EMBO reports

*Cr*RLK1L receptor-like kinases HERK1 and ANJEA are female determinants of pollen tube reception

Sergio Galindo-Trigo, Noel Blanco-Touriñán, Thomas A. DeFalco, Eloise S. Wells, Julie E Gray, Cyril Zipfel, Lisa M Smith

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 June 2019

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the interest of your findings and consider the data overall solid and convincing. Nevertheless, all three referees also have a number of suggestions for how the study should be strengthened, which should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7)) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

9) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

The manuscript "CrRLK1L receptor-1 like kinases HERCULES RECEPTOR KINASE 1 and ANJEA are female determinants of pollen tube reception" is adding novel aspects of the highly interesting gene family of the CrRLK1L gene family. It adds the importance of the redundantly acting HERK1 and ANJ for fertilization on the female side. The authors nicely combine genetics with (quantitative) cell biology, biochemistry (protein-protein interaction assays) and physiology (ROS assays). With these experimental approaches the authors could show the HERK1 and ANJ localization in synergids and there the filiform apparatus. Most of the phenotypes (reduced seed set, pollen overgrowth, NTA localization after fertilization) of the HERK1/ANJ double mutants are similar to the well characterized FER mutants. However the ROS level was not affected in HERK1/ANJ double mutants, which is different from FER mutants where the ROS level is lower in ovules upon fertilization. The authors also report conflicting to published data on the interdependence of FER, LRE, NTA regarding subcellular localization in the synergids and present convincing quantitative data on the independence of HERK1/ANJ and LRE for the localization of FER, LRE, HERK1, ANJ and NTA in unfertilized ovules. Finally the authors include some experiments to assess protein-protein interactions between HERK1, ANJ, FER and LRE und two heterologous systems - yeast to hybrid - with truncated versions of the proteins and in Nicotiana benthamiana leaves transiently expressing full length and GFP or HA-tagged proteins. The interaction between HERK1 and FER was shown in Arabidopsis seedlings expressing HERK1-GFP and using a FER specific antibody. These analyses let the authors to conclude that the main interaction platform are the extracellular domains (although the kinase domains in the yeast two hybrid systems exhibited some weak interactions too) there might be two complexes one with ANJ, FER, LRE and the other with HERK1, FER, LRE. Here I cannot follow the rational why such a complex could not consist of all the four proteins ANJ, HERK1, FER and LRE. In the supplemental material the authors provide genetic evidence that the kinase dead versions of HERK1 and ANJ are still able to rescue a phenomenon which has been published earlier for the CrRLK1L family.

Here are some suggestions how to further improve the manuscript.
- I would recommend including a model on the two additional players in female dependent fertilization.
- Cr in CrRLK1L should be written italic - comes from Catharantus roseus
- distance between number and dimension checked
- primer for FER expression would result in a large fragment of 1542 bp - but on the agarose gel it does not look like this size. FER RT-PCR rv is a forward primer in relation to the orientation of the FER gene and FER RT-PCR fw a reverse one.
- where is the primer position for expression analyses in relation to the T-DNA insertion? Is there any expression behind the T-DNA insertion comprising part of the ECD, exJM, TM and kinase domain?
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- Figure 5C - loading control of ANJ-GFP not visible and pattern in the IP is very similar to the HERK1-GFP lane. Why is in the last lane the GFP signal of the negative control Lti6b-GFP higher than in the input?
- Do you can anticipate a reason why FER and HERK1 can from homodimers and ANJ not? ANJ seems to have a generally a weaker interaction. Where the protein in yeast similarly expressed?
- a sentence would be nice to explain why the pFER::HERK1-GFP is expected to behave like a pHERK1::HERK1-GFP line

Referee #2:

Fine-tuning regulation of each steps during double fertilization is essential for success in fertilization and thus for the successful domination of angiosperms on the earth. Complex communications between male and female tissues heavily control these fine-tuning regulations, in which many receptor-like kinases from CrRLK1L family have been reported playing critical roles. The manuscript by Galindo-Trigo et al. revealed a new functional role of HERK1, a previously reported CrRLK1L receptor, in pollen tube reception in cooperation of another CrRLK1L receptor ANJEA (ANJ), through forming a receptor complex with FERONIA, which is very much similar to the CrRLK1L receptor complex BUPS1/2-ANX1/2 previously reported to be formed in pollen tubes. The authors carefully examined the loss-of-function mutants of HERK1 and ANJ for the developmental defects, compared them with the previously reported mutants fer and lre, and the protein localization and interactions in between receptors and other assisting components. In the end they came to a conclusion that more than one CrRLK1L receptors (i.e., HERK1, ANJEA and FER), with the assistance of GPI-APs (i.e., LORELEI), participate in controlling pollen tube reception. This study has provided solid evidence to support their proposed working model, revealing the flexible formation of different CrRLK1L receptor complexes for different developmental processes in different tissues. It is a competent story worthy of being published in EMBO Reports. Here I only have some suggestions for further improvement of the manuscript.

Major suggestions:

1. Although the authors showed that two more CrRLK1L receptors HERK1/ANJ associate with FER, forming a FER-HERK1/ANJ complex with LRE, in synergid cells for pollen tube reception, the idea that a CrRLK1L receptor complex is formed had been proposed in pollen tubes, in which pollen tube rupture is controlled (Ge et. al., Science 2017). The similarity between these two receptor complexes needs to be discussed more intensively.
2. About the definition of LRE-FER-HERK1/ANJ's role in pollen tube reception. Because lre, fer and anj herk1 mutants all exhibited pollen tube overgrowth phenotype, I assume that the affected event was before the pollen tube rupture and sperm release (see Line 388-399 and Line 440-441). Therefore, I would suggest to use "pollen tube arrest" or "pollen tube reception" to replace "pollen tube burst" throughout the text.
3. Line 173-177: The structure evidence is not sufficient to show the inactivity of the mutated kinase domains. Probably need to do some experiments to confirm the kinase activity.
4. Line 191-193: in Figure S5A-D, HERK1-GUS signals in ovary walls are not clearly shown, and in Figure S5F ANJ-GUS signals are evident as spots in the silique. I suggest using zoom-in images to show detailed expression pattern of ANJ and HERK1, as these two proteins are supposed to function in those places.
5. In Figure 5C, the level of ANJ-GFP was a little too low in the input.
6. Figure S12, the genotypes of FER at DNA level are needed for the CRISPR/Cas9-generated fer mutants for readers to better understand the mutation types.
7. It would be much clearer if they could make a graphical model to describe the roles of the two receptors in pollen tube reception.

Minor suggestions:

1. Figure 1A, herk1 single mutant seems to produce shorter siliques and less seeds, any explanation?
2. Please add some general information of HERK1 in the introduction because it has been described previously (Guo et al., PNAS. 2009).
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4. The format of the citations are wrong in Line 82-83, two groups of citations, should merge into the same one group.
5. Line 90-93, I think the original description of LRE function in pollen tube reception should be cited here (Capron et. al., Plant J. 2008).
6. Figure 5C-D: 'CBB' in the legend needs explanation.
7. Line 177-178, I suggest to remove "cellular localisation", since the information of cellular localization is in the next part.
8. In the text, it seems there is no citations for Figure S8B and Figure S9.
9. In the introduction, please cite the most recent reviews about fertilization and CrRLK1L receptors (Johnson et al., Annu. Rev. Plant Biol. 2019; Zhong et al., Curr. Opin. Plant Biol. 2019; Ge et al., New Phytol. 2019; Franck et al., Annu. Rev. Plant Biol. 2018).

Referee #3:

The authors have performed a convincing set of experiments to show that two closely related CrRLKs1L HERK1 and ANJ interact with FER in a complex to mediate female-male gametophyte interaction during fertilization events. They have used a screen for unfertilized ovules in stable double homozygous mutants in different combinations of CrRLK1Ls and identified the double mutant in HERK1 and ANJ. No further developmental defects were found in the double mutant. Phenotyping of the single mutants showed that both acted in a redundant way. Pollen tube staining and reciprocal crosses showed that both HERK1 and ANJ acted on the female side of the fertilization event. Reintroduced tagged proteins rescued the phenotype in the mutants, which proved that they were active protein-fusions and the knocked-out genes were responsible for the observed phenotypes. As for FER, the kinase domain of both proteins was not required for their function in fertilization. Transcriptional fusions showed specific expressions in unfertilized ovules, whereas translational fusions showed that the proteins strongly localized to the filiform apparatus of the synergid cells. Elegant experiments where translational fusion lines were introduced and analysed in mutants showed that the correct localisation of FER, LRE, HERK1, ANJ and NTA was not dependent on the presence of HERK1/ANJ, yet, NTA relocalisation upon pollen tube perception was disturbed in herk1anj double mutants. The ROS-sensitive dye H2DCF-DA was used to show that ROS production in herk1anj ovules was not affected. A complementary set of experiments then showed interactions between HERK1-ANJ-FER-LRE and showed that they could form a receptor complex in the filiform apparatus of the synergid cells to mediate pollen tube perception.

The experiments seem to be conducted in a very solid and reliable way, including all proper controls. I have nevertheless some remarks.

1. Given the importance of the ECD, how sure are the authors that herk1 is in fact a knock-out mutant? Was it verified whether the ECD was expressed or not as the T-DNA inserted after the ECD-sequence (Fig. S1A)?
2. Figure 1: Only the rates of unfertilized ovules or aborted seeds were shown for WT and HERK1 and ANJ mutants. Would it be possible to include the data for the other CrRLK1L mutant combinations or single mutants as well? Are other combinations showing a similar response?
3. Was growth of pollen tubes analysed in detail in the mutants as HERK1 seems expressed in them?
4. Figure 3B: In the experiment where NTA relocalisation was examined in the herk1anj background, how can one be sure that the ovule was fertilised? It is not clear on the image where the pollen tube was drawn whether it entered or grew behind (as this is exactly the problem with this double mutant).
5. The Yeast and Co-IP work convincingly showed potential interactions between HERK1-LRE-FER-ANJ. Is it possible to show this using super resolution imaging or TEM?
6. In the very thorough discussion, it is suggested that FER and the other CrRLK1Ls HERK1 and ANJ probably bind similar ligands (pectins/RALFs) through their ECD. However, if they do so, naively one would say they should act redundantly, which they clearly don't do. If the kinase domain is not needed for the described responses in this paper, how can different downstream signals be generated as they all work in a complex? Can this be discussed?
7. Several figures seem to have very faint grey squares around them, at least in my pdf version.

1st Revision - authors' response 20 September 2019

Referee #1:

The manuscript "CrRLK1L receptor-1 like kinases HERCULES RECEPTOR KINASE 1 and ANJEA are female determinants of pollen tube reception" is adding novel aspects of the highly interesting gene family of the CrRLK1L gene family. It adds the importance of the redundantly acting HERK1 and ANJ for fertilization on the female side. The authors nicely combine genetics with (quantitative) cell biology, biochemistry (protein-protein interaction assays) and physiology (ROS assays). With these experimental approaches the authors could show the HERK1 and ANJ

localization in synergids and there the filiform apparatus. Most of the phenotypes (reduced seed set, pollen overgrowth, NTA localization after fertilization) of the HERK1/ANJ double mutants are similar to the well characterized FER mutants. However the ROS level was not affected in HERK1/ANJ double mutants, which is different from FER mutants where the ROS level is lower in ovules upon fertilization. The authors also report conflicting to published data on the interdependence of FER, LRE, NTA regarding subcellular localization in the synergids and present convincing quantitative data on the independence of HERK1/ANJ and LRE for the localization of FER, LRE, HERK1, ANJ and NTA in unfertilized ovules. Finally the authors include some experiments to assess protein-protein interactions between HERK1, ANJ, FER and LRE und two heterologous systems - yeast to hybrid - with truncated versions of the proteins and in Nicotiana benthamiana leaves transiently expressing full length and GFP or HA-tagged proteins. The interaction between HERK1 and FER was shown in Arabidopsis seedlings expressing HERK1-GFP and using a FER specific antibody. These analyses let the authors to conclude that the main interaction platform are the extracellular domains (although the kinase domains in the yeast two hybrid systems exhibited some weak interactions too) there might be two complexes one with ANJ, FER, LRE and the other with HERK1, FER, LRE. Here I cannot follow the rational why such a complex could not consist of all the four proteins ANJ, HERK1, FER and LRE. In the supplemental material the authors provide genetic evidence that the kinase dead versions of HERK1 and ANJ are still able to rescue a phenomenon which has been published earlier for the CrRLK1L family.
Authors' response: We thank the reviewer for their positive comments and address their specific comments below.

Here are some suggestions how to further improve the manuscript.

- I would recommend including a model on the two additional players in female dependent fertilization.
Authors' response: Thank you for your suggestion. We have now included a model as Figure 6, which includes possible scenarios for FER kinase activity being required for pollen tube reception in the absence of HERK1 and ANJ kinase activity.

- Cr in CrRLK1L should be written italic - comes from Catharantus roseus
Authors' response: Thank you for pointing this out. This has now been corrected throughout the manuscript.

- distance between number and dimension checked
Authors' response: Thank you – we have now checked that we are consistent in spacing between numbers and dimensions including molarity (space before), percentages (no space) and °C (no space).

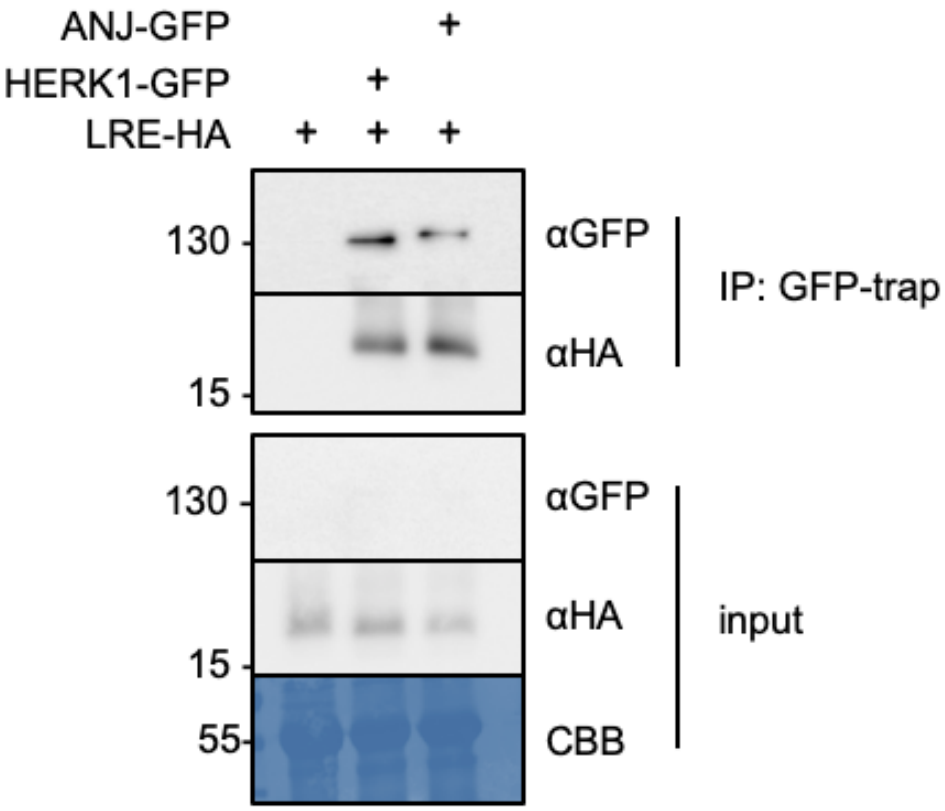
- primer for FER expression would result in a large fragment of 1542 bp - but on the agarose gel it does not look like this size. FER RT-PCR rv is a forward primer in relation to the orientation of the FER gene and FER RT-PCR fw a reverse one.
Authors' response: You are correct that the orientation was reversed in relation to the orientation of the gene, therefore we have reversed the labelling of the primers to make this clearer. We have double checked where the primers bind using Primer-BLAST at NCBI. The forward primer FER RT-PCR fw matches from 1618 nt and FER RT-PCR rv from 2076 nt, giving a product of 459 bp. This was therefore consistent with the agarose gel image. However, as we have now added qPCR data into the manuscript, we have removed the RT-PCR data.

- where is the primer position for expression analyses in relation to the T-DNA insertion? Is there any expression behind the T-DNA insertion comprising part of the ECD, exJM, TM and kinase domain?
Authors' response: We have now added RT-qPCR expression that shows that there no expression of ANJ either 5' or 3' of the T-DNA. We have however found that *herk1-1* still produces transcripts both 5' and 3' of the T-DNA insertion and added this and our interpretation thereof into the manuscript. Primer positions are now indicated

in Figure EV1C.

- Line 314 - with both HERK1-GFP probably independent transformants?
Authors' response: Apologies for the error. This is indeed the correct interpretation and has been corrected.

- Figure 5C - loading control of ANJ-GFP not visible and pattern in the IP is very similar to the HERK1-GFP lane. Why is in the last lane the GFP signal of the negative control Lti6b-GFP higher than in the input?
Authors' response: Unfortunately, ANJ-GFP accumulates to very low levels when transiently expressed in *Nicotiana benthamiana* (particularly in comparison to our negative control Lti6b-GFP), and thus can only be detected in the input with a very long exposure; however, it can be detected more easily after enrichment by IP. We have repeated this assay in triplicate and see ANJ-LRE interactions each time. Please see below for one of the other repeats where accumulation of both HERK1-GFP and ANJ-GFP was extremely low in the input (to the point of not being detectable), and yet the interaction with LRE-HA in the immunoprecipitation was clear.



The GFP signal from the GFP-trap IP is higher than in the input as the immunoprecipitation has concentrated the GFP-tagged proteins. The Lti6b-GFP control expresses to a much higher level than HERK1 or ANJ, and thus the signal is generally higher. Apparent higher MW bands of the Lti6b-GFP control in the IP are very likely artefacts simply due to the high amount of loaded protein and exposure (required to detect the ANJ-GFP band on the same blot).

- Do you can anticipate a reason why FER and HERK1 can from homodimers and ANJ not? ANJ seems to have a generally a weaker interaction. Where the protein in yeast similarly expressed?
Authors' response: We have now added in a Western blot that shows that ANJ is less strongly expressed in yeast (Figure S9A), which likely explains the weaker

interactions seen with ANJ. It is therefore possible that ANJ does form homodimers in yeast but we haven't been able to confirm this due to lower expression.

- a sentence would be nice to explain why the pFER::HERK1-GFP is expected to behave like a pHERK1::HERK1-GFP line
Authors' response: We have now added in a sentence to explain our reasoning here: *"FERONIA*'s promoter presents a broad expression pattern in ovules [27], and given the maternal origin of the reproductive defect in *herk1 anj* plants, we decided to use *pFER::HERK1-GFP* to test for complementation."

Referee #2:

Fine-tuning regulation of each steps during double fertilization is essential for success in fertilization and thus for the successful domination of angiosperms on the earth. Complex communications between male and female tissues heavily control these fine-tuning regulations, in which many receptor-like kinases from CrRLK1L family have been reported playing critical roles. The manuscript by Galindo-Trigo et al. revealed a new functional role of HERK1, a previously reported CrRLK1L receptor, in pollen tube reception in cooperation of another CrRLK1L receptor ANJEA (ANJ), through forming a receptor complex with FERONIA, which is very much similar to the CrRLK1L receptor complex BUPS1/2-ANX1/2 previously reported to be formed in pollen tubes. The authors carefully examined the loss-of-function mutants of HERK1 and ANJ for the developmental defects, compared them with the previously reported mutants fer and lre, and the protein localization and interactions in between receptors and other assisting components. In the end they came to a conclusion that more than one CrRLK1L receptors (i.e., HERK1, ANJEA and FER), with the assistance of GPI-APs (i.e., LORELEI), participate in controlling pollen tube reception. This study has provided solid evidence to support their proposed working model, revealing the flexible formation of different CrRLK1L receptor complexes for different developmental processes in different tissues. It is a competent story worthy of being published in EMBO Reports. Here I only have some suggestions for further improvement of the manuscript.
Authors' response: We thank the reviewer for their favourable summary of our manuscript and address each of their points below.

Major suggestions:

1. Although the authors showed that two more CrRLK1L receptors HERK1/ANJ associate with FER, forming a FER-HERK1/ANJ complex with LRE, in synergid cells for pollen tube reception, the idea that a CrRLK1L receptor complex is formed had been proposed in pollen tubes, in which pollen tube rupture is controlled (Ge et. al., Science 2017). The similarity between these two receptor complexes needs to be discussed more intensively.
Authors' response: We thank the reviewer for the suggestion. We have added an additional paragraph to the discussion to cover the recent *Cr*RLK1L structural studies and have further strengthened the links between the pollen tube growth and pollen tube reception mechanisms throughout the discussion.

2. About the definition of LRE-FER-HERK1/ANJ's role in pollen tube reception. Because lre, fer and anj herk1 mutants all exhibited pollen tube overgrowth phenotype, I assume that the affected event was before the pollen tube rupture and sperm release (see Line 388-399 and Line 440-441). Therefore, I would suggest to use "pollen tube arrest" or "pollen tube reception" to replace "pollen tube burst" throughout the text.
Authors' response: Thank you for the suggestion. We have now made this replacement throughout the text as appropriate. There are occasions where we have been observing pollen tube burst, therefore have kept this nomenclature where appropriate.

3. Line 173-177: The structure evidence is not sufficient to show the inactivity of the mutated kinase domains. Probably need to do some experiments to confirm the kinase activity.
Authors' response: We have now included an *in vitro* kinase assay (Figure EV2A) that confirms the inactivity of the mutated cytosolic domains, both in autophosphorylation and in transphosphorylation (using myelin basic protein as a generic substrate). Wild-type HERK1 was included, along with wildtype BAK1 and BAK1 kinase dead as controls, but unfortunately wild-type ANJ failed to clone in multiple attempts therefore was unable to be included in this assay.

4. Line 191-193: in Figure S5A-D, HERK1-GUS signals in ovary walls are not clearly shown, and in Figure S5F ANJ-GUS signals are evident as spots in the silique. I suggest using zoom-in images to show detailed expression pattern of ANJ and HERK1, as these two proteins are supposed to function in those places.
Authors' response: We have now included two further panels in Figure S5 (now Appendix Figure S5) to show more detail of the silique.

5. In Figure 5C, the level of ANJ-GFP was a little too low in the input.
Authors' response: (see also response to Reviewer #1 above) Unfortunately, ANJGFP is poorly accumulated when transiently expressed in *Nicotiana benthamiana* therefore the signal is weak despite the maximum volume possible being loaded into the well for the input western. This assay has been repeated three times and this was the repeat with the highest expression of ANJ-GFP so was selected for Figure 5C.

6. Figure S12, the genotypes of FER at DNA level are needed for the CRISPR/Cas9-generated fer mutants for readers to better understand the mutation types.
Authors' response: We have now included the genotyping data for each CRISPR line in Figure S12 (now Figure EV5).

7. It would be much clearer if they could make a graphical model to describe the roles of the two receptors in pollen tube reception.
Authors' response: Thank you for the suggestion. We have now added in a model as Figure 6 and included a description of the model in the discussion.

Minor suggestions:

1. Figure 1A, herk1 single mutant seems to produce shorter siliques and less seeds, any explanation?
Authors' response: This was simply an artefact of the silique photos that we had chosen to include, where the *herk1-1* silique had towards the lower end of the normal range of seeds (and length is closely related to number of seeds). We have therefore replaced the photos with siliques that are more representative (i.e. middle of the normal range of number of seeds).

2. Please add some general information of HERK1 in the introduction because it has been described previously (Guo et al., PNAS. 2009).
Authors' response: We have now added a short paragraph into the introduction on the roles of *Cr*RLK1L receptors beyond fertilisation, however we feel a more extensive treatment of the other roles of *Cr*RLK1L receptors would detract from our focus on fertilisation.

3. Line 71-74, LURE should be "AtLURE1", the attractants in Arabidopsis is specifically named as AtLURE1. There is newly published Science paper about the AtLURE1 attractants and their receptor PRK6, I think it should be cited here.
Authors' response: Thank you for the suggestions. We now make reference to AtLURE1 and have added in the Science paper.

4. The format of the citations are wrong in Line 82-83, two groups of citations, should merge into the same one group.
Authors' response: This has now been corrected.

5. Line 90-93, I think the original description of LRE function in pollen tube reception should be cited here (Capron et. al., Plant J. 2008).
Authors' response: Thank you for picking up this omission. We have now added the citation.

6. Figure 5C-D: 'CBB' in the legend needs explanation.
Authors' response: This has now been added in to the legend of Figure 5.

7. Line 177-178, I suggest to remove "cellular localisation", since the information of cellular localization is in the next part.
Authors' response: Thank you for the suggestion. This has now been removed.

8. In the text, it seems there is no citations for Figure S8B and Figure S9.
Authors' response: Thank you for picking up on this error. Many figures have now been renumbered or assigned to the Expanded View format and we believe the citations of the figures are now complete.

9. In the introduction, please cite the most recent reviews about fertilization and CrRLK1L receptors (Johnson et al., Annu. Rev. Plant Biol. 2019; Zhong et al., Curr. Opin. Plant Biol. 2019; Ge et al., New Phytol. 2019; Franck et al., Annu. Rev. Plant Biol. 2018).
Authors' response: Thank you for the suggestion. We now cite Franck in the introduction as a recent comprehensive review of *Cr*RLK1L receptors. Given the very high number of recent reviews of this topic we feel it would be inappropriate to cite all.

Referee #3:

The authors have performed a convincing set of experiments to show that two closely related CrRLKs1L HERK1 and ANJ interact with FER in a complex to mediate female-male gametophyte interaction during fertilization events. They have used a screen for unfertilized ovules in stable double homozygous mutants in different combinations of CrRLK1Ls and identified the double mutant in HERK1 and ANJ. No further developmental defects were found in the double mutant. Phenotyping of the single mutants showed that both acted in a redundant way. Pollen tube staining and reciprocal crosses showed that both HERK1 and ANJ acted on the female side of the fertilization event. Reintroduced tagged proteins rescued the phenotype in the mutants, which proved that they were active protein-fusions and the knocked-out genes were responsible for the observed phenotypes. As for FER, the kinase domain of both proteins was not required for their function in fertilization. Transcriptional fusions showed specific expressions in unfertilized ovules, whereas translational fusions showed that the proteins strongly localized to the filiform apparatus of the synergid cells. Elegant experiments where translational fusion lines were introduced and analysed in mutants showed that the correct localisation of FER, LRE, HERK1, ANJ and NTA was not dependent on the presence of HERK1/ANJ, yet, NTA relocalisation upon pollen tube perception was disturbed in herk1anj double mutants. The ROS-sensitive dye H2DCF-DA was used to show that ROS production in herk1anj ovules was not affected. A complementary set of experiments then showed interactions between HERK1-ANJ-FER-LRE and showed that they could form a receptor complex in the filiform apparatus of the synergid cells to mediate pollen tube perception.
The experiments seem to be conducted in a very solid and reliable way, including all proper controls. I have nevertheless some remarks.
Authors' response: We thank the reviewer for their generous evaluation of our study. Please find a point-by-point response to your specific queries below.

1. Given the importance of the ECD, how sure are the authors that herk1 is in fact a knock-out mutant? Was it verified whether the ECD was expressed or not as the TDNA

inserted after the ECD-sequence (Fig. S1A)?
Authors' response: We have now added RT-qPCR expression that shows *herk1-1* still produces transcripts both 5' and 3' of the T-DNA insertion and added this and our interpretation thereof into the manuscript. Primer positions are now indicated in Figure EV1C. This is an interesting finding given the previous evaluation of the same *herk1* allele as a knockout mutant by Guo *et al.* PNAS 2009, Figure 4C.

2. Figure 1: Only the rates of unfertilized ovules or aborted seeds were shown for WT and HERK1 and ANJ mutants. Would it be possible to include the data for the other CrRLK1L mutant combinations or single mutants as well? Are other combinations showing a similar response?
Authors' response: We apologise if our wording was misleading – this was a qualitative screen and we don't show rates of unfertilised ovules or aborted seeds anywhere in the manuscript (including for *herk1 anj*). We have made it clearer in the results that this was a qualitative screen:
"Stable double homozygous lines were qualitatively examined for fertility. Through this screen, we identified that double mutants in *HERCULES RECEPTOR KINASE 1 (HERK1)* and *AT5G59700* (hereafter referred to as *ANJEA/ANJ*) have high rates of unfertilised ovules or seeds that abort very early in development, and shorter siliques (Figure 1A). The qualitative nature of our preliminary screen for fertility defects in *Cr*RLK1L mutants does not preclude the involvement of additional *Cr*RLK1Ls in reproduction as quantitative investigation may uncover more subtle fertility defects among the mutants of this family of receptors."
We instead present in the manuscript counts of normal seeds, and rates of pollen tube overgrowth. We have now also measured the rates of pollen tube overgrowth in *lre-5*, *fer-4*, triple *herk1 anj fer* and quadruple *herk1 anj fer lre* mutants and included this data in Figure EV5 and Figure 4.

3. Was growth of pollen tubes analysed in detail in the mutants as HERK1 seems expressed in them?
Authors' response: HERK1 may be expressed in pollen tubes, however we saw no defects in pollen tube growth by aniline blue staining (Appendix Figure S2), and there was no pollen tube reception defect evident when *herk1-1 anj-1* was used as the male parent in crosses (Figure 1D). Given the functional redundancy between *Cr*RLK1L receptors, it is possible that HERK1 may act with an as yet unidentified *Cr*RLK1L receptor in pollen tube growth, however that is beyond the scope of this study.

4. Figure 3B: In the experiment where NTA relocalisation was examined in the herk1anj background, how can one be sure that the ovule was fertilised? It is not clear on the image where the pollen tube was drawn whether it entered or grew behind (as this is exactly the problem with this double mutant).
Authors' response: In Figure 3B, we have imaged ovules around the time of pollen tube reception, when NTA is normally relocalised. We are interested in the time point here of reception, when the pollen tube has just entered the micropyle, rather than pollen tube burst or fertilisation which occur a bit later. If the pollen tube had not entered the micropyle, we would not be able to see it in the same imaging plane as the synergid cell. The issue with the double mutant is that after pollen tube reception, the pollen tube doesn't just grow a bit more before bursting but continues to grow within the ovule, it is not that it does not enter the ovule. The later stages of pollen tube burst or overgrowth are shown in Appendix Figure S6.

5. The Yeast and Co-IP work convincingly showed potential interactions between HERK1-LRE-FER-ANJ. Is it possible to show this using super resolution imaging or TEM?
Authors' response: This would indeed be possible, although beyond the scope of the current manuscript. We have acknowledged the potential use of Cryo-EM and superresolution imaging by adding the following sentence in to the discussion.
"Further verification of the protein-protein interactions described here could be done via Förster Resonance Energy Transfer (FRET) analysis, cryo-electron microscopy

[58], or super-resolution microscopy techniques such as Stimulated Emission-Depletion Measurements (STED; [59]).”

6. In the very thorough discussion, it is suggested that FER and the other CrRLK1Ls HERK1 and ANJ probably bind similar ligands (pectins/RALFs) through their ECD. However, if they do so, naively one would say they should act redundantly, which they clearly don't do. If the kinase domain is not needed for the described responses in this paper, how can different downstream signals be generated as they all work in a complex? Can this be discussed?
Authors’ response: We have now added in a model as Figure 6 and included a description of the model in the discussion that covers how signalling could work in the absence of kinase activity from either HERK1 or ANJ.

7. Several figures seem to have very faint grey squares around them, at least in my pdf version.
Authors’ response: We have also observed this sometimes on pdfs and the grey squares disappear when the zoom level changes and are not printed. We have tried to replace any affected panels (that we have identified) to stop this from appearing.

2nd Editorial Decision 4 November 2019

Thank you for the submission of your revised manuscript to EMBO reports. It was sent back to former referee 1 and 3 and we have now received their reports (copied below).

As you will see, both referees are very positive about the study and support publication in EMBO reports. Please note that your manuscript will be published as "Article" and it is therefore not necessary to shorten the text. Moreover, per our editorial policies all methods must remain part of the manuscript.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

- Thank you for supplying source data. This is much appreciated but please note that we need one source data file per figure. Therefore, please combine all excel files into one file per figure with different sheets showing the data for the individual panels and do the same for the powerpoint files.

- Source data: I noticed inconsistent labeling of plates in the source data for Figure 5B ("-BD control": bottom-left sector states LRE (23-138)-BD on slide 2, while the same sector is labeled as -BD control in slide 1. Please double-check.

- The Source data for Fig. EV4A, B does not match the figure. Please correct and please also make sure that all source data refers to the correct panels.

- Our data editors from Wiley have already inspected the Figure legends for completeness and accuracy. Please see the required changes in the attached Word file. I have also taken the liberty to make a small change to the Title, which may not exceed 100 characters (incl. spaces).

- Figure EV5C: in the column "Change in FER mRNA" you refer the reader to panel E in two cases but panel E does not give information on FER mRNA. Could you please double-check whether this information is correct?

- Appendix Figure S1: Please clarify whether the definition of the scale bar size applies to all panels or only to panel G.

- Appendix figure S5: Panel B and G are not specified in the legend. Please double-check this legend for accuracy.

- Please remove all figures from the manuscript text when you upload the final version. The

manuscript .docx file should only contain text. All figures are supplied as separate files.

We look forward to seeing a final version of your manuscript as soon as possible.

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REFEREE REPORTS

Referee #1:

The authors answered all my questions and included now more data. My suggestions for shortening would transfer the methods for the expanded views into the supplement (i.e. qPCR, Western blot description for protein expression in the Y2H experiment, recombinant protein expression and kinase assays). Another possibility to shorten the manuscript would reduce the description of future possible experiments in the discussion (i.e. to proof importance of the kinase domain of FER for ROS production).

Referee #3:

In this revised version of the manuscript and the accompanying rebuttal, the authors have done their best to provide answers/explanations to all remarks/criticisms made by the reviewers. Moreover, they have provided additional data that backs up their conclusions and even included a model that should explain the involvement of HERK1 and ANJ in pollen tube reception. One small remark is that it still remains puzzling that the constructs for pHERK::HERK, pFER:HERK-GFP and pHERK::HERK-KD-GFP could be cloned in bacteria and pHERK::HERK-GFP seemed toxic.

2nd Revision - authors' response 8 November 2019

The authors performed all minor editorial changes.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Lisa Smith
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48466

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	A minimum of three biologicial replicates (plants) were used for each analysis. Sample sizes are indicate either on each figure or in the associated figure legend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples were only discarded from the pollen tube overgrowth analysis if aniline blue stained siliques were unable to be scored due to poor uptake of stain, poor fertilisation, or presence of so many pollen tubes that pollen tube overgrowth could not be confidently scored. Ovules with visible damage due to sample preparation were also excluded from any quantification in the ROS assays and in the localisation of fluorescent proteins. Two T1 lines were excluded from the study due to non-complementation of the seed set phenotype, presumably due to non-expression or silencing of the genes encoded on the constructs.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Where possible, quantification and pollen tube overgrowth scoring were done with blinding of the investigator.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests were selected based on sample size and parameters. Student's t tests and Chi-squared tests were mostly used. One-way ANOVA analysis followed by Bonferroni's post-hoc comparison of means was used when comparing more than five samples
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Where data was non-parametric (e.g. catagorical), chi-square tests were used instead of Student's t-tests.

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Is there an estimate of variation within each group of data?	Standard deviations are included for bar charts as appropriate.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	A few antibodies were used in the study and the details are given in the methods: anti-FER (rabbit polyclonal; Xiao et al., 2019) and anti-Rabbit IgG (whole molecule)–HRP (Sigma A0545). : α-GFP-HRP (B-2, sc-9996, Santa Cruz); α-HA-HRP (3F10, Roche).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Our protein-protein interaction data is being submitted to the EBI IntAct database.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	See above. Raw datasets are also being provided as source file data.
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA