Appendix for *Cr*RLK1L receptor-like kinases HERCULES RECEPTOR KINASE 1 and ANJEA are female determinants of pollen tube reception

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Table of contents

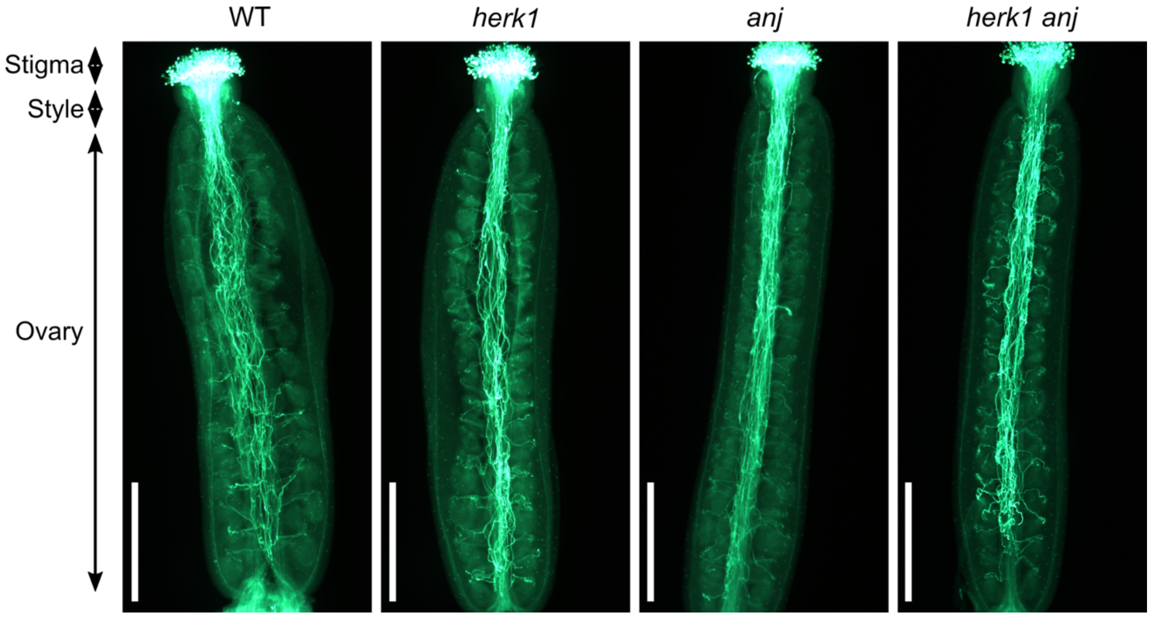
Appendix Figure S1. Growth comparison of WT and *herk1 anj* plants. All scale bars = 1.5 cm.

A,B Representative wild-type plants at 10 and 21 days old.

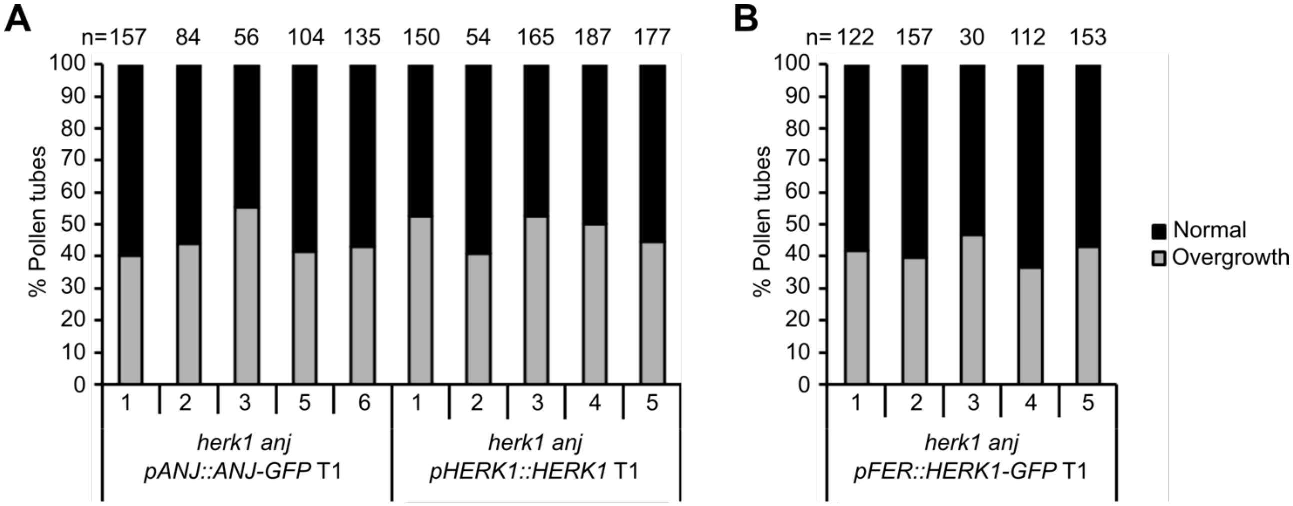
C,D Representative *herk1 anj* plants at 10 and 21 days old.

E,F Representative *fer-4* plants at 10 and 21 days old.

G Representative wild-type and *herk1 anj* plants (left and right, respectively) at 5 weeks old.



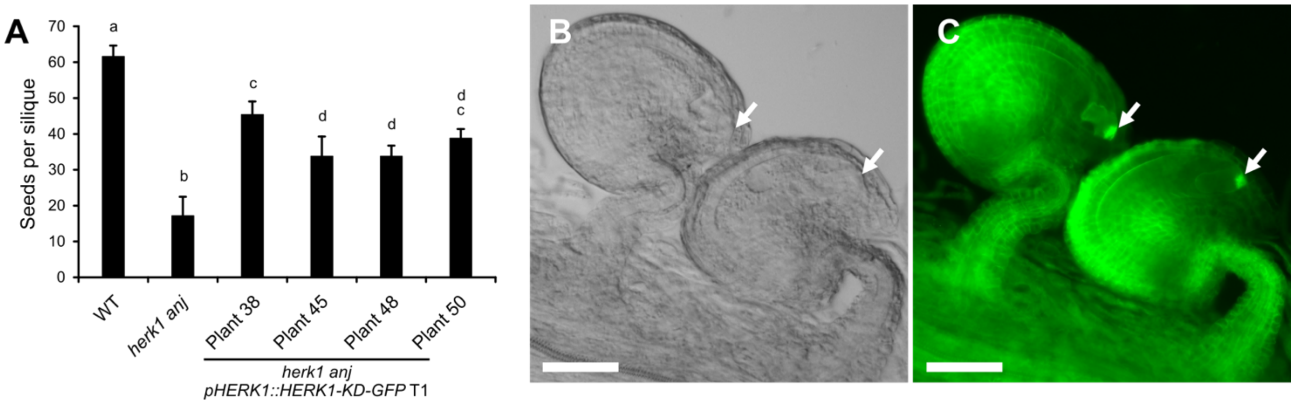
Appendix Figure S2. Pollen tube growth and targeting of ovules is not altered in *herk1 anj* plants. Aniline blue staining of pollen tubes in self-pollinated stage 16 flowers in wild-type, *herk1*, *anj* and *herk1 anj* plants. Scale bars = 500 µm.



Appendix Figure S3. The *herk1 anj* defect in pollen tube reception can be complemented by expression of *HERK1* and *ANJ* constructs*.*

A Percentage of pollen tubes with normal reception at the female gametophyte (black bars) and displaying overgrowth (grey bars) in siliques of five independent T1 *herk1 anj* plants transformed with *pANJ::ANJ-GFP*, and *pHERK1::HERK1*. Pollen tube reception was scored for ovules in at least three siliques per line. *pANJ::ANJ-GFP* T1 line 4 was excluded from the figure due to non-complementation of the seed phenotype.

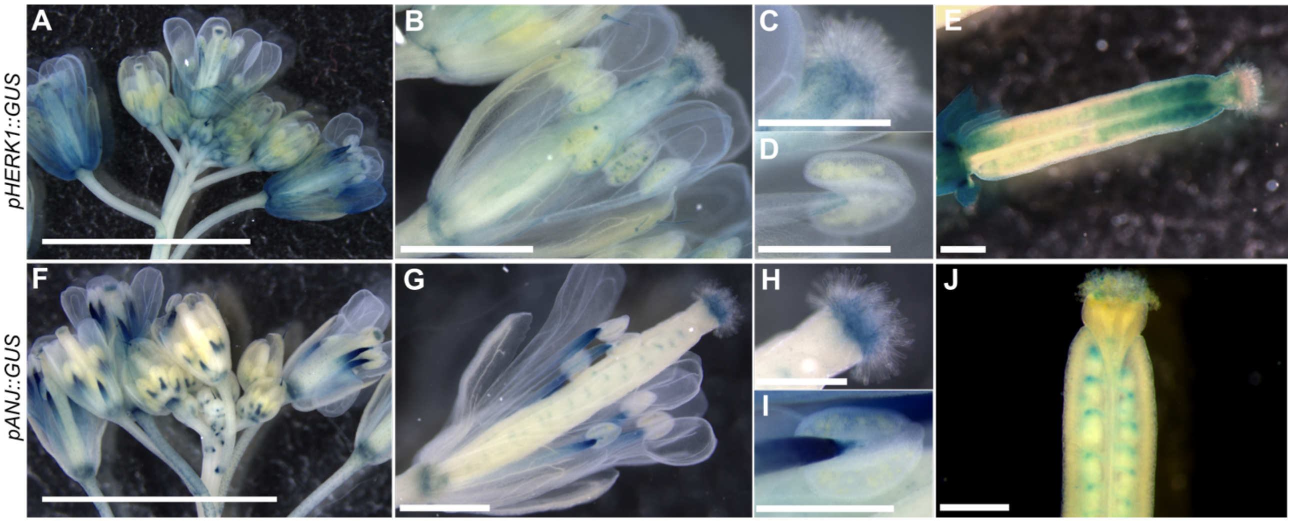
B Percentage of pollen tubes with normal reception at the female gametophyte (black bars) and displaying overgrowth (grey bars) in siliques of five independent T1 *herk1 anj* plants transformed with *pFER::HERK1-GFP*.



Appendix Figure S4. Kinase dead HERK1 expressed under its own promoter can complement the seed phenotype.

A Developing seeds per silique in wild-type, *herk1 anj*, and *pHERK1::HERK1-KD-GFP* T1 plants. Fully expanded siliques were cleared and photographed under a stereomicroscope. Four independent T1 lines were examined and seeds in five siliques per plant counted. Data presented are means per silique ± SD (n = 5). Letters (a-d) mark statistically significant differences between samples in one-way ANOVA analysis followed by Bonferroni's post-hoc comparison of means ($p<0.05$). *pHERK::HERK1-KD-GFP* T1 line 49 was excluded from the figure due to non-complementation of the seed phenotype.

B,C Corresponding DIC image (B) and localisation of HERK1-KD-GFP (C) in the synergid cell from the *pHERK1::HERK1-KD-GFP* construct. Scale bars = 50 µm. White arrows, filiform apparatus.

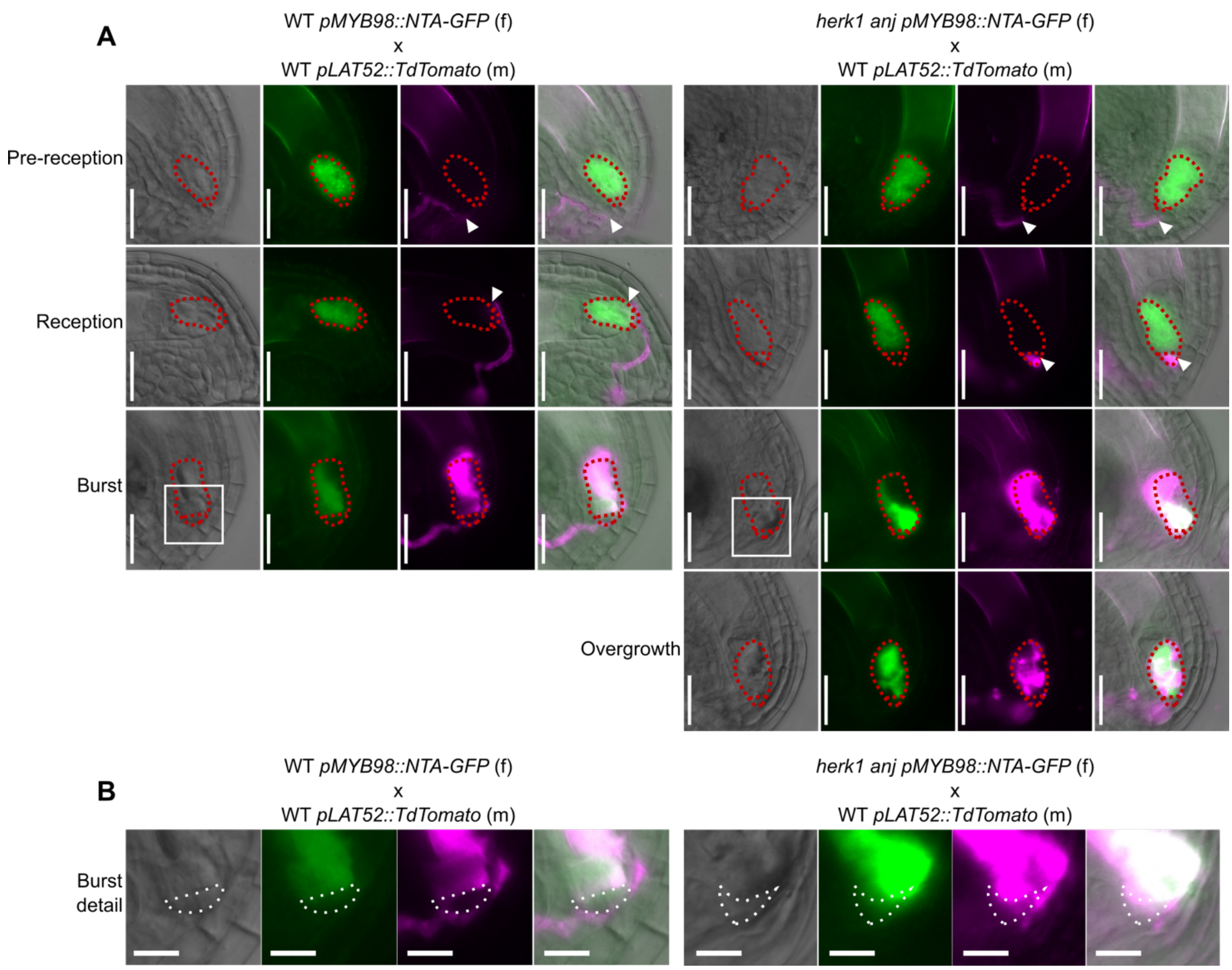


Appendix Figure S5. Expression pattern of *HERK1* and *ANJ* in flowers.

A,B Representative image of the expression pattern in inflorescences and flowers of *HERK1* as shown by *pHERK1::GUS*. Details of a mature stigma, an anther, and a silique are shown in (C), (D), and (E) respectively.

F,G Representative image of the expression pattern in inflorescences and flowers of *ANJ* as shown by *pANJ::GUS*. Details of a mature stigma, an anther, and a silique are shown (H), (I), and (J), respectively.

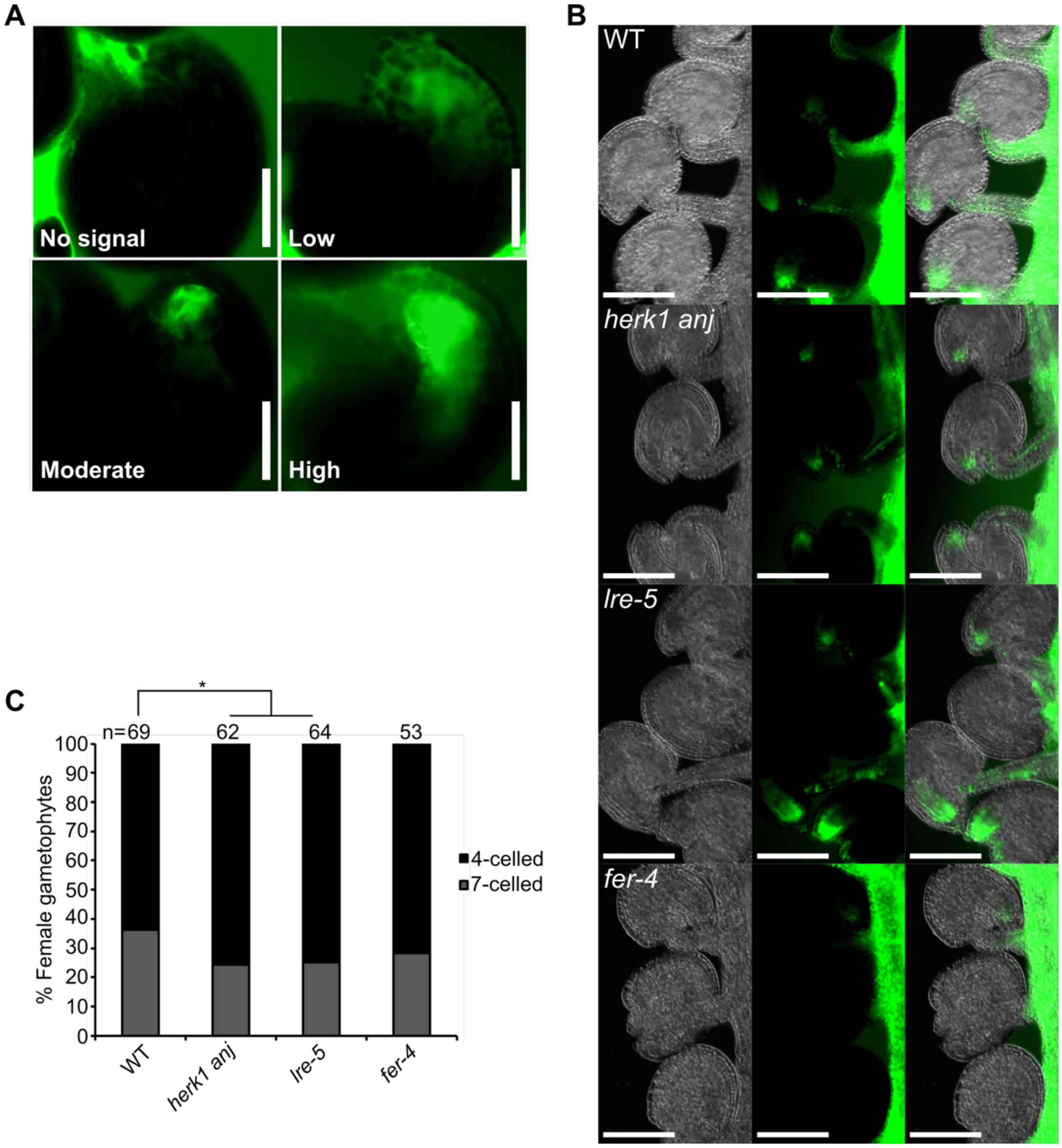
GUS activity in at least four T1 lines was examined. Scale bars = 5 mm in (A,F) 1 mm in (B,G); 0.5 mm in (C,D,E,H,I,J).



Appendix Figure S6. NTA localisation in the synergid cells of WT and *herk1 anj* at different stages of pollen tube reception.

A DIC images are shown in grey. In green is NTA-GFP fluorescence in ovules expressing *pMYB98::NTA-GFP*. In magenta, TdTomato fluorescence from pollen tubes expressing *pLAT52::TdTomato*. On the right are merged DIC and fluorescence images. Red dotted lines delineate the synergid cells. White arrowheads indicate the pollen tube tip. Scale bars = 25 µm.

B Detailed images of the filiform apparatus corresponding to the areas highlighted with white squares in (A). White dotted lines delineate the filiform apparatus. Scale bars = 10 µm.

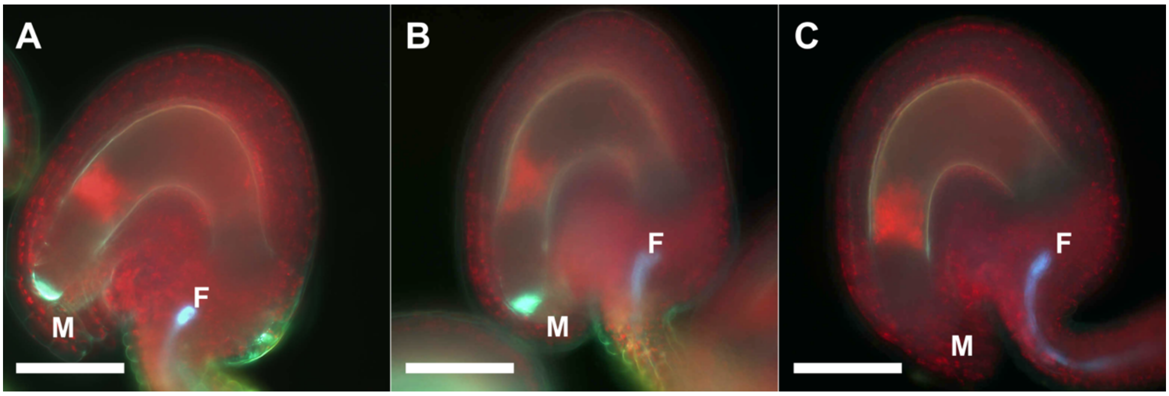


Appendix Figure S7. **H_2DCF-DA measurements of ROS production in *herk1 anj* ovules.**

A Images of H_2DCF-DA fluorescence in representative ovules corresponding to each category used in the ROS assays presented in this study. Scale bars = 25 µm.

B Representative images of H_2DCF-DA staining of ROS in three ovules from wild-type, *herk1 anj*, *lre-5* and *fer-4* plants at 20 hours after emasculation (HAE). Scale bars = 100 µm.

C Female gametophyte development stage in ovules from stage 14 flowers at 20 hours after emasculation (HAE) in wild-type, *herk1 anj*, *lre-5* and *fer-4* as assessed by confocal microscopy as per [1]. Ovules analysed from five siliques per line. * $p<0.05$ (χ-square tests).



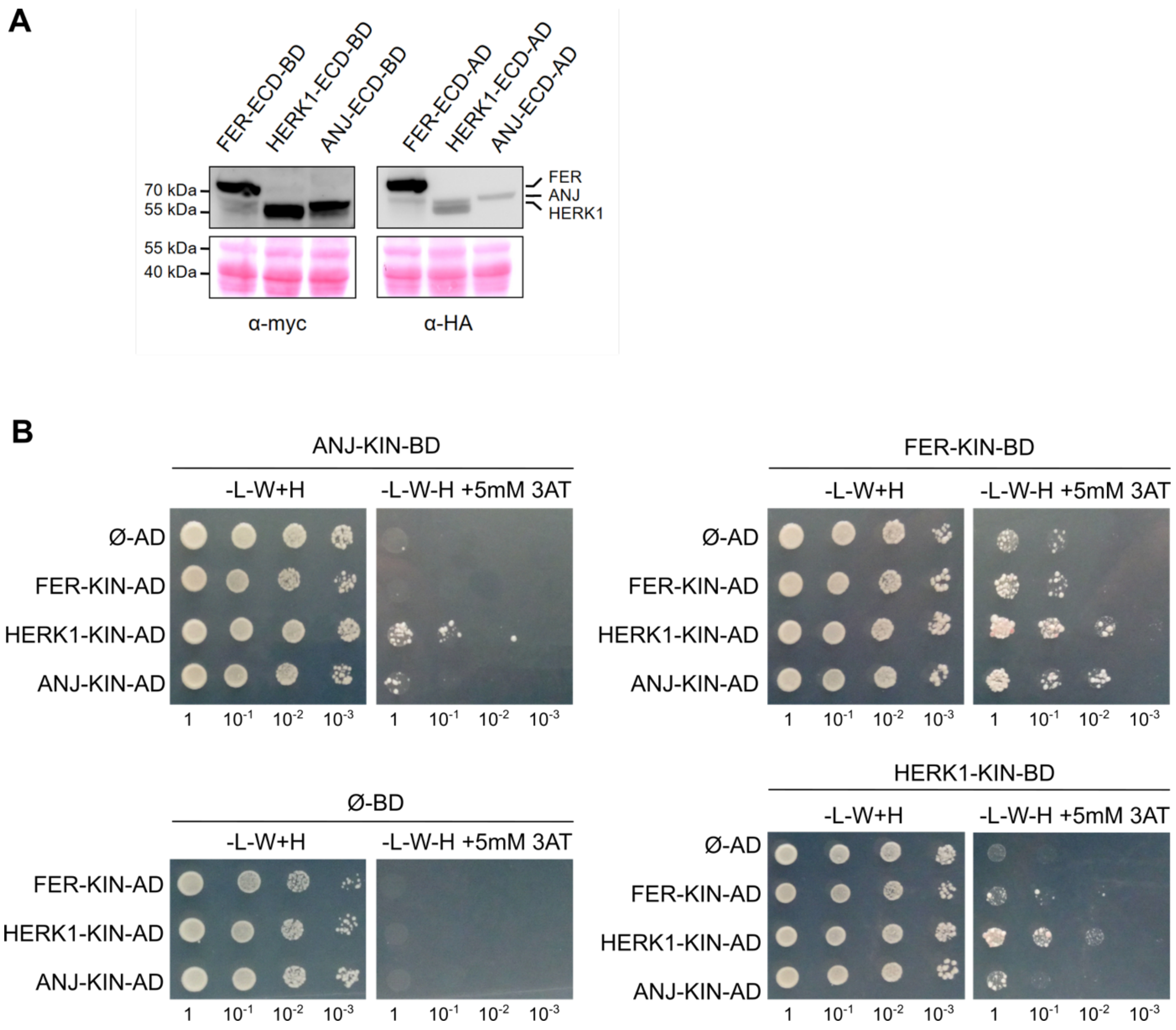
Appendix Figure S8. **Callose accumulation at the filiform apparatus in *herk1 anj* mutants.**

A Representative image of a mature ovule from a wild-type plant. SR2200 white fluorescence at the filiform apparatus indicates accumulation of callose.

B Representative image of a mature ovule from a *herk1 anj* plant. SR2200 white fluorescence at the filiform apparatus indicates accumulation of callose.

C Representative image of the background autofluorescence present in mature ovules.

Chlorophyll red autofluorescence can be seen in all cell layers in the ovule. Blue autofluorescence from the xylem lignin within the funiculus can also be observed. Scale bars = 25 µm. M, micropyle. F, funiculus.



Appendix Figure S9. Yeast two hybrid assays between HERK1, ANJ and FER domains.

A Western blots showing expression of the FER, HERK1 and ANJ ECD domains in yeast as detected by anti-Myc and anti-HA antibodies. Ponceau red staining of the membrane is included below as a loading control.

B Yeast two hybrid assays with the intracellular kinase domains of HERK1, ANJ and FER (HERK1-KIN, ANJ-KIN and FER-KIN, respectively). Ø represents negative controls where no sequence was cloned into the activating domain (AD) or DNA-binding domain (BD) constructs. -L-W-H, growth medium depleted of leucine (-L), tryptophan (-W) and histidine (-H). Plates were supplemented with 5 mM 3-Amino-1,2,4-triazole (3 AT) due to yeast growth autoactivation in several of these constructs.

Appendix Table S1. List of Arabidopsis lines use in this study. Sources and NASC stock identifiers are listed where relevant.

Experimental Models: Organisms/Strains		
Arabidopsis thaliana: Col-0	NASC	N1092
Arabidopsis thaliana: herk1-1	NASC	N657488
Arabidopsis thaliana: anj-1	NASC	N654842
Arabidopsis thaliana: fer-4	Prof. A. Cheung [2]	NASC ID: N69044
Arabidopsis thaliana: lre-5	Dr. R. Palanivelu [3]	NASC ID: N66102
Arabidopsis thaliana: herk1-1 anj-1	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 lre-5	This study	N/A
Arabidopsis thaliana: Col-0 CRISPR *fer*	This study	N/A
Arabidopsis thaliana: herk1-1 anj CRISPR *fer*	This study	N/A
Arabidopsis thaliana: Col-0 *herk1 anj lre-5* CRISPR *fer*	This study	N/A
Arabidopsis thaliana: Col-0 *pHERK1::GUS*	This study	N/A
Arabidopsis thaliana: Col-0 *pANJ::GUS*	This study	N/A
Arabidopsis thaliana: Col-0 *pHERK1::H2B-tdTomato*	This study	N/A
Arabidopsis thaliana: Col-0 *pANJ::H2B-tdTomato*	This study	N/A
Arabidopsis thaliana: Col-0 *pHERK1::HERK1*	This study	N/A
Arabidopsis thaliana: Col-0 *pANJ::ANJ-GFP*	This study	N/A
Arabidopsis thaliana: Col-0 *pFER::HERK1-GFP*	This study	N/A
Arabidopsis thaliana: Col-0 *pHERK1::HERK1-KD-GFP*	This study	N/A

Arabidopsis thaliana: Col-0 *pLRE::LRE-Citrine*	This study	N/A
Arabidopsis thaliana: Col-0 *pMYB98::NTA-GFP*	This study	N/A
Arabidopsis thaliana: Col-0 *pFER::FER-GFP*	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pHERK1::HERK1	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pANJ::ANJ-GFP	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pFER::HERK1-GFP	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pLRE::LRE-Citrine	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pMYB98::NTA-GFP	This study	N/A
Arabidopsis thaliana: herk1-1 anj-1 pFER::FER-GFP	This study	N/A
Arabidopsis thaliana: lre-5 pHERK1::HERK1	This study	N/A
Arabidopsis thaliana: lre-5 pANJ::ANJ-GFP	This study	N/A
Arabidopsis thaliana: lre-5 pLRE::LRE-Citrine	This study	N/A
Arabidopsis thaliana: lre-5 pMYB98::NTA-GFP	This study	N/A
Arabidopsis thaliana: lre-5 pFER::FER-GFP	This study	N/A
Arabidopsis thaliana: Col-0 *pLAT52::TdTomato*	Dr. M. Bayer (unpublished)	N/A

Appendix Table S2. List of primers used for cloning, genotyping PCR or qPCR.

Primer name	**Oligonucleotides (5' - 3')**
HERK1 genotyping fw	GTTGCTCGCGGTAGTCTTCT
HERK1 genotyping rv	CTGTCCTGAATTCCGCAAGC
ANJEA genotyping fw	CTCCTCTGTAGCAAAACCAGGA
ANJEA genotyping rv	CTCACGTTTACTCCCTCGGG
LRE genotyping fw	AAGCCAGTTTTAGAGTACGAAGA
LRE genotyping rv	TCAAGTCAACACTAACAAAGCAAAAACAGCGG
FER genotyping fw	CGGATCCATGAAGATCACAGAGGGACGATTC
FER genotyping rv	CGCAGATCTAGCACCAAACACACAAAACCC
SALK LB genotyping primer	ATTTTGCCGATTTCGGAAC
GABI LB genotyping primer	GTGGATTGATGTGATATCTCC
HERK1 qPCR fw (5’ of T-DNA)	GTAGGCGGTAACTCTGGCTC
HERK1 qPCR rv (5’ of T-DNA)	AGACGAACCCAATGACGACC
HERK1 qPCR fw (3’ of T-DNA)	CGTCACCGCCATTTGGTTTC
HERK1 qPCR rv (3’ of T-DNA)	CAAGCCGTTGTTTCCAAGTCA
ANJ qPCR fw (5’ of T-DNA)	TAATGAAATCCTCGCCGCCA
ANJ qPCR rv (5’ of T-DNA)	GGCCTCTAGCAACGGAGAAT
ANJ qPCR fw (3’ of T-DNA)	CTGTCTATGGTACTTGCACC
ANJ qPCR rv (3’ of T-DNA) (underlined bases do not match genome)	CCACCAACATTCTTCTTAGTGGTTG
ACTIN2 qPCR fw	CCAGAAGGATGCATATGTTGGTG

ACTIN2 qPCR rv	GAGGAGCCTCGGTAAGAAGA
pHERK1 fw	TAGGTACCTAGAATGTTTTTCTCAAGTTTTCTTCC
HERK1 rv	TAAGGATCCTCTTCCTTCAGATTTCACCAGTTGTG
pANJ fw	TTAGGTACCTTGTGGAATCATGAAATCGTAGTGT
ANJ rv	TAGGATCCACGTCCCTCAGATTTGATCAGCTGCG
pFER fw	TAGGTACCCGAGTTGTAAAAGGCCTGGC
FER rv	TAAGGATCCACGTCCCTTTGGATTCATGA
HERK1-KD fw	AGAAACGTGAGATCTGCAAACATATTGCTTGACGA
HERK1-KD rv	AGATCTCACGTTTCTGTGAATGACCGGTTTCGAGT
ANJ-KD fw	AGAAACGTCAGATCCGCCAACATATTGCTTGA
ANJ-KD rv	GGATCTGACGTTTCTGTGAATCACGGGTTTCG
pHERK1 pentrdtopo fw	CACCTAGAATGTTTTTCTCAAGTTTTCTTCC
pHERK1 pentrdtopo rv	AACCTGGAAATGGAACAGATC
pANJ pentrdtopo fw	CACCTTGTGGAATCATGAAATCGTAGT
pANJ pentrdtopo rv	TTCACAAAACCTGGAAATTTTAAATAATT
HERK1exJM Y2H (324-406) fw	GGATATTGATCTTAGCACTCTTGTGG
HERK1exJM Y2H (324-406) rv	AACCCGAGATTACTCTTACTGCT
ANJexJM Y2H (324-406) fw	GCTTGATCTGAGCTCTTATTTATCCA
ANJexJM Y2H (324-406) rv	CCACCAACATTCTTCTTAGTGGTTG
LRE Y2H (23-138) fw	GATATCGGATGGTGTGTTTGAATCA
LRE Y2H (23-138) rv	CCGGCGTTTAGGTTATGTGAATAGAG
HERK1 ECD Y2H (24-405) fw	GGATTCACACCTGTGGATAATTAC
HERK1 ECD Y2H (24-405) rv	TTACCCGAGATTACTCTTACTGCT

ANJ ECD Y2H (25-405) fw	TACGTACCAGTGGATAATTACCTC
ANJ ECD (25-405) rv	TTAACCAACATTCTTCTTAGTGGTTG
FER ECD Y2H (28-446) fw	GCTGATTACTCTCCAACAGAGA
FER ECD Y2H (28-446) rv	TTACGTATTGCTTTTCGATTTCCTAG
HERK1 kin Y2H (429-830) fw	GAAGAAGCGGAAACGTGGC
HERK1 kin Y2H (429-830) rv	CCTCTTCCTTCAGATTTCACCAGTTGTG
ANJ kin Y2H (429-830) fw	GAAGAAACGAGGACGAGACC
ANJ kin Y2H (429-830) rv	CCTCCACGTCCCTCAGATTTGATCAGCTGCG
FER kin Y2H (470-895) fw	GGCTTACCGCAGACGTAAGC
FER kin Y2H (470-895) rv	CCACGTCCCTTTGGATTCATGA
FER CRISPR construct 1 Out fw (5’ gRNA 1; target FER sequence underlined)	ATATATGGTCTCGATTGTTCTACCCAAACTCGTACGAGTT
FER CRISPR construct 1 In fw (5’ gRNA 1)	TGTTCTACCCAAACTCGTACGAGTTTTAGAGCTAGAAATAGC
FER CRISPR construct 1 In rv (3’ gRNA 1)	AACCGAGTCCGTCACATTCCCTTCAATCTCTTAGTCGACTCTAC
FER CRISPR construct 1 Out rv (3’ gRNA 1)	ATTATTGGTCTCGAAACCGAGTCCGTCACATTCCCTTCAA
FER CRISPR construct 2 Out fw (5’ gRNA 2)	ATATATGGTCTCGATTGAAAAGGAGTATGCGGTGACAGTT
FER CRISPR construct 2 In fw (5’ gRNA 2)	TGAAAAGGAGTATGCGGTGACAGTTTTAGAGCTAGAAATAGC

FER CRISPR construct 2 In rv (3’ gRNA 2)	AACCGGAAGGCGAGATATCATTCCAATCTCTTAGTCGACTCTAC
FER CRISPR construct 2 Out rv (3’ gRNA 2)	ATTATTGGTCTCGAAACCGGAAGGCGAGATATCATTCCAA
CRISPR-Cas9 *fer* mutant genotyping inner primer fw	GATGAAGATCACAGAGGGACG
CRISPR-Cas9 *fer* mutant genotyping inner primer rv	CATTGACGCGATTCATGTTT
CRISPR-Cas9 *fer* mutant genotyping outer primer fw	TAACAGCTGCAACTGGTGC
CRISPR-Cas9 *fer* mutant genotyping outer primer rv	GCAGGACTTGGCACGAAATG
Control gDNA region for genotyping fw	CTGCCTTACGAGCATTGGTT
Control gDNA region for genotyping rv	TAACGCTTCCCAAGGTGATT
HERK1 429-830 pOM fw	AAGTTCTGTTTCAGGGCCCGAAGAAGCGGAAACGTGGC
HERK1 429-830 pOM rv	ATGGTCTAGAAAGCTTTATCTTCCTTCAGATTTCAC
ANJ 429-829 pOM fw	AAGTTCTGTTTCAGGGCCCGAAGAAACGAGGACGAGAC
ANJ 429-829 pOM rv	ATGGTCTAGAAAGCTTTAACGTCCCTCAGATTTG

Appendix Table S3. **List of plasmids used in the present study and their corresponding sources.**

Recombinant DNA	**Reference**
pHERK1::HERK1 in pGreen-IIS	This study
pANJ::ANJ-GFP in pGreen-IIS	This study
pFER::FER-GFP in pGreen-IIS	This study
pHERK1:HERK1-KD in pGreen-IIS	This study
pHERK1:HERK1-KD-GFP in pGreen-IIS	This study
pANJ::ANJ-KD-GFP in pGreen-IIS	This study
pHERK1::GUS in pGWB433	This study
pANJ::GUS in pGWB433	This study
pHERK1::H2B-tdTomato in pAH21	This study
pANJ::H2B-tdTomato in pAH21	This study
pFER::HERK1-GFP in pMDC111	Prof. U. Grossniklaus [4]
pMYB98::NTA-GFP in pMDC83	Dr. S. Kessler [5]
p35S::HA-LRE in pSK	Dr. C. Li [6]
p35S::HA-LRE in pMLBart	This study
pLRE::LRE-Citrine in pMDC99	Prof. U. Grossniklaus [7]
pU6-26::FER 5' gRNA 1; pU6-29::FER 3' gRNA 1	This study
pU6-26::FER 5' gRNA 2; pU6-29::FER 3' gRNA 2	This study
pGreen-IIS – Cterm GFP	[8]
pGWB433	[9]

pGADT7	Clontech
pGBKT7	Clontech
pAH21\GW	Dr. M. Butenko
pBEE401E	Prof. D. Goring [10]
pCBC-DT1T2	Prof. D. Goring [10]

References

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2. Duan Q, Kita D, Johnson EA, Aggarwal M, Gates L, Wu HM, Cheung AY (2014) Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in Arabidopsis. *Nat Commun* **5:** 3129

3. Tsukamoto T, Qin Y, Huang YD, Dunatunga D, Palanivelu R (2010) A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development. *Plant Journal* **62:** 571-588

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7. Lindner H, Kessler SA, Muller LM, Shimosato-Asano H, Boisson-Dernier A, Grossniklaus U (2015) TURAN and EVAN mediate pollen tube reception in Arabidopsis Synergids through protein glycosylation. *PLoS Biol* **13:** e1002139

8. Mathieu J, Warthmann N, Kuttner F, Schmid M (2007) Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. *Current Biology* **17:** 1055-1060

9. Nakagawa T, Suzuki T, Murata S, Nakamura S, Hino T, Maeo K, Tabata R, Kawai T, Tanaka K, Niwa Y*, et al.* (2007) Improved Gateway Binary Vectors: High-Performance Vectors for Creation of Fusion Constructs in Transgenic Analysis of Plants. *Bioscience, Biotechnology, and Biochemistry* **71:** 2095-2100

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