NaCl medium (120 mM) were calculated. Values are means ± SD (n=3), $^{**}p < 0.01$ (Student's *t*-test).

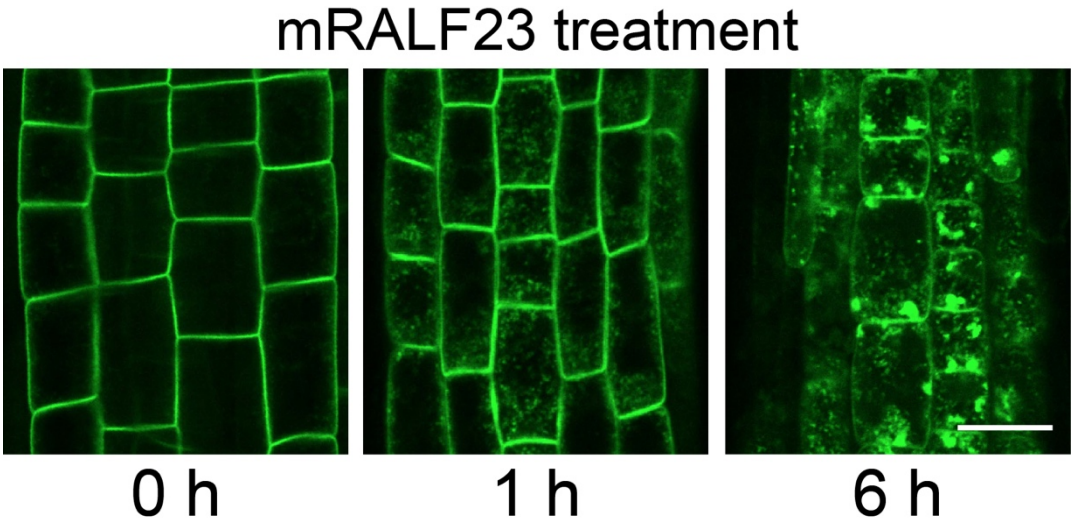

Fig. S6. Mature RALF23 peptide promotes the internalization of FER protein.
pFER::FER-GFP transgenic plants were treated with synthesized mature RALF23 (mRALF23) (1 μM) peptide. Fluorescence in root cells was detected at the indicated time point after treatment by using confocal microscopy. Identical parameters, including the same laser strength and the same pinhole, were applied for all samples. Scale bars, 10 μm.

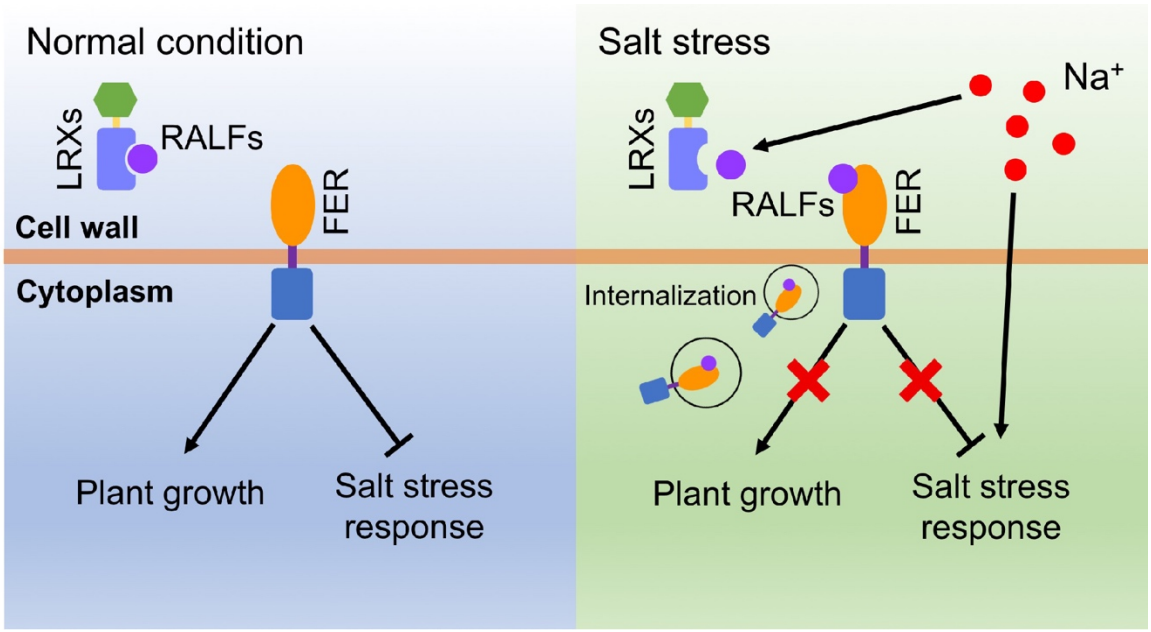

Fig. S7. A working model for the role of LRXs, RALFs and FER in regulating plant growth and stress response.

Under normal conditions, the LRX3/4/5 proteins associate with RALF22/23 in the cell wall to prevent the binding of RALF22/23 to the plasma membrane-localized FER, which promotes plant growth and represses stress responses. Under salt stress, LRX3/4/5 and RALF22/23 are disassociated through an as yet undefined mechanism. The released RALF22/23 peptides bind FER and induce its internalization, as part of a homeostatic mechanism to halt growth. The internalization of FER would also allow the activation of stress responses. The enhanced cell death of the *lrx345* and *fer-4* mutants under salt stress is probably caused by overactivation of salt stress reponse or by the defects in cell wall integrity.

Table S1. Primers used in this study.

Primer name	Sequence	Usage
RALF22 RT-LP	CTCTTCTCTGGATGCACAGGAT	Quantitative real-time PCR
RALF22 RT-RP	GTTGTAGTACGACGCACCACGC	
RALF23 RT-LP	CGTCGCCGTATCTTCTCAATCC	Quantitative real-time PCR
RALF23 RT-RP	TCCTCTCCCATTTCTCCTCCGC	
Actin RT-LP	ATGACTCAGATCATGTTTGAGACC	Quantitative real-time PCR
Actin RT-RP	TCAGTAAGGTCACGACCAGCAA	
LB1.3	ATTTTGCCGATTTCGGAAC	Genotyping
LBa (GABI)	ATATTGACCATCATACTCATTGC	Genotyping
SALK_094400-LP	AGAATATACTGGCGGAGGAGG	Genotyping
SALK_094400-RP	AATCGGAAGATTCGTACCGTC	
GABI_017A08-LP	CAAATCGGTTCGATAGCAAAC	Genotyping
GABI_017A08-RP	CATTTCCATGGTTGTATTCCG	
SALK_013968-LP	AATAGGAGAGCTCGGAGTTGG	Genotyping
SALK_013968-RP	ACGCTGACATTGCTGGTTATC	
LRX3-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAAGAAGACGATTCAAATCC	Transgenic plants Split-LUC assay
LRX3-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGAACGGC GGTGGTGGAGGAG	
LRR3-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAAGAAGACGATTCAAATCC	Co-IP assay Split-LUC assay
LRR3-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCACAGAACGG CCACAACTAAA	
LRX4-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAAGAACAACACCACTCAAT	Transgenic plants Split-LUC assay
LRX4-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGAAGGGT GGTGGTGGGGGA	
LRR4-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAAGAACAACACCACTCAAT	Co-IP assay Split-LUC assay
LRR4-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGGTTTAACAA CAGAACGACC	
LRX5-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAAGACGAAGATGATGATGA	Transgenic plants
LRX5-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAGAAGGGA GGCGGCGGAGGAGA	
RALF22-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGACGAACACTCGCGCGATCT	Transgenic plants Co-IP assay Split-LUC assay
RALF22-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCACGCCTGCACC TAGTGATGGTG	
RALF23-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGAGAGGACTCTCCAGAAACTC	Transgenic plants Co-IP assay Split-LUC assay
RALF23-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGAGCGCCGGC AGCGAGTGATGG	
RALF24-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTCTA GATCCTTAGCTCTCG	Split-LUC assay
RALF22-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCAGTCTTGATGT CGTTAGTGTCTC	
RALF31-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGTTTA ACTCTACAGCGTTGG	Split-LUC assay
RALF31-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGTTGATGT CGTTTGTGTCTC	
ectoFER-P1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGAT AGAACCATGGATTACTCTCCAACAGAGAAAA	Co-IP assay
ectoFER-P2	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTATTGCTTT TCGATTTCCTA	
LRR3-P3	ACGCGTCGACTCCTCTCAATCTCCTCTGACGGCG	Pull down assay
LRR3-P4	AATGCGGCCGCTCACGACACAGAACGGCCACAACTA	
mRALF22-P1	CGCGGATCCGCACAGAAGAAGTACATTAGCT	Pull down assay
mRALF22-P2	CCGCTCGAGTCAACGCCTGCACCTAGTGATG	

Table S2. Peptide sequences used in this study.

Peptide name	Sequence
flg22	QRLSTGSRINSAKDDAAGLQIA
RALF22	AQKKYISYGAMRRNSVPCSRRGASYYNCQRGAQANPYSRGCSTITRCRR
RALF23	ATRRYISYGALRRNTIPCSRRGASYYNCRRGAQANPYSRGCSAITRCRRS

Additional data table S1 (separate file)

IP-MS data generated from *LRX* transgenic plants.