PLANT SCIENCE

RALF4/19 peptides interact with LRX proteins to control pollen tube growth in *Arabidopsis*

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The communication of changes in the extracellular matrix to the interior of the cell is crucial for a cell's function. The extracellular peptides of the RAPID ALKALINIZATION FACTOR (RALF) family have been identified as ligands of receptor-like kinases of the *Cr*RLK1L subclass, but the exact mechanism of their perception is unclear. We found that *Arabidopsis* RALF4 and RALF19 redundantly regulate pollen tube integrity and growth, and that their function depends on pollen-expressed proteins of the LEUCINE-RICH REPEAT EXTENSIN (LRX) family, which play a role in cell wall development but whose mode of action is not understood. The LRX proteins interact with RALFs, monitoring cell wall changes, which are communicated to the interior of the pollen tube via the *Cr*RLK1L pathway to sustain normal growth.

RAPID ALKALINIZATION FACTORs (RALFs) are secreted peptides serving as extracellular signals that are transduced to the inside of the cell (*1*–*3*) (Fig. 1A). Spread throughout the plant kingdom, with 36 members in *Arabidopsis* (*1*), RALFs regulate developmental and physiological processes (*1*–*6*). FERONIA (FER), a member of the *Cr*RLK1L family of receptor-like kinases (RLKs) that is at the nexus between extracellular and intracellular events, is a receptor of several RALF peptides (*3*, *7*).

We used the pollen tube, which displays rapid and polarized growth, to study the function of RALF peptides in signal transduction (*1*). Although pollen tube growth depends on RALF peptides, the underlying mechanisms are unknown (*4*, *8*, *9*). Using publicly available expression data, we identified eight RALFs that were expressed in pollen (fig. S1), belonging to three phylogenetic clades (fig. S2). We focused on clade I with two members, *RALF4* and *RALF19* (fig. S3), and confirmed their specific expression by reverse transcription polymerase chain reaction (RT-PCR) and reporter gene analyses (fig. S4).

To investigate the biological function of *RALF4/19*, we generated plants expressing an artificial microRNA (amiRNA) (Fig. 1A), *amiRRALF4/19*, under a pollen-specific promoter (*10*); 50% and 25% of the independent T1 plants displayed short and intermediate silique lengths, respectively (*n* = 40). Droplet digital PCR (ddPCR) confirmed down-regulation of *RALF4* and *RALF19* in the *amiRRALF4/19* lines; expression of the nontargeted *RALF9* was unaffected (Fig. 1, A and B, and fig. S5D). The homozygous *amiRRALF4/19* line #23, with 10% of the wild-type seed set (Fig. 1, C and D), was used for further studies. Semi–in vivo pollen germination of *amiRRALF4/19* or wild-type pollen, on wild-type or *amiRRALF4/19* carpels, demonstrated that *amiRRALF4/19* pollen failed to grow on any carpel; this result indicates that the reduced *RALF4/19* expression in pollen was responsible for the low seed set (fig. S6). Moreover, in vivo only 3.5% of *amiRRALF4/19* pollen tubes reached the ovules (fig. S7). Nearly 70% of in vitro germinated pollen tubes of *amiRRALF4/19* plants burst, suggesting a role of *RALF4/19* in regulating pollen tube integrity and growth (Fig. 1, E and F). The observed in vivo and in vitro phenotypes of *amiRRALF4/19* line are reminiscent of double mutants affecting the redundant ANXUR1 (ANX1) and ANX2 *Cr*RLK1Ls (*11*, *12*) and the redundant reactive oxygen species–producing NADPH oxidases RbohH and RbohJ (*13*) (Fig. 1C), respectively, and mutants affecting the receptor-like cytoplasmic kinase (RLCK) MARIS (MRI) (*14*), the latter three acting downstream of *ANX1/2* in the signal transduction pathway.

To investigate the relevance of *RALF4/19* for pollen tube growth, we characterized the insertion mutant *ralf4-1* (Fig. 1, B to F, fig. S8, and table S1). Homozygous *ralf4-1* plants were fertile (Fig. 1C and fig. S8) but 47% of in vitro germinated pollen tubes burst, versus 19% in the wild type (Fig. 1, E and F, and fig. S9), perhaps because of excessive growth soon after germination (fig. S9). These results suggest redundancy of *RALF4/19* for in vivo pollen tube growth. To confirm this, we complemented *amiRRALF4/19* lines with *rRALF4*, an amiRNA-resistant version of *RALF4* (Fig. 1A) driven by its own promoter (p*RALF4:rRALF4*), carrying mutations that reduce the interaction with *amiRRALF4/19* without affecting the RALF4 amino acid sequence (Fig. 1, A and B). Transgenic plants (*rRALF4* lines) had a restored, normal fertility (22 of 32 independent lines; Fig. 1, C and D). In contrast, *rRALF4* pollen tubes behaved like those of *ralf4-1* mutants (49% bursting) (Fig. 1, E and F). These results confirm that reduction of either *RALF4* or *RALF19* leads to pollen tube bursting in vitro, whereas in vivo *RALF4* and *RALF19* act redundantly.

Plants with *RALF4* overexpression showed a skewed segregation ratio in crosses with transgenic pollen (tables S2 and S3) as well as reduced pollen germination and seed set (fig. S10). Synthetic RALF4 peptide can repress pollen tube growth (*4*), although a scrambled RALF4 peptide, which contains the same amino acids but in random sequence, could not (fig. S11, A and B). Together, these results suggest that high levels of RALF4 affect pollen germination and pollen tube growth.

To determine whether RALF4/19 peptides bind to specific regions of growing pollen tubes, we added synthetic RALF4 and RALF19 labeled with fluorescein isothiocyanate (FITC) to in vitro growing wild-type pollen tubes. Both bound along the length of the pollen tubes, with stronger signals at the tip (Fig. 1, G and H). The FITC-RALF4 Scrambled peptide did not show binding at the tip (fig. S11, C and D). After incubation, FITC-labeled RALF4/19 peptides were observed in vesicles inside the cell, as reported for other peptides binding pollen tubes (*15*, *16*) and consistent with binding to receptors that become internalized after activation.

Pollen tube growth cannot occur unless the cell wall at the tip is sufficiently soft to allow cell expansion but rigid enough to resist turgor pressure (*17*–*20*). Because *amiRRALF4/19* pollen tubes showed impaired growth, we analyzed cell wall components and found that, relative to the wild type, *amiRRALF4/19* pollen tubes were deficient in callose deposition (Fig. 2, A and D), accumulated acidic pectin in bulges (Fig. 2, B and E, and fig. S12), and showed a broader distribution of esterified pectins, usually localized at the pollen tube tip (Fig. 2, C and F). These results indicate that down-regulation of *RALF4/19* leads to changes in cell wall composition that are correlated with the formation of bulges, where acidic pectins accumulate and pollen tubes burst.

RALF peptides can bind FER to regulate various signaling pathways (*3*, *7*, *21*). Because ANX1 and ANX2 show the highest similarity to FER (*22*) and because the *amiRRALF4/19* phenotype resembles that of mutants in the *ANX1/2* pathway (*11*, *12*), we performed genetic analyses to place *RALF4/19* in this signaling pathway. Overexpression of *ANX1* or *MRI* in pollen tubes represses pollen germination and pollen tube elongation (*13*, *14*). Expressing *ANX1-YFP* or *MRI-YFP* in an *amiRRALF4/19* background under a pollen-specific promoter showed that neither *ANX1* (31 independent lines)

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nor *MRI* (50 independent lines) could rescue the pollen tube bursting phenotype of *amiRRALF4/19* (Fig. 2, G to J); these results imply that the *ANX1* and *MRI* overexpression phenotypes depend on *RALFL4/19*. However, expression of a dominant version of MRI, *MRI*^{R240C}*-YFP*, which suppresses the *anx1/2* double mutant (*14*), also suppressed the *amiRRALF4/19* phenotype (39 of 46 independent lines; Fig. 2, G to J). These results indicate that *RALF4* and *RALF19* act upstream of *ANX1/2* in the signaling pathway, consistent with a putative function as ligands for the activation of ANX1/2.

A pollen-specific RALF was found to interact with an LRX protein from tomato pollen in a yeast two-hybrid screen (*8*). LRX proteins play a role in cell wall development, and *lrx* mutants show defects in tip-growing cells, such as root hairs (*23*). Four members of the LRX family (*LRX8* to *LRX11*) are expressed in pollen (*24*), and higher-order mutants show a pollen tube bursting phenotype reminiscent of *amiRRALF4/19* lines (*25*). This suggests that RALF4/19 peptides may control pollen tube growth in conjunction with LRX proteins. To test this hypothesis, we treated single, double, and triple *lrx* mutant pollen tubes with synthetic RALF4 peptides that repress wild-type pollen tube growth (fig. S11). Growth of wild-type and all *lrx* mutant pollen tubes was not affected when scrambled RALF4 peptides were used (Fig. 3A and movie S1). However, when RALF4 peptides were added, wild-type, single, and double but not triple *lrx* mutant pollen tubes stopped growing (Fig. 3A and movie S1), which demonstrates that *LRX* proteins are redundant and are required for growth inhibition by RALF4 peptides. Thus, the perception of RALF4 depends on the presence of pollen-expressed LRX proteins.

To test whether RALF peptides directly interact with LRX proteins, we performed coimmunoprecipitation experiments with several LRX-Citrine [CIT, a version of green fluorescent protein (GFP)] and RALF4–hemagglutinin (HA) fusion proteins in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. After immunoprecipitation with GFP-Trap agarose beads, mature RALF4-HA was detected when LRX8-CIT, LRX10-CIT, or LRX11-CIT were coexpressed (Fig. 3B and fig. S13). Complementary results were obtained using anti-HA agarose beads, showing coimmunoprecipitation of LRX8-CIT, LRX10-CIT, and LRX11-CIT (fig. S13). When coimmunoprecipitation was performed using a scrambled RALF4-HA peptide, no interaction was observed (fig. S13). A yeast two-hybrid assay

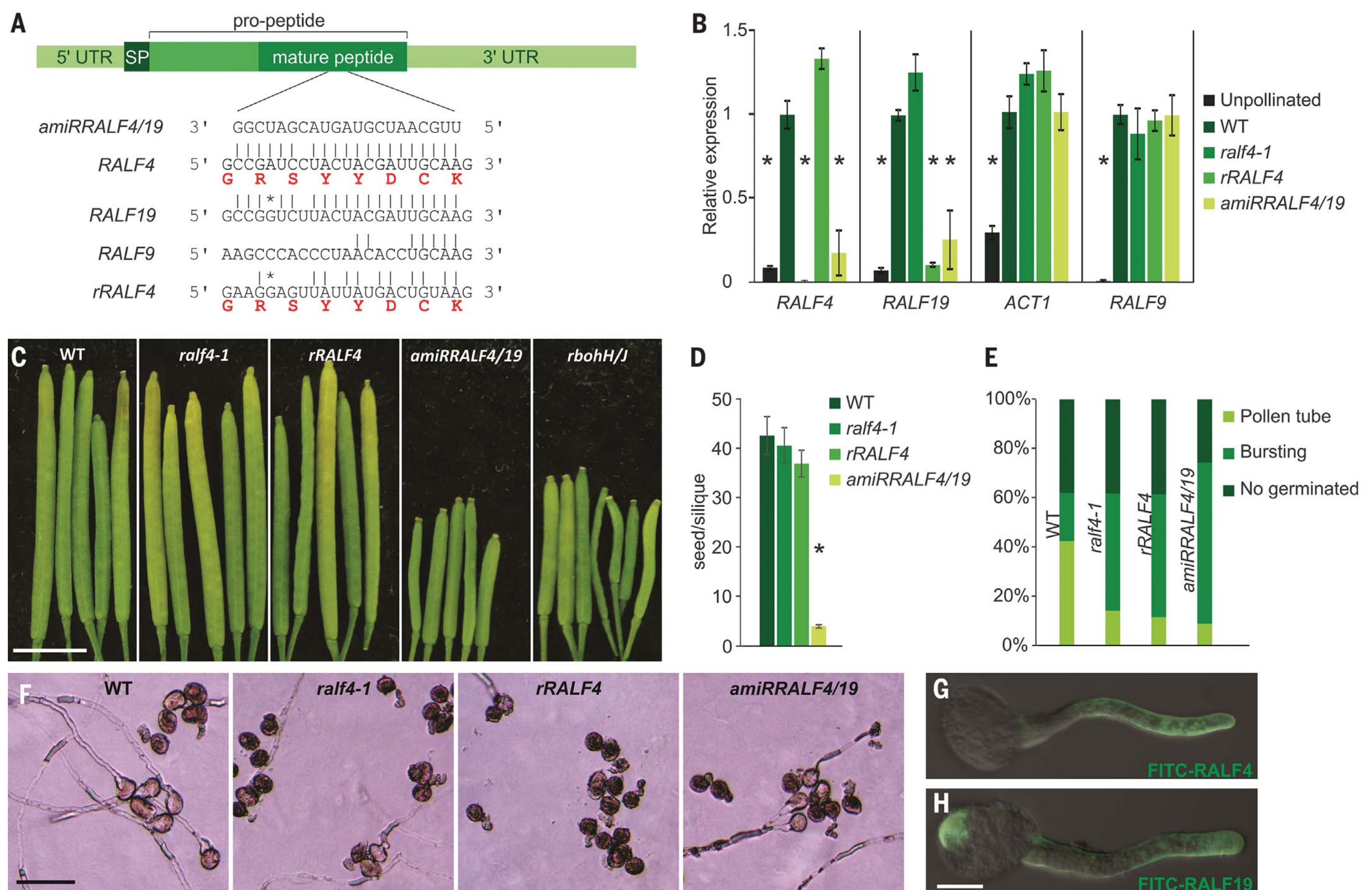

Fig. 1. *RALF4* and *RALF19* are important for pollen tube integrity and growth. (**A**) Sequence alignment of the mature amiRNA against *RALF4* and *RALF19* (*amiRRALF4/19*) with a target site in the coding regions of *RALF4* and *RALF19* mRNAs; asterisks indicate G:U base pairs. Alignment of *RALF9* and a resistant version of *RALF4* (*rRALF4*) shows that silent mutations prevent amiRNA regulation but do not change the amino acid sequence (red). SP, signal peptide. Amino acid abbreviations: C, Cys; D, Asp; G, Gly; K, Lys; R, Arg; S, Ser; Y, Tyr. (**B**) ddPCR of *RALF4* and *RALF19* levels in open flowers of wild-type (WT), *ralf4-1* mutant, *rRALF4*, and *amiRRALF4/19* plants. Unpollinated carpels were used as a control for pollen-specific expression. Expression was calculated as copies relative to *ARTUMES* (*AT1G61790*) mRNAs; WT was used as reference. *RALF9* and *ACT1* were used as internal reference genes. Data are means ± SEM of three biological replicates. $*P < 0.01$, one-way analysis of variance (ANOVA). (**C**) Siliques from the primary inflorescence of WT, *ralf4-1*, *rRALF4*, *amiRRALF4/19*, and *rbohH/J* mutant plants. Scale bar, 0.5 cm. (**D**) Seeds per silique of WT, *ralf4-1*, *rRALF4*, and *amiRRALF4/19* lines. Data are means ± SEM of three biological replicates ($n = 20$). $*P < 0.01$, one-way ANOVA. (**E**) Analysis of in vitro pollen germination of WT, *ralf4-1*, *rRALF4*, and *amiRRALF4/19*. (**F**) In vitro pollen germination of WT, *ralf4-1*, *rRALF4*, and *amiRRALF4/19*. Scale bar, 100 μm. (**G** and **H**) Binding of FITC-conjugated RALF peptides on WT pollen tubes. Both FITC-RALF4 (G) and FITC-RALF19 (H) peptides bind to growing pollen tubes. Scale bar, 10 μm.

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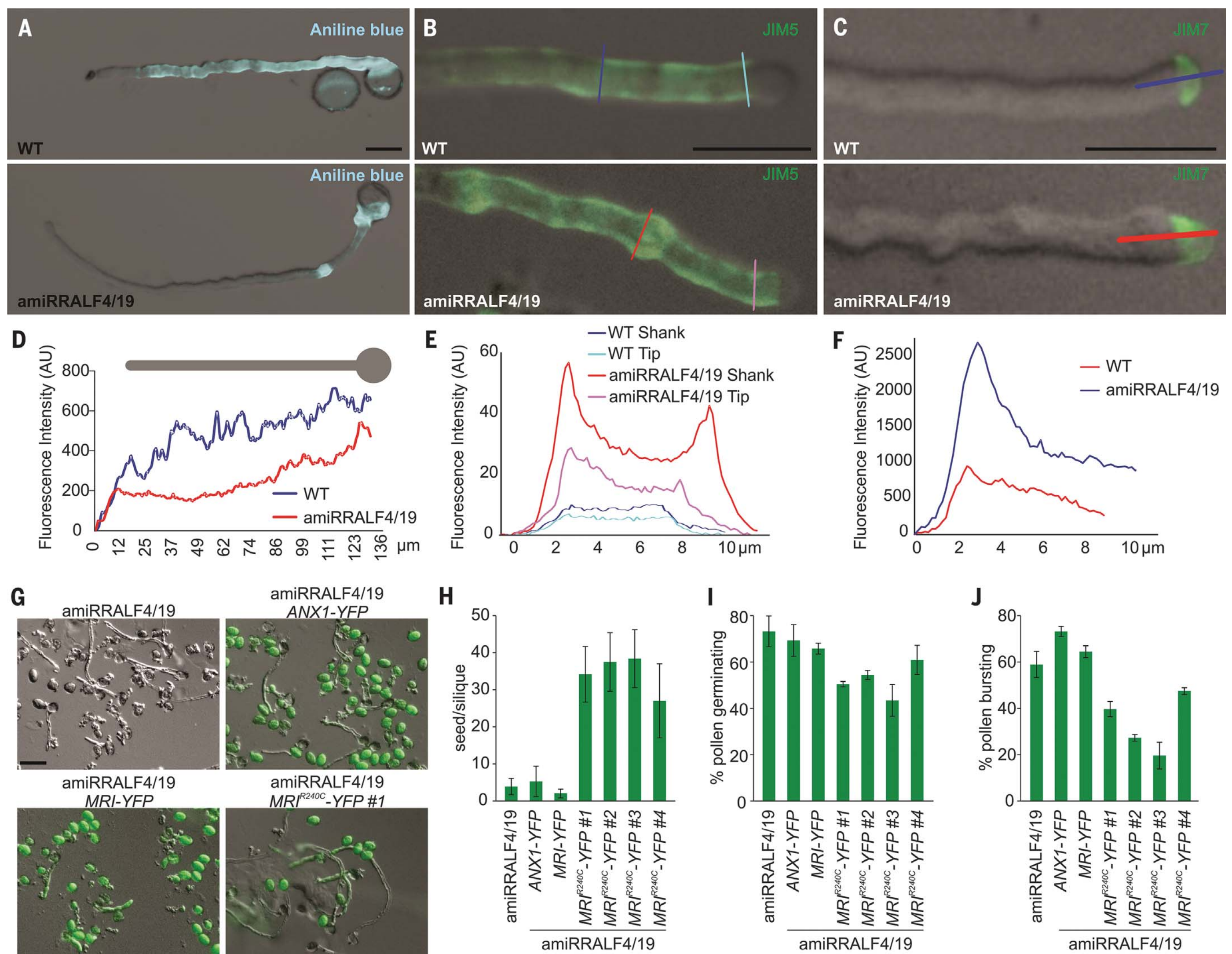

Fig. 2. *RALF4* and *RALF19* control cell wall composition and act upstream of *ANX1* and *MRI* in the *ANX1/2* pathway. (**A**) Aniline blue staining of callose deposition in WT and *amiRRALF4/19* pollen tubes. Scale bar, 20 μm. (**B**) JIM5 immunostaining of acidic pectins in WT and *amiRRALF4/19* pollen tubes. Scale bar, 20 μm. (**C**) JIM7 immunostaining of esterified pectins in WT and *amiRRALF4/19* pollen tubes. Scale bar, 20 μm. (**D**) Histogram of fluorescence intensity of aniline blue staining along pollen tubes of WT and *amiRRALF4/19* plants (n = 20). (**E**) Histogram of transversal sections of fluorescence accumulation in WT and *amiRRALF4/19* pollen tubes (n = 20). (**F**) Histogram of longitudinal sections of fluorescence accumulation in WT and *amiRRALF4/19* pollen tubes (n = 20). (**G**) In vitro pollen germination of *amiRRAL4/19* lines expressing *ANX1-YFP*, *MRI-YFP*, or *MRI^{R240C}-YFP*. Scale bar, 50 μm. (**H**) Number of seeds per silique of *amiRRALF4/19* lines expressing *ANX1-YFP*, *MRI-YFP*, or *MRI^{R240C}-YFP*. Data are means ± SEM of two biological replicates with 15 siliques analyzed per experiment. (**I**) Percentages of in vitro germinated pollen of *amiRRALF4/19* lines expressing *ANX1-YFP*, *MRI-YFP*, or *MRI^{R240C}-YFP*. Data are means ± SEM of two biological replicates with 200 pollen grains analyzed per experiment. (**J**) Percentages of bursting pollen tubes after in vitro germination of *amiRRALF4/19* lines expressing *ANX1-YFP*, *MRI-YFP*, or *MRI^{R240C}-YFP*. Data are means ± SEM of two biological replicates with 200 pollen tubes analyzed per experiment.

confirmed interactions of LRX8 and LRX9 with RALF4 (fig. S13), and quantification of the LRX8-RALF4 interaction by biolayer interferometry resulted in a dissociation constant K_D of 900 nM (fig. S14). Moreover, incubation of wild-type and *lrx8/9/11* pollen tubes with FITC-RALF4 peptides showed reduced binding of RALF4 to the triple mutant pollen tubes (Fig. 3, C to E). Thus, RALF4 interacts with LRX proteins to control pollen tube integrity and growth.

Our results show that RALF4/19 peptides and LRX proteins physically interact in the cell wall and are required to activate the *ANX1/2*-mediated signal transduction pathway regulating pollen tube growth. Both the RALF and LRX proteins are secreted by the pollen tube itself, whose integrity and growth they control. We propose that pollen tubes use autocrine signaling to explore their extracellular space, which may influence the composition, level, and modification of these secreted proteins, thereby providing information about the female tissues through which the pollen tubes grow and navigate. *Cr*RLK1Ls have two malectin domains that are thought to bind oligosaccharides (*26*) and interact with a variety of proteins. FER, for example, binds different RALF peptides and interacts with many other proteins, including RLKs, glycosylphosphatidylinositol-anchored proteins, phosphatases, and small guanosine triphosphatases (*27*, *28*). Thus, it seems that *Cr*RLK1Ls form a signaling hub by bringing together secreted peptides in the cell wall with various membrane-associated proteins to control specific cellular processes. Future investigations will elucidate how this multitude of interactions is regulated at the biochemical level

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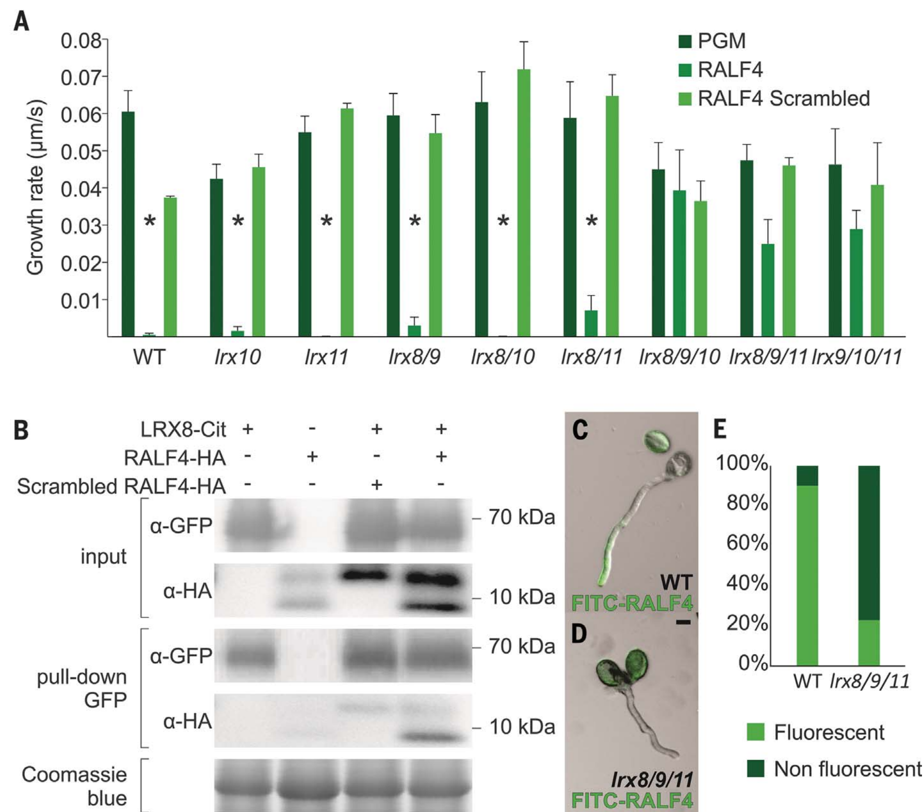

Fig. 3. RALF4 and RALF19 interact with LRX proteins to coordinate pollen tube integrity and growth. (**A**) Growth rate of WT and *lrx* single, double, and triple mutant pollen tubes in pollen germination medium (PGM), mature RALF4 synthetic peptides (250 nM), or RALF4 Scrambled synthetic peptides (250 nM) (n = 29). *$P < 0.05$, one-way ANOVA. (**B**) Western blot analysis of coimmunoprecipitated RALF4-HA after immunoprecipitation of LRX8-CIT using antibody against GFP (α-GFP). Predicted sizes: RALF4-HA and Scrambled RALF4-HA propeptide, 14.8 kDa; mature RALF4-HA, 8.3 kDa; LRX8-Cit, 70 kDa. (**C** and **D**) Binding of FITC-RALF4 conjugated peptides on WT (C) and *lrx8/9/11* triple mutant (D) pollen tubes. Scale bar, 10 µm. (**E**) Percentage of pollen tubes with FITC-RALF4 binding (n = 50).

to direct the diverse processes controlled by *Cr*RLK1L signaling pathways.

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SUPPLEMENTARY MATERIALS

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Timing a switch in tissue integrity

In plants, sperm cells travel through the pollen tube as it grows toward the ovule. Successful fertilization depends on the pollen tube rupturing to release the sperm cells (see the Perspective by Stegmann and Zipfel). Ge *et al.* and Mecchia *et al.* elucidated the intercellular cross-talk that maintains pollen tube integrity during growth but destroys it at just the right moment. The signaling peptides RALF4 and RALF19, derived from the pollen tube, maintain its integrity as it grows. Once in reach of the ovule, a related signaling peptide, RALF34, which derives from female tissues, takes over and causes rupture of the pollen tube.

Science, this issue p. 1596, p. 1600; see also p. 1544

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