PLANT SCIENCE

Pollen PCP-B peptides unlock a stigma peptide–receptor kinase gating mechanism for pollination

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Sexual reproduction in angiosperms relies on precise communications between the pollen and pistil. The molecular mechanisms underlying these communications remain elusive. We established that in *Arabidopsis*, a stigmatic gatekeeper, the ANJEA–FERONIA (ANJ–FER) receptor kinase complex, perceives the RAPID ALKALINIZATION FACTOR peptides RALF23 and RALF33 to induce reactive oxygen species (ROS) production in the stigma papillae, whereas pollination reduces stigmatic ROS, allowing pollen hydration. Upon pollination, the POLLEN COAT PROTEIN B-class peptides (PCP-Bs) compete with RALF23/33 for binding to the ANJ–FER complex, leading to a decline of stigmatic ROS that facilitates pollen hydration. Our results elucidate a molecular gating mechanism in which distinct peptide classes from pollen compete with stigma peptides for interaction with a stigmatic receptor kinase complex, allowing the pollen to hydrate and germinate.

In angiosperms, multiple layers of male-female interactions occur throughout pollination (*1*, *2*). During this process, pollen grains land on the stigma and germinate, each producing a pollen tube that elongates into the stylar transmitting tissue and grows toward the ovule for fertilization (*3*). Immediately after adhering to the stigma, compatible pollen must be distinguished from incompatible pollen and other unwanted invasive agents, such as pathogenic spores (*4*, *5*). In response to compatible pollen, stigmas initiate a basal response pathway that transfers water to the desiccated pollen grain for pollen hydration and germination (*6*). The signaling components and the underlying mechanism that regulate this first crucial event in pollination are largely unknown (*6*).

Numerous cysteine-rich peptides (CRPs) have been identified in plant reproduction (*7*). In *Arabidopsis thaliana*, a highly polymorphic family of pollen-borne CRPs, the POLLEN COAT PROTEIN B-class peptides (PCP-Bs), play a role in pollen hydration. The loss of PCP-Bs notably slows pollen hydration and germination (*8*). In a wide spectrum of angiosperms, considerable amounts of reactive oxygen species (ROS) have been detected in the mature, unpollinated stigmas (*9*). In this work, we established that pollination in wild-type (WT) *Arabidopsis* triggered a significant reduction of ROS levels in stigmatic papilla cells in the first 20 min after pollination (MAP) (Fig. 1A and fig. S1A) (*9*). Decreased ROS levels were detected at the pollen adhesion site as early as 1 MAP (Fig. 1B), and by 10 MAP, obvious ROS decline extended to the adjacent area along the papillar cell edge (fig. S1B). Upon pollination by pollen from loss-of-function *PCP-B* mutants (*pcp-bγ* and *pcp-bβ/γ*), WT stigmas showed significantly slower pollen hydration and a significantly reduced capacity to suppress stigmatic ROS levels compared to WT stigmas pollinated by WT pollen (Fig. 1C).

We then expressed and purified the PCP-Bβ/γ peptides from insect cells and obtained chemically synthesized PCP-Bγ (cPCP-Bγ). Mass spectrometry analysis showed that both forms of the peptides had molecular masses that were 8 Da lower than expected, suggestive of four disulfide bonds: one between Cys1 and 2; one between Cys7 and 8; one connecting Cys3, 4, or 5; and one connecting Cys6 and either one of Cys3, 4, or 5 (fig. S1, C to E). The latter two we were not able to pinpoint because Cys3, 4, and 5 are too close. WT stigmas treated with PCP-Bγ showed a significant and dose-dependent reduction in ROS (Fig. 1D and fig. S2, A and B). The PCP-B$\gamma_{C30A/C32S/C33A}$ (mPCP-Bγ) did not induce a significant reduction in stigmatic ROS and did not restore pollen hydration (Fig. 1, D and E). We also performed a pistil feeding assay (*10*) with an ROS production inhibitor and ROS scavengers (fig. S2, C and D). It has been reported that the first 10 min of pollen hydration determines the rate of pollen germination (*11*). The ROS-repressed pistils displayed significantly faster pollen hydration in the initial 10 min (Fig. 1F), as well as longer pollen tubes by 4 hours after pollination, probably because of a head start in germination (fig. S2E). These results suggest that pollen PCP-Bs induce a decrease in stigmatic ROS levels that facilitates pollen hydration.

We next explored the signaling components on the stigma that recognize PCP-Bs. Many receptor-like kinases (RLKs) have been identified in male-female interactions functioning to perceive peptide signals (*12*, *13*). Therefore, stigmatic RLKs could very likely be involved in mediating pollen PCP-B signals to initiate the stigmatic response. A whole-genome transcriptional analysis has revealed that the ANJEA (ANJ) receptor kinase is highly expressed in the stigma (*14*). *ANJ* belongs to the 17-member *Catharanthus roseus* receptor-like kinase 1–like (CrRLK1L) gene family (*15*). FERONIA (FER), the most extensively studied member of this family, functions as a receptor for several RAPID ALKALINIZATION FACTOR (RALF) CRPs, which control cell growth and immune responses (*16*–*18*). By mining the Genevestigator and TraVA databases, we established that *ANJ* and *FER* are highly expressed in the stigma (fig. S3A), as confirmed by an analysis of *pANJ::GUS* and *pFER::GUS* transgenic plants (fig. S3B).

We identified two *anj* mutant alleles, both knockouts of *ANJ* as revealed by reverse transcription polymerase chain reaction (RT-PCR) analysis (fig. S3C). Compared to the WT, compatible pollen hydrated more rapidly in the *anj* and *fer* single mutant and most rapidly in the *anj-1 fer-4* double-mutant stigmas. The phenotypes were rescued in mutant lines complemented with either the *pANJ::ANJ*–green fluorescent protein (*GFP*) or *pFER::FER-GFP* transgenic constructs (Fig. 2, A and B; fig. S3D; and movie S1). Longer pollen tubes were observed in the *anj-1 fer-4* pistils than in WT pistils 4 hours after pollination (fig. S3E). Lower ROS levels were detected in the *anj* and *fer* mutant stigmas than in WT stigmas, and these were restored by treatment with H_2O_2 (Fig. 2C and fig. S3, F to G). Moreover, regardless of whether pollen from WT or *pcp-bγ* was used for pollination, the *anjfer* stigmas showed faster pollen hydration and longer pollen tubes relative to the WT stigmas (Fig. 2D and fig. S4A).

The similar expression patterns of *ANJ* and *FER* and the similar phenotypes of the *anj* and *fer* mutants suggested that ANJ and FER might function in a protein complex. To test this hypothesis, we performed co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation assays and established that kinase domain–deleted FERΔK and ANJΔK interacted with each other (fig. S4, B and C). Luciferase complementation assays showed that cytoplasmic domain–deleted ANJΔC and FERΔC also interacted (fig. S4D). Furthermore, a pull-down assay established that the extracellular domains (ECDs) of ANJ and FER interact with each other (fig. S4E). These results demonstrate that ANJ and FER function in a complex and are consistent with yeast two-hybrid assay results in a previous study that focused on late pollination events (*19*). We observed that ANJ and FER were expressed in the stigmatic papillae from flower stage 11 to 14, during which the stigma matures

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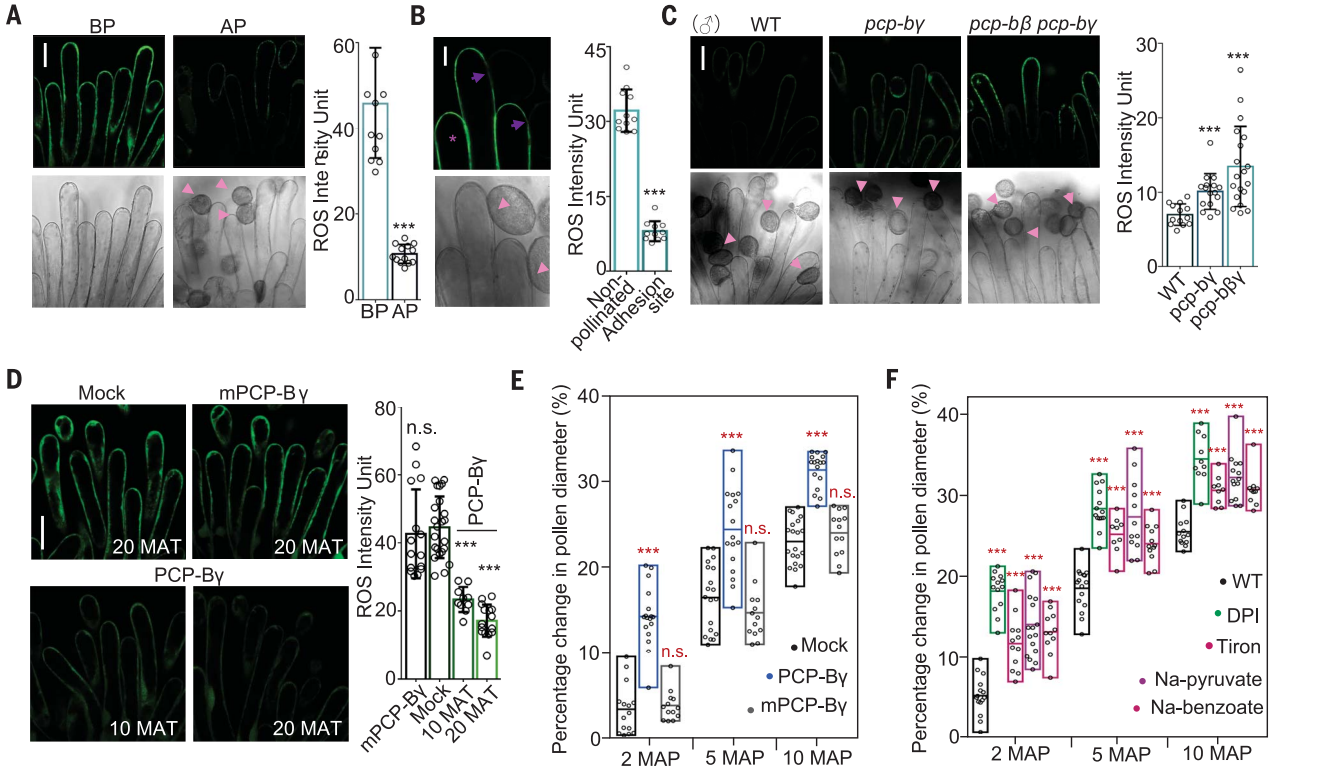

Fig. 1. PCP-Bs in pollen trigger reductions in stigmatic ROS levels. (**A**) ROS dye H_2DCF-DA staining (left) and quantification (right) of ROS level of stigmatic papilla cells from WT *Arabidopsis* before pollination (BP) and 20 min after pollination (AP) with compatible pollen. Bottom, bright-field images. (**B**) ROS dye DHE staining of stigmatic papillae after 1 min of pollination by a single pollen grain. Arrowheads point to the pollen grain adhesion site. Asterisk indicates nonpollinated papilla. (**C**) H_2DCF-DA staining and quantification of ROS levels of papilla cells 20 MAP with *pcp-b*γ and *pcp-b*βγ pollen. Arrowheads indicate pollen grains (A to C). (**D**) H_2DCF-DA staining showing stigmatic ROS levels upon treatment with 0.5 μM PCP-Bγ or mPCP-Bγ. MAT, minutes after treatment. (**E** and **F**) Altered pollen hydration profiles in WT stigmas treated with PCP-Bs (E) or with the NADPH oxidase inhibitor DPI or the ROS scavengers (F). (A) to (F): means ± SD. ***$P < 0.001$ (two-tailed Student's *t* test); n.s., not significant. n = 13 (A), 11 (B), 14 (C), 10 (D), 15 (E), or 10 (F) stigmas. Scale bars, 20 μm (A, C, and D) or 10 μm (B).

and becomes receptive to pollen (fig. S4F). Given that the ANJ–FER complex and PCP-Bs are both involved in pollination-induced reductions in ROS levels, we hypothesized that ANJ–FER senses the pollen-derived PCP-Bs for compatible pollen recognition. We established that PCP-Bs, but not the mPCP-Bγ, efficiently pulled down maltose-binding protein (MBP)–FER_{ecd}/ANJ_{ecd} (Fig. 2E and fig. S4, G and H). We also detected an interaction between nLuc-FERΔC/ANJΔC and PCP-Bγ-cLuc in a luciferase complementation assay (Fig. 2F). These results indicate that the ANJ–FER complex interacts with PCP-Bs and so could function as the receptor complex that senses the PCP-Bs.

RALF peptides have been characterized as the ligand of FER in different tissues (*16*, *17*, *20*). Based on Genevestigator data and the quantitative RT-PCR analysis, *RALF23* and *RALF33* are the most highly expressed RALF genes in the stigma (fig. S5, A and B). FER and RALF23 are known to interact (*17*, *18*), and our pull-down and Co-IP assays showed that FERΔK and ANJΔC interacted with RALF33 (Fig. 3, A and B, and fig. S5C). On the basis of structural information (*18*), we mutated the amino acids in FER and analogous positions in ANJ that are critical for FER–RALF–LLG complex formation and observed that the resulting proteins showed a much weaker interaction with RALF33 (Fig. 3B). The interactions of nLuc-FERΔC/ANJΔC with RALF33-cLuc in a luciferase complementation assay further confirmed their interactions (fig. S5D). To investigate the function of RALF33 in stigmatic papillae, we obtained the published *ralf33-2* mutant (*17*) and characterized *ralf33-3*, an independent knockout mutant of RALF33 (fig. S5E). Similar to the phenotypes of *anj*- and *fer*-related mutants, *ralf33* mutants produced pistils that supported more rapid pollen hydration, which led to longer pollen tubes (fig. S5, F and G). ROS staining showed that loss of RALF33 resulted in reduced ROS accumulation in the stigmatic papilla cells (Fig. 3C and fig. S5H), whereas treatment with 1 μM RALF33 significantly induced ROS production in stigmatic papillae (Fig. 3D).

FER and its co-receptor LLG1 activate the guanine nucleotide exchange factors (GEFs) of Rho-like GTPases from plants (RAC/ROP) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thereby inducing ROS production in roots and root hairs (*21*, *22*). Because ANJ–FER binds to RALF33 and RALF33 induces ROS production, we propose a pathway in which RALF33 stimulates the ANJ–FER receptor complex to activate ROS production through the RAC/ROP–NADPH oxidase pathway. RALF33 treatment did not induce a marked increase in ROS levels in *anj-1 fer-4* mutant stigmas (Fig. 3D). Based on a transcriptional analysis of the *LORELEI* family genes, we established that LLG1 is the most abundant member of that family in the stigma (fig. S6A). Similar to the *fer-4* mutant, the *llg1-2* mutant showed reduced ROS accumulation in stigmatic papillae (fig. S6B). RAC/ROP-regulated NADPH oxidases play a major role in cytoplasmic ROS production (*23*). Based on Genevestigator data and the RT-PCR analysis, we identified RBOHD as the most abundantly expressed gene of the RBOH family in the stigma (fig. S6C). We obtained the published *rbohd* mutant (*24*) and a knockout mutant of RBOHD, *rbohd-1*, both of which

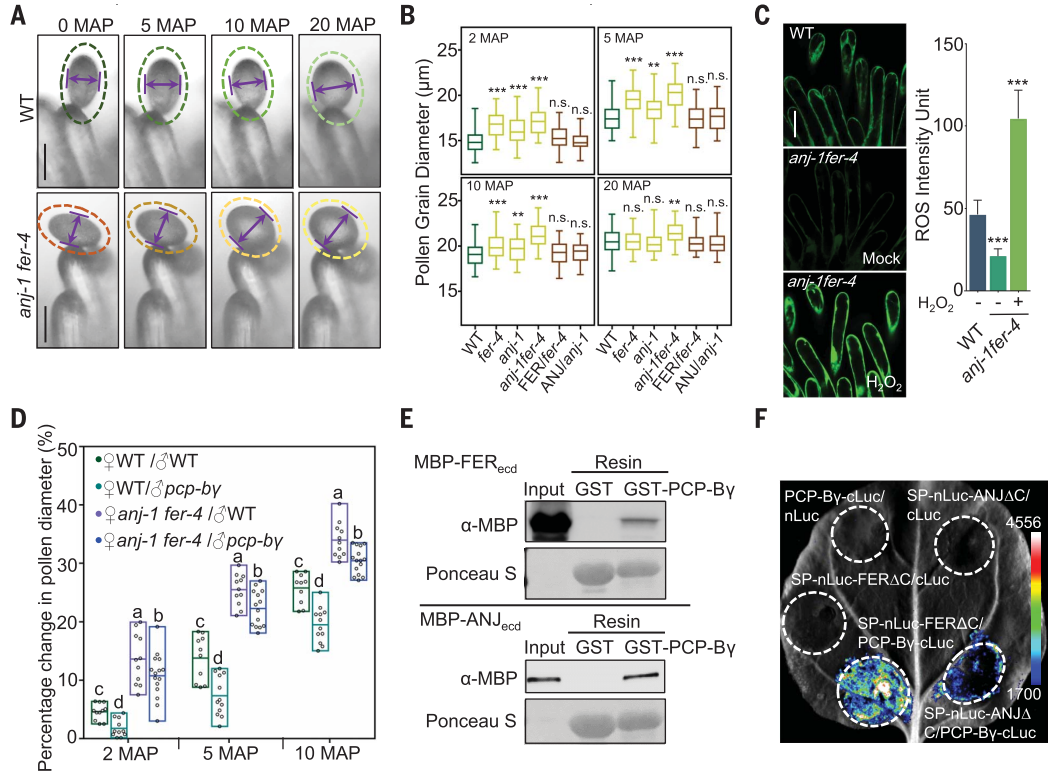

Fig. 2. ANJ and FER are crucial for pollen hydration and PCP-Bγ–induced decreases in ROS levels in stigmatic papilla cells. (**A**) Pollination-induced pollen hydration on stigmas. Equatorial diameters of pollen grains were measured as indicated. (**B**) Pollen hydration rates on stigmas from *fer-4* and *anj-1* single and double mutants with and without genetic complementation. (**C**) H_2DCF-DA staining of *anj-1 fer-4* stigma papillae with and without 10 mM H_2O_2 treatment. (B) and (C): data are means ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$ (two-tailed Student's *t* test). (**D**) Both WT and *pcp-bγ* pollen had faster hydration rates on *anj-1 fer-4* stigmas. One-way analysis of variance ($P < 0.01$). (**E**) Pull-down assay showing interaction of GST-PCP-Bγ with MBP-FER_{ecd} and ANJ_{ecd}. (**F**) Luciferase complementation assay showing interaction between PCP-Bγ-cLuc and nLuc-FERΔC–ANJΔC. SP, signal peptide. In (A) to (C), WT pollen was used for pollination. Scale bars, 20 μm (A and C). *n* = 7 (B), 5 (C), or 16 (D) stigmas.

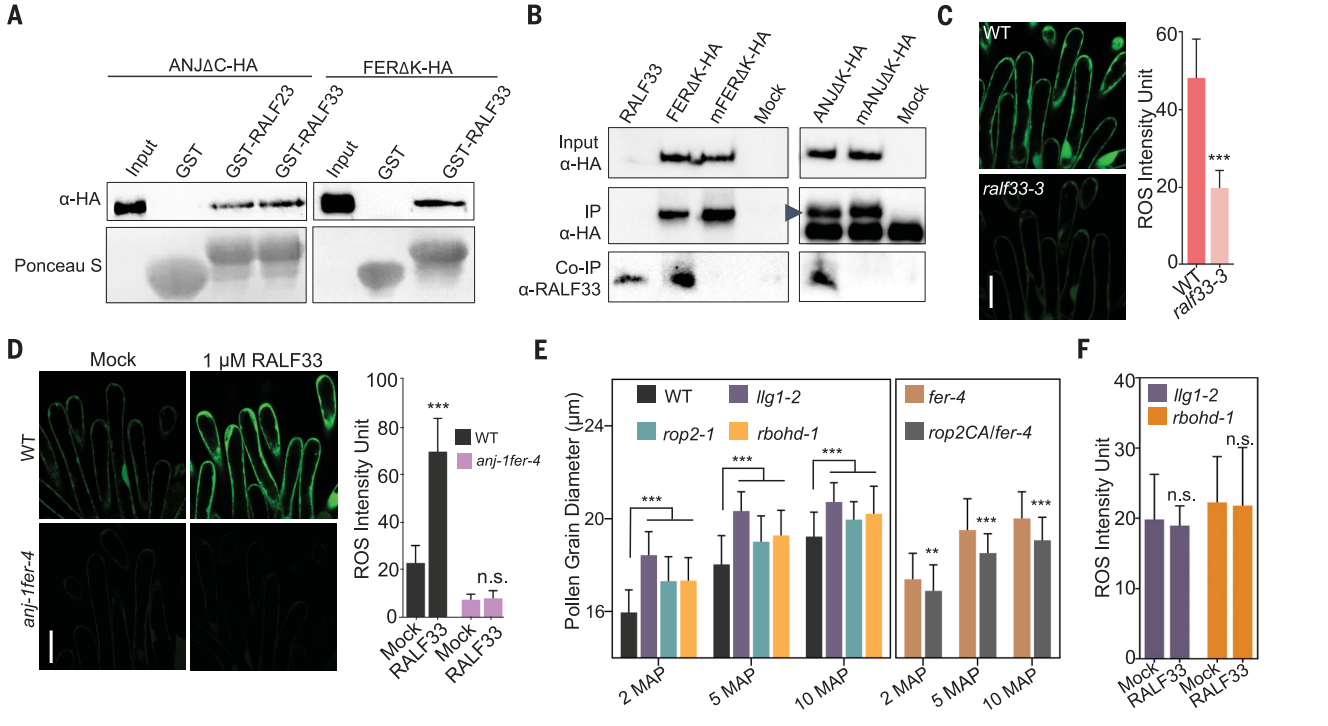

Fig. 3. RALF33 stimulates the ANJ–FER receptor complex to induce ROS production through the RAC/ROP–NADPH oxidase pathway. (**A**) Pull-down assay showing interaction of RALF23/33 with ANJΔC-hemagglutinin (HA). (**B**) Co-IP showing interaction of RALF33 with FERΔk/mFERΔk/ANJΔk/mANJΔk-HA. Arrowhead indicates immunoglobulin heavy chain. (**C**) H_2DCF-DA staining of the stigmatic papillae of *ralf33-3*. (**D**) ROS levels in WT and *anj-1 fer-4* stigmas treated with 1 μM RALF33 for 20 min. (**E**) Pollen hydration profiles of WT, *llg1-2*, *rop2-1*, *rbohd-1*, and *pROP2::rop2CA fer-4* stigmas. (**F**) ROS levels in *llg1-2* and *rbohd-1* papilla cells with or without (Mock) treatment with 1 μM RALF33 for 20 min. (C) to (F): means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ (two-tailed Student's *t* test). Scale bars, 20 μm (C and D). *n* = 16 (C), 10 (D), 10 (E), or 11 (F) stigmas.

showed lower stigmatic ROS than the WT plants in the unpollinated pistils (fig. S6, D and E). Next, we found that a mutant of the most abundant stigmatic RAC/ROP, ROP2, had reduced stigmatic ROS levels before pollination (fig. S6, F and G). Similar to *fer-4* mutant stigmas, the *llg1-2*, *rop2-1*, and *rbohd-1* mutant stigmas all supported significantly more rapid pollen hydration than WT stigmas (Fig. 3E). By contrast, a transgenic complementation mutant of ROP2 (*ROP2p::rop2CA*) showed partial reversion of the faster pollen hydration and reduced stigmatic ROS of the *fer-4* mutant, consistent with rop2CA being a constitutive activator of RBOH (22) (Fig. 3E and fig. S6G). Moreover, the *llg1-2* and *rbohd-1* mutants were both less responsive to RALF33-induced ROS elevation than the WT, as shown earlier (Fig. 3F). These results suggest that the ANJ–FER complex senses the autocrine RALF33 peptides and then activates ROS production in stigmatic papillae through the ROP2–RBOHD pathway, which suppresses pollen hydration.

Next, we examined how the ANJ–FER complex coordinates PCP-Bs and RALF33 to regulate ROS levels in the stigma. We mixed PCP-Bγ and RALF33 to co-treat stigmas in ROS and pollen hydration assays. The application of PCP-Bγ suppressed the effect of RALF33 in inducing stigmatic ROS in a dose-dependent manner (Fig. 4A). Consistent with this, RALF33 treatment reversed the faster pollen hydration of *ralf33-3* stigmas, and the addition of PCP-Bγ, but not mPCP-Bγ, countered this effect (fig. S7A). In a luciferase complementation assay that used the nLuc-FERΔC/RALF33-cLuc combination, the addition of cPCP-Bγ significantly and dose-dependently reduced the luciferase signal (fig. S7B). Pull-down assays showed that PCP-Bγ, but not mPCP-Bγ, competed dose-dependently with glutathione *S*-transferase (GST)–RALF33 for interaction with His-FER_{ecd} (Fig. 4B and fig. S7, C and D). Microscale thermophoresis confirmed that RALF23 and RALF33 each interacted with FER_{ecd} and ANJ_{ecd} (fig. S7E). FER_{ecd} and ANJ_{ecd} interacted with PCP-Bγ with dissociation constants (K_d) of 0.34 and 0.35 μM, respectively, but showed K_d of only 4.63 and 6.38 μM when interacting with mPCP-Bγ (Fig. 4C). Furthermore, fluorescein isothiocyanate (FITC)–RALF33 interacted with FER_{ecd} with a K_d of 0.1604 μM, and the PCP-Bγ added to the mixture competed with the RALF33–FER_{ecd} interaction with an inhibition constant (K_i) of 2.5099 μM (Fig. 4D). Together, these results suggest that PCP-Bγ competes with RALF33 for binding to FER_{ecd}/ANJ_{ecd}, which results in the suppression of RALF33-induced ROS production in the stigma.

Once pollen lands on a stigma, signal communications between pollen and the stigma papilla are initiated; this is thus likely to be the earliest checkpoint in pollen-stigma interactions. In this work, we identified a RALF33–ANJ/FER receptor kinase–RAC/ROP–RBOHD signaling pathway that controls the generation of stigmatic ROS. Our findings further uncovered an intricate regulatory mechanism by which pollen-derived PCP-Bs compete with stigma-produced RALF33, thereby repressing stigmatic ROS levels to facilitate pollen hydration (Fig. 4E). This work demonstrates the versatility of a receptor kinase in being able to perceive and switch interactions with different types of peptide ligands. Together with the earlier findings on peptide competitions in stomatal patterning (*25*), pollen tube burst (*20*), and pollen germination (*26*), our work reinforces the concept that antagonistic peptide–receptor kinase pairs fine-tune plant developmental processes (*27*). As CrRLK1L, RALFs, PCP-Bs, RAC/ROPs, and NADPH oxidases are all conserved in the plant kingdom, the antagonistic control of ROS levels by PCP-Bs and RALFs through CrRLK1L might represent a fundamental mechanism by which compatible pollen disengages stigma gating in flowering plants.

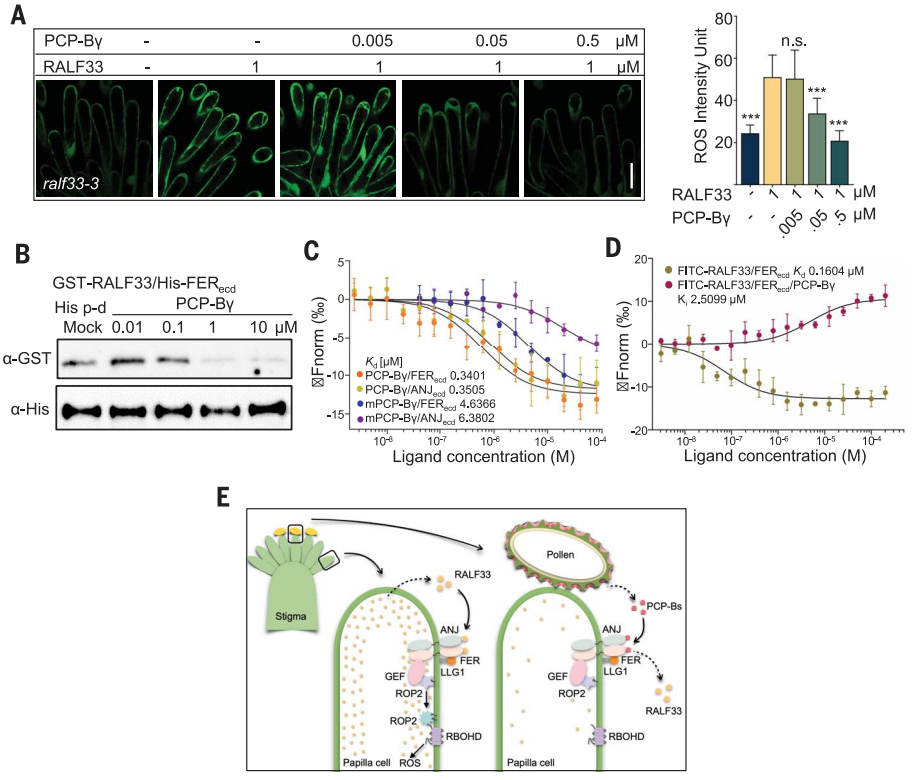

Fig. 4. PCP-Bs compete with RALF23/33 for interaction with the ANJ–FER receptor complex. (**A**) H_2DCF-DA staining and quantification of ROS levels of *ralf33-3* mutant stigma co-treated with PCP-Bγ and RALF33 (means ± SD; n = 10 stigmas). ***P < 0.001 (two-tailed Student's t test). Scale bar, 20 μm. (**B**) Pull-down assay showing competition by PCP-Bγ with GST-RALF33 in interaction with His-FER_{ecd}. His-FER_{ecd} was used to pull down GST-RALF33. (**C**) Quantification of binding affinity between fluorescently labeled FER_{ecd}/ANJ_{ecd} and PCP-Bγ/mPCP-Bγ by microscale thermophoresis (MST). (**D**) MST analysis of the inhibition of PCP-Bγ in the interaction of FITC-RALF33 with FER_{ecd}. (**E**) Model of pollen peptide repression of stigma-peptide–induced ROS production by means of receptor kinases. Before pollination, RALF23/33 induces ROS production in the stigmatic papilla cells through an ANJ–FER–ROP2–RBOHD pathway. Upon pollination, PCP-Bs from the pollen coat compete with RALF23/33 for interaction with the ANJ–FER complex, repressing ROS production and initiating stigmatic responses.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S7
Tables S1 and S2
References (*28*–*35*)
Movie S1
MDAR Reproducibility Checklist

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Science

Pollen PCP-B peptides unlock a stigma peptide–receptor kinase gating mechanism for pollination

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Competing signal peptides hold the key

When a pollen grain lands on a receptive flower's pistil, a complex dance leading to sexual reproduction begins. Liu *et al.* show some of the early steps that help to distinguish a compatible pollen grain from a random piece of dust. Normally, a stigmatic gatekeeper, the ANJEA–FERONIA receptor kinase complex, perceives signaling peptides produced by the stigma that drive the production of reactive oxygen species at the stigma papillae. Upon pollination, POLLEN COAT PROTEIN B-class peptides compete with those stigmatic peptides for binding to the stigmatic receptor kinase complex. The subsequent decline of stigmatic reactive oxygen species production allows hydration and opens the gates to pollen germination.

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