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Plant immunity suppression via PHR1-RALF-FERONIA shapes the root microbiome to alleviate phosphate starvation

Jing Tang, Dousheng Wu, Xiaoxu Li, Lifeng Wang, Ling Xu, Yi Zhang, Fan Xu, Hongbin Liu, Qijun Xie, Shaojun Dai, Devin Coleman-Derr, Sirui Zhu, and Feng Yu
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Corresponding author: Feng Yu (feng_yu@hnu.edu.cn)

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **31st Jul 2021**

Dear Prof. Yu,

Thank you again for the submission of your manuscript entitled "PHR1 suppresses plant immunity to shape root microbiome through the RALF-FERONIA complex under PSR" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, referees are in general rather positive towards your work but point out to a variety of concerns that will need to be addressed before your manuscript can be published in The EMBO Journal. These concerns not only refer to experimental issues, but they are also related to the way the data are presented.

Based on the overall interest expressed in the reports, I would like to invite you to address the comments of all referees in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve all main concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but we are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, so please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

David

David del Alamo, PhD.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

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 response 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 (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed

under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can 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 or a single pdf per main figure 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 at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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: .

- Additional Tables/Datasets should be labelled 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.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. For additional details, please visit .

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 29th Oct 2021:

Link Not Available

Referee #1:

This is a comprehensive and thoroughly performed work and it is well illustrated. Provides a large body of scientific data that are mostly rigorously analyzed and interpreted.
I have some concerns though regarding the plant material analyzed and the generalization at the levels of plant species and organs used in the different assays and discussion. The authors link PHR1-RALF/FER molecular pathway with Pi starvation.

There are differences in immunity between the root and the leaf. The authors used often seedlings or leaf for their immune assays (ROS, MAPK and gene expression) but roots (? Unclear from the description) for the bacterial infection. Can they elaborate on this?
The abstract is too general. The authors have worked with one plant species. Please avoid any generalization at this point, also it took me a while to find out with which organisms the authors worked. As it turned out this is Arabidopsis. So I am not sure that the first sentence of the abstract fits this host because forward genetic screenings are possible in this system.
This is true also for the introduction. The sentence "under phosphate starvation responses (PSRs), plants shape the root microbiome to alleviate the PSRs" is supported by papers that refers all to Arabidopsis. I would therefore be careful in assuming that this is true for all plants, this is valid for Arabidopsis and should be specified. The authors should also specify the plant host in the intro when writing about specific genes and pathways. Please add this information whenever possible.
The results are mainly based on the differences between the responses on High Pi and Low Pi media but it is unclear how the authors have prepared the media, 1/2 MS has 1.25mM Monopotassium phosphate (KH_2PO_4) 170 mg/l inside but the low Pi medium is not further elaborated. Is this a different medium? How is the pH in the two media? Are there other differences beside Pi in nutrients? Can they elaborate on this?
Figure 1A: The author have measured phosphorylation of the MAPKs. This is activation not accumulation, please change also in the main text and intro. How was the activation of the MAPKs in the roots? The authors have used seedlings here.
Figure 1B: How is the ROS production in roots? This is the organ used for the colonization.
Figure 1F: The CFU/seedling at time point 0 is missing (equal inoculation?). Roots and shoots should be analyzed separately or clarified better how the experiment was performed (to avoid leaf contamination).
Line 135: I would refer here to differentially regulated genes involved in immunity which were enriched (to avoid confusion). Also I see up and down regulated genes in Figure 1C, I would be carefully here to conclude that this suggests similar immunity "inhibition"? (Line 141). Rather regulation!
Figures 3C and 3E: The letters shown for the statistical analyses cannot be correct, here something is missing (letters: e and d in ae and ad)?
Figure 3D why the authors used RALF34 here?
Line 225: Is this per root or seedling? (root in the main text and seedling in the figure). This is a bit confusing throughout the paper.
Line269: fer-4 mutants have reduced root hairs. This is a strong root phenotype as far as I recall from Duan et al. 2010 and can of course affect bacterial entry as previously shown.
Line271: Why here the DC3000 has no negative effects on Arabidopsis growth on 1/2 MS which was shown previously in Kong et al., 2020? Can the author elaborate on this? The plants have more lateral roots as possible bacterial entry but they clearly look diseased and smaller than non inoculated plants in Kong et al., I would not call this beneficial effect (see also discussion line 362).
Line 308: Please add: in Arabidopsis.

Additional comments
I would avoid using abbreviations in the title (e.g. PSR).
Line67 remove that before triggered
Line73 remove to before crosstalk
Line77 this is activation not accumulation and ROS is not following the MAPK activation. The description of PTI is not fully correct, please rephrase from line 77 to line 79.
Line90 a ref at the end of the sentence is missing.
Line 177 Pseudomonas does not grow faster under Pi conditions, it forms more colonies or what was here the read out?
Line 128 add in seedlings
Line 129 add in leaves

Referee #2:

It is known that the symbiotic association between plants and beneficial microbes is promoted under phosphate (Pi) starvation conditions. However, a generalized model for the interaction between plant and pathogenic microbes (plant immunity) under Pi-deprived conditions remains established. This study attempts to address an important issue in this direction by integrating PHR1-mediated PSR signaling and RALFs/FERONIA-mediated defense response.

The major limitation of this study is that the central concept appears to contradict current understanding in the field. In addition, several experimental conditions and designs are not described or presented clearly, making it difficult to distinguish between genuine differences from experimental artifacts.

Major concerns:
1 The current understanding suggests that Pi starvation alone (Khan et al. 2016 Plant Physiol) or a combination of Pi starvation and PAMP elicitation promote defense responses (Castrillo et al. 2017 Nature; Morcillo et al. 2020 EMBO J). However, this study observed the opposite (Fig. 1A, 1B, 1E, 1F, S1C). Although PHR1, a key transcriptional activator of PSR, suppresses the immune responses (Castrillo et al., 2017), there is no evidence showing repression of immune responses by Pi starvation. The

enhanced immune responses observed in phr1;phl1 are independent of Pi status (Castrillo et al., 2017). It seems that the authors did not comprehend this notion correctly. Therefore, the first sentence, "To confirm that PSR inhibits the plant immune response, ...", in the Result is inappropriate because the statement "PSR inhibits the plant immune response" has not been established.
2 Throughout the whole manuscript (including the title), authors often stated "under PSR conditions", "during the PSR", "PSR-mediated". These words are confusing. PSR (Pi starvation response) is a collective term to describe overall responses to Pi starvation, including developmental, physiologic, and molecular responses. The authors should use more specific words (for example, Pi starvation) instead.
3 In many figures and supplemental figure legends, they end a sentence "Three biological replicates were evaluated, each yielding similar results." It is unclear if the data shown are from one representative replicate or three replicates. Additional replicates should be shown in the supplement.
4 The citation for RALF microarray data (Fig. 2C) is unclear (line 169). What does the number on the right mean? It looks the changes are negligible (less than two folds?). According to the published dataset (Castrillo et al. 2017), RALF genes are in general unaffected or mildly suppressed under Pi-starvation. The opposite is observed in Fig. 2B. In the CHIP-PCR analysis (Fig. 2G), WT may not be a good control. WT expressing an irrelevant protein tagged with Myc will be better.
5 Fig. S2A and B: are these Pi-starvation/RALF23 differentially expressed genes induced or repressed genes? The authors should indicate them separately. Also, this will be a good chance for the authors to examine how the global immune responses are affected by Pi starvation.
6 Inconsistent bacteria growth assays:
- Usually, fls2 and bak1 mutants are susceptible when grown under nutrient-rich media. However, this is not observed in Fig. 3F.
- The current manuscript indicated that Col-0 is more susceptible under "LP" conditions. The difference in susceptibility is huge in Fig. 1F (>2-orders) but becomes very subtle in Fig. 3C. Also, the presentation of Fig. 1C is unclear. What do the number and "Sig" on the right mean?
7 For the analysis of the rhizosphere microbiome in Fig. 4, the result is skeptical because microorganisms in the inoculum (from local soil) are uncharacterized. And, it is not known how general the result can be applied when different inoculum is used.
8 It is unclear what samples/tissues were analyzed in Fig. 5C. WT? Under HP or LP? Then, what will be the result if phr1 and fer-4 are analyzed? A recent report by Finkel et al. (PLoS Biology 2019, The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response) is a very relevant reference. The authors should discuss it.
9 The authors should elaborate or explain why the root microbiota of fer-4 can improve the growth under LP. What factor can change the root microbiota? Does fer-4 show any PSR? The experiment in Fig. 6D is under HP or LP?

Minor Concerns:
1. Several data need quantification, for example, ROS production (Fig. 1B), change of root morphology (Fig. S8), western blots (Fig. 1A, 2A, 3A, 3B, 3D, S5D, S5E)
2. Line 326, there is no Pi uptake data shown.
3. Line 380, unclear citation.
4. There is no figure title in all the supplemental figures.

Referee #3:

In this research article, authors characterized a molecular mechanism to verify the correlation between plant defense mechanism and Pi-starvation stress response. RALF was activated by PHR1 under Pi-starvation conditions and abolished the complex formation between FLS2 and BAK1 for inhibition of plant defense signaling. The suppression of plant defense mechanism can result in beneficial bacteria enhanced colonization of beneficial bacteria and activation PSI gene expression during Pi-starvation stress. Rhizosphere microbiome, established from FER knock-out mutants, contributed to increase plant growth performance under Pi-starvation conditions. This article includes very interesting molecular pathways to reveal how PHR1 regulates the plant defense mechanism to activate phosphate stress response.

* Major concerns:
1. Authors revealed the role of FER in suppression of plant defense mechanism under Pi-starvation conditions. In the text, they mentioned "Recent studies have highlighted the role of FER in plant immunity regulation. We hypothesized that FER is involved in PSR-mediated immunity suppression". Even though authors provide evidence to support their hypothesis in this manuscript, authors need to explain the logic how they make this hypothesis or why they think FER function in PSR. It is hard to find out the logic why authors chose FER to test its role in PSR.
2. Authors used PHR1-OE to provide an evidence regarding inhibition of flg22-induced FLS2-BAK1 interaction. As control, it is necessary for authors to provide a result of FLS2-BAK1 interaction without flg22 treatment under both Pi-sufficient and starvation conditions. And a complementation assay with PHR1-OE in phr1 mutant background is necessary to confirm whether FLS2-BAK1 formation is restored in phr1 mutant with PHR1 under the control of native PHR1 promoter.
3. Figure 4B, 4D, 4F and 4H showed the role of RALF23 in colonization of Pseudomonas, Bacillus, Geodematophilus and Methylobacillus under Pi-starvation condition. It will be a good information for authors to provide a result of colonization with these bacteria after RALF23 treatment under Pi-sufficient conditions.
4. FER functions in FLS2-BAK1 complex formation and appears to play a role in colonization of beneficial bacteria in Arabidopsis. I presume that complex formation of FLS-BAK1 can be affected under Pi-sufficient and deficient conditions,

depending on the absence/presence of FER. No biochemical result was provided in this manuscript regarding FLS-BAK1 complex formation in fer-4 (mutant) and FER/fer-4 (complemented line) under Pi-sufficient and deficient conditions.

* Minor concerns:
1. Authors got consistent results regarding suppression of plant defense response under Pi-starvation conditions (Fig S1). As the major concept was already reported in Castrillo et al., 2017, it would be better to include the reference in the main text (Line 106 - 119).
2. Authors used DAB staining methods to detect ROS in plants. However, it is hard to see difference between HP and LP or Col-0 and fer-4. Quantification will help to make clear with this result.
3. Authors showed the similar trend (not same) of differential gene expression under Pi-starvation and RAFP23 treated conditions. Authors did not provide the evidence of these gene expression pattern in Pi-sufficient and No RALF23 treated plants.
4. Authors showed repression of flg22-induced FRK1 expression in RALF23-OE under Pi-sufficient and deficient conditions. It would be better to have the result of FRK1 expression in RALF23-OE, without flg22 treatment, under Pi-sufficient and deficient conditions as Appendix figure.
5. Authors need to check the legend of Figure S5. It is hard to understand it due to mismatching the information to figures.
6. In text, authors explained relative bacterial abundance of Fig 4 with "upregulated" for some results. Do you mean enhanced bacterial growth?
7. Figure 5A: It is hard to see plant phenotype, especially fer-4. Authors can magnify each plant in Figure 5A.
8. Line 264 - 266: "both Pto DC3000 and B. subtilis inoculation significantly alleviated Pi-starvation phenotypic characteristics and promoted LR growth of WT plants and phr1 mutants under LP conditions" - For readers who are not working in PSR research, it would be better to explain more about what "alleviated Pi-starvation phenotypic characteristics" authors were observed in the text.
9. Figure S7: Based on the main text, it would be better to change the order of figure sections. For example, Fig S7C, S7E and S7F can be changed to S7B, S7C, and S7D, respectively.
10. Line 380 - 381: I don't find out this citation in the reference.

Thank you and the reviewers for constructive comments that have greatly improved our manuscript during this revision. We have studied reviewer's comments carefully and revised our manuscript according to those comments. Additional experiments were performed according to requirements and we made our point-to-point response as below with our response highlighted in blue. The figure shown in the response to reviewers were named as Fig R + number. The corresponding modifications in the manuscript are marked by yellow highlight. Here we would like to submit our revised manuscript and hope this version will be suitable for publication in *EMBO Journal* now.

Response to Comments of Reviewer #1

I have some concerns though regarding the plant material analyzed and the generalization at the levels of plant species and organs used in the different assays and discussion. The authors link PHR1-RALF/FER molecular pathway with Pi starvation. There are differences in immunity between the root and the leaf. The authors used often seedlings or leaf for their immune assays (ROS, MAPK and gene expression) but roots (? Unclear from the description) for the bacterial infection. Can they elaborate on this?

Response: We appreciate your professional comments and suggestions that help us to improve the manuscript. In the revised manuscript, additional experiments were performed to validate the immune response in roots and we also indicated that the plant species (i.e., *Arabidopsis*) in the revised text. Please see responses below and the revised manuscript for details.

The abstract is too general. The authors have worked with one plant species. Please avoid any generalization at this point, also it took me a while to find out with which organisms the authors worked. As it turned out this is Arabidopsis. So, I am not sure that the first sentence of the abstract fits this host because forward genetic screenings

are possible in this system.

Response: We appreciate this professional comment. We have modified the first sentence of the abstract based on your comments, and please see lines 26-28. We have also stated the plant species *Arabidopsis thaliana* in the abstract (see lines 30, 31).

This is true also for the introduction. The sentence "under phosphate starvation responses (PSRs), plants shape the root microbiome to alleviate the PSRs" is supported by papers that refers all to *Arabidopsis*. I would therefore be careful in assuming that this is true for all plants, this is valid for Arabidopsis and should be specified. The authors should also specify the plant host in the intro when writing about specific genes and pathways. Please add this information whenever possible.

Response: Thanks. We have specified the plant species *Arabidopsis* in the introduction as well as other places of the main text when needed (see lines 58,64,67,74,87,104 and 108).

Major concerns

1.The results are mainly based on the differences between the responses on High Pi and Low Pi media but it is unclear how the authors have prepared the media; 1/2 MS has 1.25mM Monopotassium phosphate (KH_2PO_4) 170 mg/l inside but the low Pi medium is not further elaborated. Is this a different medium? How is the pH in the two media? Are there other differences beside Pi in nutrients? Can they elaborate on this?

Response: We performed phosphate stress treatment assay as described below. Arabidopsis seeds were first grown in 1/2 MS medium for 3 days and then transferred to either 1/2 MS medium containing 1.25 mM Pi (HP) or low-Pi medium containing 10 μM Pi (LP) for another 5 days. The medium formula is the same as previously described (Zheng *et al*, 2019) and the 1/2 MS media is prepared by ourselves using individual chemicals. The standard HP medium was 1/2 MS medium with 1% (w/v) sucrose and 1% (w/v) agarose (Biowest Regular Agarose G-10). For the LP medium,

KH_2PO_4 in the HP medium was replaced with K_2SO_4. The pH was adjusted with 1 M NaOH to 5.8 for both HP and LP media. We have added the detailed information in the Material and Method section (see lines 452-458).

2.Figure 1A: The author has measured phosphorylation of the MAPKs. This is activation not accumulation, please change also in the main text and intro. How was the activation of the MAPKs in the roots? The authors have used seedlings here.

Response: Thanks for your suggestion. We have replaced the word "accumulation" with "activation" in the introduction section and the result section (see lines 81, 115, 134, and 213). For the MAPKs activation in the roots, we have performed new experiments by using Col-0 and *fer-4* mutants roots and the results are similar with what we have observed with seedlings (Fig R1), and we have replaced the Figure 1A data by root data. We also specified the root tissue in the main text (see lines 116, 135).

Figure for referees removed

3.Figure 1B: How is the ROS production in roots? This is the organ used for the colonization.

Response: Thanks for your good comments. We have performed new ROS assay in Col-0 and *fer-4* roots, which is consistent with what we have observed with in leaf tissue. We updated the Figure 1B and Figure 1C with root results. We also updated the text (lines 138-139)

4.Figure 1F: The CFU/seedling at time point 0 is missing (equal inoculation?). Roots and shoots should be analyzed separately or clarified better how the experiment was performed (to avoid leaf contamination).

Response: Thanks for your good comments.

We performed the *Pto* DC3000 infection assay as described below. The *Pto* DC3000 strain was grown overnight in King's B medium (10 g/L proteose peptone, 1.5 g/L anhydrous K2HPO4, 5 g/L MgSO4) with shaking at 28°C. Bacteria were collected and resuspended in deionized water to an OD_{600} = 0.2. Seven-day-old seedlings grown in either HP or LP agar medium were only root soak inoculated with bacterial suspension for 2 min and then transfer to HP or LP medium (only put the roots on the medium and the shoot should stand in the air. One can achieve this by discarding about 1/4 or 1/3 agar medium from one side of the petri dish) without sucrose, and bacterial amount from individual seedlings, which also represent bacterial amount of individual root (what we have sampled in the initial submission) were quantified at 3 days or 5 days after inoculation (dpi). According to your comment, we have

performed new experiments and quantified bacteria in individual root. Twenty-seven seedling roots were collected for each treatment individually and ground with a drill-adapted pestle. Serial dilutions were plated on LB agar and colonies were counted 2 days later. We have replaced the Figure 1G with the new data that also includes the data of time point 0 (Fig R2). We have also updated the text (see lines 163-165).

Figure for referees removed

5. Line 135: I would refer here to differentially regulated genes involved in immunity which were enriched (to avoid confusion). Also, I see up and down regulated genes in Figure 1C, I would be carefully here to conclude that this suggests similar immunity "inhibition"? (Line 141). Rather regulation!

Response: We appreciate your professional comments and suggestions that help us to improve the manuscript. We have changed to “differentially regulated genes” (lines

144-147). We also used “regulate” instead of “inhibition” (line 152).

6. Figures 3C and 3E: The letters shown for the statistical analyses cannot be correct, here something is missing (letters: e and d in ae and ad)?

Response: Thanks for your good comments. We have corrected the labeling of the statistical analysis according to your comments and updated the Figure 3C and 3E (Fig R3).

Figure for referees removed

7. Figure 3D why the authors used RALF34 here?

Response: Thanks for your good comments.

Based on our data in Figure 2, PHR1-regulated RALFs include RALF1, RALF4, RALF23, RALF33 and RALF34. Both RALF34 and RALF23 have been reported to be involved in the regulation of immunity (Stegmann *et al*, 2017), so RALF23 and RALF34 were selected as representative RALFs for testing. We have explained this in

the revised text and please see lines 218-220.

8. Line 225: Is this per root or seedling? (root in the main text and seedling in the figure). This is a bit confusing throughout the paper.

Response: We are sorry for the confusion. We measured the bacterial amount in root tissue. We corrected the description in the Figure 3C and Figure 3E legend.

9. Line269: *fer-4* mutants have reduced root hairs. This is a strong root phenotype as far as I recall from Duan et al. 2010 and can of course affect bacterial entry as previously shown.

Response: Thanks for your good comments.

We agree with you that less root hairs in general will reduce the bacterial entry in the roots. And yes, the *fer-4* mutants have reduced root hairs as Duan *et al*. have shown. However, the colonization amount of *Pto* DC3000 in *fer-4* mutants was significantly higher than of wild-type (Stegmann *et al*, 2017) (Figure 3G), and a recent study also have shown that the root hair deficient phenotype (*ark1-1* mutants) is not related to *Arabidopsis-Pseudomonas* interactions in roots (Song *et al*, 2021). This indicates that under certain cases, the suppression of plant immunity rather than root hair numbers plays a more important role in bacterial growth. To further clarify this, we will do more research to explore the effect of *fer-4* root hair on the growth of different rhizosphere microorganisms in the future.

10. Line271: Why here the DC3000 has no negative effects on Arabidopsis growth on 1/2 MS which was shown previously in Kong et al, 2020? Can the author elaborate on this? The plants have more lateral roots as possible bacterial entry but they clearly look diseased and smaller than non-inoculated plants in Kong et al, I would not call this beneficial effect (see also discussion line 362).

Response: Thanks for your good comments.

Pto DC3000 is a pathogenic bacterium because it can cause wilting in leaves and affect *Arabidopsis* growth. In Kong *et al*, 2020, the authors inoculated the whole

Arabidopsis seedlings including leaves in a medium containing *Pto* DC3000 broth for 5 days. In this case the *Pto* DC3000 will colonize the leaves and cause the disease and thus affect the growth of the plants. In our *Pto* DC3000 inoculation experiment, only the roots of *Arabidopsis* seedlings were inoculated, and we observed no leaf disease phenotype after 4 weeks co-cultivatation. It is possible that *Pto* DC3000 may slightly affect *Arabidopsis* roots at the early inoculation stages. However, the plants might have recovered from early stage stunting caused by *Pto* DC3000 after 4 weeeks co-cultivation, the time point that we chose for phenotype observation. That's why *Pto* DC3000 did not show negative effects on *Arabidopsis* in our experimental system. We have explained this in the revised discussion (lines 417-422).

11. Line 308: Please add: in *Arabidopsis*.

Response: We have added 'in *Arabidopsis*' in the line 353. Thanks.

Minor concerns

1. I would avoid using abbreviations in the title (e.g. PSR).

Response: Thanks for your good comments. We used the abbreviations 'PSR' in the title because the upload system has a limitation of 100 characters for the title. We hope this is fine.

2. Line67 remove that before triggered.

Response: We have removed 'that' before 'triggered' based on your comments, and please see line 70.

3. Line73 remove to before crosstalk.

Response: We have corrected the sentence based on your comments, and please see line 78.

4. Line77 this is activation not accumulation and ROS is not following the MAPK activation. The description of PTI is not fully correct, please rephrase from line 77 to line 79.

Response: We appreciate your professional comments. We have rephrased this sentence based on your comments, and please see lines 79-83.

5. Line90 a ref at the end of the sentence is missing.

Response: Thanks for pointing this out. We have added the references in the revised manuscript (line 95).

6. Line 117 *Pseudomonas* does not grow faster under Pi conditions, it forms more colonies or what was here the read out?

Response: We appreciate your professional comments. *Pseudomonas* grow faster under Pi conditions means it forms more colonies. We compared the colonization in the *Arabidopsis* roots between HP and LP condition as the read out. Statistic data of *Pseudomonas* growth were shown in Appendix Fig S1D and the text was updated, please see lines 122-124.

7. Line 128 add in seedlings.

Response: We have performed new experiments with root tissue and added 'in roots' in lines 138.

8. Line 129 add in leaves.

Response: We appreciate your professional comments. We have performed new experiments with root tissue and added 'in roots' in line 139.

Response to Comments of Reviewer #2

Major concerns

1. The current understanding suggests that Pi starvation alone (Khan et al. 2016 Plant Physiol) or a combination of Pi starvation and PAMP elicitation promote defense responses (Castrillo et al. 2017 Nature; Morcillo et al. 2020 EMBO J). However, this study observed the opposite (Fig. 1A, 1B, 1E, 1F, S1C). Although PHR1, a key transcriptional activator of PSR, suppresses the immune responses (Castrillo et al.,

2017), there is no evidence showing repression of immune responses by Pi starvation. The enhanced immune responses observed in *phr1;phl1* are independent of Pi status (Castrillo et al., 2017). It seems that the authors did not comprehend this notion correctly. Therefore, the first sentence, "To confirm that PSR inhibits the plant immune response, ...", in the Result is inappropriate because the statement "PSR inhibits the plant immune response" has not been established.

Response: Thanks for your good comments. We agree with you that our findings are somewhat different with what have been reported in the above-mentioned articles. However, there are some differences between our experimental settings and published papers, which likely cause the 'opposite' results.

Firstly, Khan *et al*, reported that Pi-deficient *Arabidopsis* plants showed enhanced JA levels in WT plants and enhances resistance to insect herbivory (Khan *et al*, 2016). In our study, we focus on the plant immune responses against rhizosphere microbiome and this is different with immune response triggered by insects. Actually, there is a signaling antagonism between SA mediated immunity towards biotrophic pathogens and JA mediated immunity towards herbivory and necrotrophic pathogens. So it is not surprising that LP enhances immunity to insect herbivory while suppresses immunity to bacterial pathogens. Besides, a previous study showed that the RALF23-FER-MYC2 signaling pathway negatively contributes to plant immunity through elevation of JA signaling to promote bacterial colonization (Guo *et al*, 2018). And we found that the RALF23-FER signaling is activated under LP condition. The different conclusion might because different organisms were studied.

Secondly, Morcillo *et al*. revealed that diacetyl from the bacteria enhances phytohormone-mediated immunity under Pi starvation conditions (Morcillo *et al*, 2020). Diacetyl is a volatile compound, and it is totally different with immune elicitors such as flg22. Therefore, diacetyl mediated immune regulation is likely different with PAMP-triggered immunity. So that is probably why our results are different with previous observations.

Castrillo *et al*. revealed that PHR1, the master transcriptional regulator of PSR, is

involved in immune suppression (Castrillo *et al*, 2017). The authors focused on defensive-associated genes. In our manuscript, we used the common PTI marker responses including MAPK activation, ROS burst and PTI marker genes induction as output to study the impact of Pi starvation on PTI. We agree with you that the term 'confirm' is not correct here since no one has done this before and we are sorry for that. We have corrected it in the revised manuscript with sentence 'To determine the impact of Pi starvation on plant immune response' (line 114). We have also discussed the above "opposite results" in our revised manuscript (lines 366-370).

2. Throughout the whole manuscript (including the title), authors often stated "under PSR conditions", "during the PSR", "PSR-mediated". These words are confusing. PSR (Pi starvation response) is a collective term to describe overall responses to Pi starvation, including developmental, physiologic, and molecular responses. The authors should use more specific words (for example, Pi starvation) instead.

Response: We appreciate your professional suggestions. We have corrected the "PSR" to "Pi starvation" throughout the manuscript (lines 51, 54, 58, 60, 64, 70, 74, 107, 122, 128, 171, 177, 183, 212, 215, 237, 247, 259, 285, 301, 302, 310, 338, 345, 353, 361, 365, 382, 383, 387, 403 and 407).

3. In many figures and supplemental figure legends, they end a sentence "Three biological replicates were evaluated, each yielding similar results." It is unclear if the data shown are from one representative replicate or three replicates. Additional replicates should be shown in the supplement.

Response: Thanks for your good comments and we are sorry for the confusion. The data shown in figures or supplemental figures are only from one representative experiment. One biological replicate means one independent experiment. Within each biological replicate, three technical replicates were used. To avoid confusion, we modified this sentence to "All experiments were repeated three times with similar results" in the revised figure legends. If the Journal style would need the data from other independent experiments, we would be happy to provide it and integrate it into

supplemental figures.

4. The citation for RALF microarray data (Fig. 2C) is unclear (line 169). What does the number on the right mean? It looks the changes are negligible (less than two folds?). According to the published dataset (Castrillo et al. 2017), RALF genes are in general unaffected or mildly suppressed under Pi-starvation. The opposite is observed in Fig. 2B. In the CHIP-PCR analysis (Fig. 2G), WT may not be a good control. WT expressing an irrelevant protein tagged with Myc will be better.

Response: Thanks for your good comments.

We quote the microarray data to show that expression of *RALF* is induced by Pi starvation and the number on the right represents the Log foldchange value. We have added this information in the revised Figure 2C.

In the Castrillo' published RNA-seq, the authors harvested the whole seedlings for transcriptome analysis. However, we only used roots for RT-qPCR analysis. It is likely that sampling the whole seedlings will dilute the induction fold of *RALF* genes that are induced only in root tissue under Pi starvation.

Concerning the CHIP-PCR analysis, we agree with you that WT is not a good control. We have performed new experiments with another Myc-labelled transgenic plants (i.e., *CRY1-Myc* as a negative control) and similar results were observed (Fig R4). We have updated the Figure in the revised manuscript (Fig 2G).

Figure for referees removed

5. Fig. S2A and B: are these Pi-starvation/RALF23 differentially expressed genes induced or repressed genes? The authors should indicate them separately. Also, this will be a good chance for the authors to examine how the global immune responses are affected by Pi starvation.

Response: Thanks for your good suggestions. Pi-starvation and RALF23 regulated differentially expressed genes include both induced and repressed genes. We have re-analyzed the data and performed GO enrichment analysis with induced and repressed genes separately (Fig R5). We have updated the Appendix Fig S2 and lines 144-147.

Figure for referees removed

6. Inconsistent bacteria growth assays:

- Usually, *fls2* and *bak1* mutants are susceptible when grown under nutrient-rich media. However, this is not observed in Fig. 3F.

Response: Thanks for your good comments.

We totally agree with you that normally *fls2* and *bak1* mutants are more susceptible in leaves. However, the data shown in Fig. 3F (revised Fig. 3G) were in root tissue, which is different with leaf tissues. So that's may be why the differences were observed. In support with our data, a recent study (Song *et al*, 2021) reported that *Pseudomonads* colonize similarly in the root of *fls2* and *bak1* mutants and WT (Fig R6). We hope our explanation would help to understand the issue.

Figure for referees removed

- The current manuscript indicated that Col-0 is more susceptible under "LP" conditions. The difference in susceptibility is huge in Fig. 1F (>2-orders) but becomes very subtle in Fig. 3C. Also, the presentation of Fig. 1C is unclear. What do the number and "Sig" on the right mean?

Response: Thanks for your good comments.

The Fig. 1F (updated Fig. 1G) and Fig. 3C are two independent experiments. To our experience and knowledge, it is sometimes normal that independent experiments

would produce slightly different results. But it is clear both experiments showed statistical differences. So, we hope the statistical significance would be fine here.

In Fig. 1C (updated Fig. 1D), the number on the right means the Log foldchange value and "Sig" means significance. We have indicated the significance in the revised Figure 1D legend.

7. For the analysis of the rhizosphere microbiome in Fig. 4, the result is skeptical because microorganisms in the inoculum (from local soil) are uncharacterized. And, it is not known how general the result can be applied when different inoculum is used.

Response: Thanks for your good comments. We are sorry for forgetting the negative control data in the manuscript. We used the same inoculum which ideally should contain a identical microbiome for all treatment. We have also measured the bulk soil from different treatments (the soil control that inoculated only with the microbiome but not Pi stressed plants in) and the result showed that the relative abundance of *Pseudomonas*, *Bacillus*, *Geodermatophilus* and *Methylobacillus* were similar between LP and HP treatment or mock and RALF23 treatment (Fig R7). We have updated the Appendix Fig S9 E and S9 F and lines 268-272 and 278-281. Therefore, we hope this is fine for our manuscript here.

Figure for referees removed

8. It is unclear what samples/tissues were analyzed in Fig. 5C. WT? Under HP or LP? Then, what will be the result if *phr1* and *fer-4* are analyzed? A recent report by Finkel et al. (PLoS Biology 2019, The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response) is a very relevant reference. The authors should discuss it.

Response: Thanks for your good comments.

In the Fig. 5C, the RT-qPCR were performed in wild type *Arabidopsis* (Col-0) seedlings under LP condition and we are sorry for not explaining clearly the data in Fig. 5C. We have performed new experiments by using Col-0, *phr1* and *fer-4* mutants roots and the results showed that the *Pto* DC3000 and *B. subtilis* inoculation significantly upregulated *PHT1:1* and *PHT1:4* expression in Col-0 and *phr1* mutants, but *fer-4* mutation attenuates almost all these responses (Fig R8). We have also updated the Fig 5C and the text (see lines 303-306).

A recent report pointed that the effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response by using a bacterial synthetic community (SynCom) (Finkel *et al*, 2019). The author also showed that in the absence of *Burkholderia* from the SynCom, plant shoots accumulated higher Pi levels than shoots colonized with the full SynCom under Pi starvation conditions (Finkel *et al*, 2019). That shows that rhizosphere microbiomes can alleviate Pi starvation under LP condition; similarly, we found the *Pto* DC3000 and *B. subtilis* can promote LR growth and Pi-uptake genes expression to alleviate Pi starvation under LP condition (Fig 5C and Appendix Fig S10), which is consistent with previous report. We have updated the Figure 5C with root results and discussed it in lines 423-430

Figure for referees removed

9. The authors should elaborate or explain why the root microbiota of *fer-4* can improve the growth under LP. What factor can change the root microbiota? Does *fer-4* show any PSR? The experiment in Fig. 6D is under HP or LP?

Response: Thanks for your good comments.

The recent report (Song *et al*, 2021) has shown that the root microbiota of *fer-4* will enrich the *Pseudomonas* in roots. Our work also confirmed it. Here, we further found that, under LP condition, the root microbiota of *fer-4* will upregulate the expression of Pi-absorbing genes (Fig. 6D). Besides, we found the genera *Flavobacterium*,

Pseudomonas and *Delftia* were more abundant in *fer-4* mutants (Appendix Fig S8F). Previous studies have shown that the genera *Flavobacterium*, *Pseudomonas* and *Delftia* were among the most-predominant genera of plant-beneficial bacteria (Lebeis *et al*, 2015; Bernal *et al*, 2017; Lally *et al*, 2017; Melnyk *et al*, 2019; Rolli *et al*, 2015). This might can explain why the microbiota of *fer-4* improve the plant growth under LP conditions (lines 316-321 and 345-348).

Concerning the factors that change the root microbiota, in Song's report, FER regulates root reactive oxygen species (ROS) to control Pseudomonads under normal growth condition (Song *et al*, 2021). In our work, we found the immune receptors such as FLS2 and BAK1 might also be involved in reshaping the root microbiota under LP condition (Fig 3G and Appendix Fig S7). Besides, we found that the secondary metabolites such as glucosinolates, which is activated upon perception of pathogen-associated molecular patterns by pattern recognition receptors of the innate immune system and is needed for broad-spectrum defense to restrict the growth of pathogens (Clay *et al*, 2019; Bednarek *et al*, 2006), was induced in *fer-4* mutants (Data not shown). We have updated the discussed points in our revised manuscript (lines 330-337).

We performed additional experiment and found that *fer-4* mutants are more sensitive to Pi deficiency (Fig R9A and R9B). We have updated it in the Appendix Figure S1E and S1F and please see lines 130-132.

Figure for referees removed

Minor concerns

1. 1. Several data need quantification, for example, ROS production (Fig. 1B), change of root morphology (Fig. S8), western blots (Fig. 1A, 2A, 3A, 3B, 3D, S5D, S5E).

Response: We appreciate your professional comments and suggestions. We have quantified the ROS data in Fig. 1B and 1C, and WB results in Fig. 1A, 2A, 3A, 3B, 3D, 3F, S1C, S4C, S6D and S6E.

2. Line 326, there is no Pi uptake data shown.

Response: Thanks for your good comments.

We are sorry for that we used the wrong description about the results. What we mean is RALF23/FER pathway can recruit a specific beneficial taxon to alleviate Pi starvation (line 376).

3. Line 380, unclear citation.

Response: Sorry. The reference has replaced and please see lines 443-444.

4. There is no figure title in all the supplemental figures.

Response: We appreciate your professional suggestions. We have added a title for each supplemental figure.

Response to Comments of Reviewer #3

Major concerns

1. Authors revealed the role of FER in suppression of plant defense mechanism under Pi-starvation conditions. In the text, they mentioned "Recent studies have highlighted the role of FER in plant immunity regulation. We hypothesized that FER is involved

in PSR-mediated immunity suppression". Even though authors provide evidence to support their hypothesis in this manuscript, authors need to explain the logic how they make this hypothesis or why they think FER function in PSR. It is hard to find out the logic why authors chose FER to test its role in PSR.

Response: We appreciate your professional comments. Our group mainly works on the receptor FER and its ligands RALF peptides. Based on current research on the molecular function of FER, this receptor is involved in many biological processes such as immunity and nutrition stress responses (Stegmann *et al*, 2017, Guo *et al*, 2018; Zhang *et al*, 2020 and Xu *et al*, 2019), and we found *fer-4* mutants are more sensitive to Pi deficiency (Fig R9A and R9B, revised Appendix Fig S1E and S1F). Benefiting from the paper by Castrolla *et al*, 2017, we tested the Pi-starvation mediated immunity inhibition in Col-0 and *fer-4* mutants. Interestingly, we found Pi-starvation mediated immune suppression is FER dependent. Therefore, we continued the research work on this project and that is basically the logic behind.

2. Authors used *PHR1-OE* to provide an evidence regarding inhibition of flg22-induced FLS2-BAK1 interaction. As control, it is necessary for authors to provide a result of FLS2-BAK1 interaction without flg22 treatment under both Pi-sufficient and starvation conditions. And a complementation assay with *PHR1-OE* in *phr1* mutant background is necessary to confirm whether FLS2-BAK1 formation is restored in *phr1* mutant with PHR1 under the control of native PHR1 promoter.

Response: Thanks for your good comments.

For FLS2-BAK1 interaction, two independent experiments in this assay were performed. Unfortunately, we cannot detect the interaction between BAK1 and FLS2 without flg22 treatment (Fig R10). It is not surprising because BAK1 and FLS2 are immune receptors and they normally only interact with each other when an immune elicitor exists (for example, upon flg22 treatment), a lot of published works also confirm this observation (Chinchilla *et al*, 2007, Sun *et al*, 2013; Koller and Bent, 2014). So, we did the experiment with flg22 treatment. Concerning the

complementation experiments, it is challenging for us to get stable transgenic lines during the revision stage. Therefore, we performed experiments with transient expression in Arabidopsis protoplasts. The result revealed that FLS2-BAK1 formation is dependent on PHR1 (Fig R11). We have integrated this into the main Figure 3B. We also explained this in the main text (lines 222-227).

Figure for referees removed

Figure for referees removed

3. Figure 4B, 4D, 4F and 4H showed the role of RALF23 in colonization of *Pseudomonas*, *Bacillus*, *Geodematophilus* and *Methylobacillus* under Pi-starvation condition. It will be a good information for authors to provide a result of colonization

with these bacteria after RALF23 treatment under Pi-sufficient conditions.

Response: Thanks for your good comments.

In our microbiome sequence, we also included RALF23 treatment under Pi-sufficient condition. We have analyzed the data according to your suggestions. The results showed that RALF23 had the same function to enrich the bacterium (*Pseudomonas*, *Bacillus*, *Geodematophilus* and *Methylobacillus*) in roots under Pi-sufficient conditions (Fig R12). We have added this information into Appendix Figure S9A-S9D and updated the text (lines 278-281).

Figure for referees removed

4. FER functions in FLS2-BAK1 complex formation and appears to play a role in colonization of beneficial bacteria in Arabidopsis. I presume that complex formation of FLS-BAK1 can be affected under Pi-sufficient and deficient conditions, depending on the absence/presence of FER. No biochemical result was provided in this manuscript regarding FLS-BAK1 complex formation in *fer-4* (mutant) and *FER*/*fer-4* (complemented line) under Pi-sufficient and deficient conditions.

Response: Thanks for your good comments. We have performed new experiments to confirm the result in the FER complementation condition. We did the experiment with flg22 treatment and found FLS2-BAK1 formation is dependent on FER (Fig R13). We have also integrated this result into Fig. 3F and updated the text (lines 239-247).

Figure for referees removed

Minor concerns

1. Authors got consistent results regarding suppression of plant defense response under Pi-starvation conditions (Fig S1). As the major concept was already reported in

Castrillo et al., 2017, it would be better to include the reference in the main text (Line 106 - 119).

Response: We appreciate your professional comments. We agree with you that Castrillo *et al*, 2017 have revealed the immune suppression under Pi starvation conditions at the transcriptional level, here we further confirmed this conclusion by testing the PTI marker responses. To acknowledge the previous work, we have cited the Castrillo *et al*, 2017 (lines 126).

2. Authors used DAB staining methods to detect ROS in plants. However, it is hard to see difference between HP and LP or Col-0 and *fer-4*. Quantification will help to make clear with this result.

Response: We appreciate your professional comments. We have performed new experiments to stain ROS with H_2DCFDA in Arabidopsis roots and we also quantified the ROS data, shown in Figure 1B and 1C.

3. Authors showed the similar trend (not same) of differential gene expression under Pi-starvation and RAFP23 treated conditions. Authors did not provide the evidence of these gene expression pattern in Pi-sufficient and No RALF23 treated plants.

Response: Thanks for your good comments.

We showed the heatmap data by using the log foldchange value of LP vs HP or RALF23-treat vs Mock to indicate that LP treatment or RALF23 treatment cause the similar trend of gene differential expression. In this analysis, the gene expression in Pi-sufficient or No RALF23 treatment were used for normalization. Therefore, we did not show the gene expression pattern in these two conditions.

4. Authors showed repression of flg22-induced *FRK1* expression in *RALF23-OE* under Pi-sufficient and deficient conditions. It would be better to have the result of *FRK1* expression in *RALF23-OE*, without flg22 treatment, under Pi-sufficient and deficient conditions as Appendix figure.

Response: We appreciate your professional comments. We have performed new

experiments and the result is shown in Fig R14. We have also integrated is data into Appendix Figure S3 and lines 153-158.

Figure for referees removed

5. Authors need to check the legend of Figure S5. It is hard to understand it due to mismatching the information to figures.

Response: We appreciate your professional comments. We have reordered the Figure legend and please see Appendix Figure S6 legend.

6. In text, authors explained relative bacterial abundance of Fig 4 with "upregulated" for some results. Do you mean enhanced bacterial growth?

Response: Thanks for your good comments.

The relative bacterial abundance of Fig 4 with “upregulate” means the bacterial had more colonization in the root of plants and we have replaced the word “upregulate” with “high abundance” in the result section (line 321).

7. Figure 5A: It is hard to see plant phenotype, especially *fer-4*. Authors can magnify each plant in Figure 5A.

Response: We appreciate your professional comments. We have magnified the seedlings in the revised manuscript (Fig. 5A) (Fig R15 A).

Figure for referees removed

8. Line 264 - 266: "both *Pto* DC3000 and B. subtilis inoculation significantly alleviated Pi-starvation phenotypic characteristics and promoted LR growth of WT plants and *phr1* mutants under LP conditions" - For readers who are not working in PSR research, it would be better to explain more about what "alleviated Pi-starvation phenotypic characteristics" authors were observed in the text.

Response: We appreciate your professional comments. Firstly, the *Pto* DC3000 and *B. subtilis* inoculation will up-regulated the phosphate transporter *PHT1;1* and *PHT1;4*

expression (Fig 5C) (Fig R15 C) to alleviate Pi-starvation phenotypic. This could be a character of Pi-stress phenotype. Secondly, leaf size, leaf color as well as plant size are characters of Pi starvation phenotype. We have updated the main text and added such explanations (lines 291-297 and 303-309).

9. Figure S7: Based on the main text, it would be better to change the order of figure sections. For example, Fig S7C, S7E and S7F can be changed to S7B, S7C, and S7D, respectively.

Response: We appreciate your professional suggestions. We have re-ordered the Figures as you suggested. We have updated the Appendix Fig S8.

10. Line 380 - 381: I don't find out this citation in the reference.

Response: Sorry. The reference has added and please see lines 443-444.

Reference:

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A & Schulze-Lefert P (2009) A Glucosinolate Metabolism Pathway in Living Plant Cells Mediates Broad-Spectrum Antifungal Defense. *Science* 323: 101–106

Bernal, Patricia, Luke P Allsopp AF and MAL (2017) The Pseudomonas putida T6SS is a plant warden against phytopathogens. ISME Journal 528: 1575–1588

Castrillo G, Teixeira PJPL, Herrera Paerdes S, Law TF, de Lorenzo L, Feltcher ME, Finkel OM, Breakfield N, Mieczkowski P, Jones CD, *et al* (2017) Direct integration of phosphate starvation and immunity in response to a root microbiome. *Nature* 543: 513–518

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JDG, Felix G & Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448: 497–500

Clay NK, Adio AM, Denoux C, Jander G & Ausubel FM (2009) Glucosinolate metabolites

required for an Arabidopsis innate immune response. *Science* 323: 347–348

Finkel OM, Salas-González I, Castrillo G, Spaepen S, Law TF, Teixeira PJPL, Jones CD & Dangl JL (2019) The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response. *PLOS Biology* 17: e3000534

Guo H, Nolan TM, Song G, Liu S, Xie Z, Chen J, Schnable PS, Walley JW & Yin Y (2018) FERONIA receptor kinase contributes to plant immunity by suppressing jasmonic acid signaling in Arabidopsis thaliana. *Current Biology* 28: 3316–3324

Khan GA, Vogiatzaki E, Glauser G & Poirier Y (2016) Phosphate deficiency induces the jasmonate pathway and enhances resistance to insect herbivory. Plant Physiology 171: 632–644

Koller T & Bent AF (2014) FLS2-BAK1 Extracellular Domain Interaction Sites Required for Defense Signaling Activation. *PLOS ONE* 9: e111185

Kong X, Zhang C, Zheng H, Sun M, Zhang F, Zhang M, Cui F, Lv D, Liu L, Guo S, *et al* (2020) Antagonistic interaction between auxin and SA signaling pathways regulates bacterial infection through lateral root in Arabidopsis. *Cell Reports* 32: 108060

Lally RD, Galbally P, Moreira AS, Spink J, Ryan D, Germaine KJ & Dowling DN (2017) Application of endophytic pseudomonas fluorescens and a bacterial consortium to brassica napus can increase plant height and biomass under greenhouse and field conditions. Frontiers in Plant Science 8: 1–10

Lebeis SL, Paredes SH, Lundberg DS, Breakfield N, Gehring J, McDonald M, Malfatti S, del Rio TG, Jones CD, Tringe SG, et al (2015) Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. Science 349: 860–864

Melnyk RA, Hossain SS & Haney CH (2019) Convergent gain and loss of genomic islands drive lifestyle changes in plant-associated Pseudomonas. ISME Journal 13: 1575–1588

Morcillo RJ, Singh SK, He D, An G, Vílchez JI, Tang K, Yuan F, Sun Y, Shao C, Zhang S, et al (2020) Rhizobacterium-derived diacetyl modulates plant immunity in a phosphate-dependent manner. The EMBO Journal 39: 1–15

Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Gandolfi C, Casati E, Previtali F, Gerbino R, et al (2015) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environmental Microbiology 17: 316–331

Song Y, Wilson AJ, Zhang X-C, Thoms D, Sohrabi R, Song S, Geissmann Q, Liu Y, Walgren L, He SY, *et al* (2021) FERONIA restricts Pseudomonas in the rhizosphere microbiome via regulation of reactive oxygen species. *Nature Plants* doi:10.1038/s41477-021-00914-0.

Stegmann M, Monaghan J, Smakowska-Luzan E, Rovenich H, Lehner A, Holton N, Belkhadir Y & Zipfel C (2017) The receptor kinase FER is a RALF-regulated scaffold controlling plant immune signaling. *Science* 355: 287–289

Xu G, Chen W, Song L, Chen Q, Zhang H, Liao H, Zhao G, Lin F, Zhou H & Yu F (2019) FERONIA phosphorylates E3 ubiquitin ligase ATL6 to modulate the stability of 14-3-3 proteins in response to the carbon/nitrogen ratio. Journal of Experimental Botany 70: 6375–6388

Yadong Sun, Lei Li, Alberto P. Macho, Zhifu Han, Zehan Hu, Cyril Zipfel, Jian-Min Zhou, J. C. (2013). Structural Basis for flg22-Induced Activation of the Arabidopsis FLS2-BAK1 Immune Complex. *Science*, 342, 624–629.

Zhang X, Peng H, Zhu S, Xing J, Li X, Zhu Z, Zheng J, Wang L, Wang B, Chen J, *et al* (2020) Nematode-Encoded RALF Peptide Mimics Facilitate Parasitism of Plants through the FERONIA Receptor Kinase. *Molecular Plant* 13: 1434–1454

Zheng Z, Wang Z, Wang X, and Liu D (2019) Blue Light Triggered-Chemical Reactions Underlie Phosphate Deficiency-induced Inhibition of Root Elongation of Arabidopsis Seedlings Grown in Petri Dishes. *Molecular Plant* 11: 1515–1523

1st Revision - Editorial Decision **8th Dec 2021**

Dear Prof. Yu,

Thank you again for the submission of your revised manuscript and for your patience during the review process. We have now received the reports from two of the original referees, which I copy below.

As you can see from their comments, both referees are rather positive towards your work but point out to some persisting, mostly minor concerns, that will require your attention before your manuscript can be published in The EMBO Journal. Although it is our policy to allow only a single major round of revision based on the overall interest expressed in the reports, I would like to invite you to address the comments of the referees in a revised version of the manuscript. Most of the concerns require some clarification in the text, but some of them may require relatively minor experimental work. Please do contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, as before follow the instructions below when preparing your manuscript for resubmission.

Also as before, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Again, please contact me at any time during revision if you need any help or have further questions.

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

David

David del Alamo, PhD.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

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 response 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 (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can 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 or a single pdf per main figure 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 at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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: .

- Additional Tables/Datasets should be labelled 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.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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Referee #2:

In this revised manuscript, the authors address and clarify the most concerns raised. The current manuscript is comprehensive enough to describe the role of RALF-FER module in Pi-deficiency induced immunity response.

There are some further comments:
Line 126: Castrillo et al. reported that PHR1 is a negative regulator for SA-responsive gene expression under the combined effect of Pi-deficiency and bacterial elicitation. However, Pi starvation alone showed a minimal effect.
1. Fig. 1A: What is the pMAPK profile in fer-4 without flg22 treatment? This might be relevant in clarifying whether LP-mediated inhibition via FER is a specific process or just due to pleiotropic effect/constitutive pMAPK activation.
2. Please explain how the 738 defense responsive genes were selected in Fig. 1D and E? In Fig. 1D, it is unclear how the color correlates with the degree of "significance"?
3. Fig. S2: please indicate the ID for each GO term. The genes involved in "Immune response" and "Defense response" are enriched in both up-regulated and down-regulated genes after RALF23 treatment. However, the genes involved in "Immune

response" and "Defense response" are only enriched in down-regulated genes after Pi starvation. This seems contradictory to the result shown in Fig. 1E.
4. Fig. 4. The figure legends stated that samples are technical replicates. How reproducible is the data?
5. The natural microbiome population is an interesting resource. May the authors elaborate on the rationale for site selection, preparation method, characterization and propagation procedure? It may be helpful to indicate the geographic coordinate (latitude and longitude).
6. Line 335-337: The authors state that the expression of genes involved in synthesis of the secondary metabolites glucosinolates is induced in fer-4 mutants. Please show the data or cite the reference.
7. Line 424-427: The authors claim that Pto DC3000 inoculation significantly promoted LR growth in WT plants and the phr1 mutants under LP conditions. The single image in Fig. S10 is not convincing enough.
8. Fig. 5, the authors state no effect on fer-4 mutant after inoculation of Pto DC3000 or B. subtilis inoculation. However, when checking the seedlings in Fig. 5A, the anthocyanin accumulation in the fer-4 mutant was alleviated by Pto DC3000 or B. subtilis inoculation under LP. Please explain.
9. It is curious why the plant growth phenotypes of the "Mock" controls in Fig. 5A and 6A are quite different.
10. In the model of Fig. 7, the arrows and lines are a bit confusing. Sometimes, it indicates action or route, but sometimes, it shows a consequence. Pi should also be transported into the cell under HP (left side). Not sure what Pi inside the bacterium means.

Minor comments:
1. Line 126: "Reposted" should be "reported".
2. Line 331: "Pseudomonads" should be "Pseudomonas" (a genus name, italic and first letter capitalized) or pseudomonads (a bacteria family, non-italic, non-capitalized).
3. Title: "...under PSR" should be replaced by "...under Pi deficiency/starvation".

Referee #3:

Authors revised and resubmitted their manuscript to propose combined mechanisms of plant defense and Pi-starvation stress responses. They addressed reviewer's questions and here additional comments below;
*Major concerns:
1. In Fig 1D and 1E, authors suggested that "Pi starvation and RALF23 regulate similar type of plant defense response at the transcriptional level". However, authors did not include the expression pattern in HP. How about these gene expression in HP?
2. Authors showed comparison of FRK1 expression in WT and RALF23-OE with flg22 treatment under HP and LP conditions at Fig 1F. However, in Fig S1A, FRK1 expression with flg22 treatment is much higher in WT, compared in that in Fig 1F, even though authors seem to use the same condition in these figures. I know it is not reality to show the exact same qRT-PCR results at this experiment, but it seems to show too much inconsistency between these figures.
3. Authors used RALF promoter-luciferase system to test PHR1-mediated RALF expression in Fig 2F. In Fig 1B and 1E, PHR1 appears not to be involved in RAFL1 expression, even though RAFL1 promoter has a P1BS motif. This result (Fig 2F) needs a negative control (e.g. promoter without P1BS) to confirm PHR1-mediated expression of the luciferase reporter.
4. Authors described reduction of the inhibitory effect of flg22-induced MAPK activation in phr mutant (Fig 3A). However, even though authors provide quantified MAPK level in this result, it is hard to conclude "The PSR inhibits flg22-induced MAPK activation in a PHR1-dependent manner".
5. Line 298: "significantly alleviated Pi-starvation phenotypic characteristics" - what phenotypes did authors examine? In authors' response, "Firstly, the Pto DC3000 and B. subtilis inoculation will up-regulated the phosphate transporter PHT1;1 and PHT1;4 expression (Fig 5C) (Fig R15 C) to alleviate Pi-starvation phenotypic. This could be a character of Pi-stress phenotype. Secondly, leaf size, leaf color as well as plant size are characters of Pi starvation phenotype. We have updated the main text and added such explanations (lines 291-297 and 303-309)." - However, I cannot find out their updated explanation regarding what alleviated Pi-starvation phenotypic characteristics they observed. I am pretty sure that most readers will get confused on this explanation. Clearly indicate it.

Minor concerns:
1. "ROS assays also confirmed a role of FER in Pi starvation mediated immunity suppression in roots (Fig 1B and 1C)" - As the description of these results are after explanation of Fig 1G, authors can move these figures after Fig 1G. It would be better to support this result if authors provide ROS assay results for RALF23-OE as supplementary information.
2. Authors showed flg22-induced MAPK activation in WT and fer-4 mutant roots under HP and LP conditions. However, I cannot see a control experiment regarding MAPK activation in fer-4 mutant roots without flg22.
3. In Fig S1D, authors concluded that "Pto DC3000 had more colonization in roots under LP conditions than under HP conditions". I am not sure how authors performed statistic analysis on this data; however, I presume readers, including me, would not agree with this data with authors statement due to unclear description in the legend. Asterisks indicate p values in this figure, but it is not clear what the p value is for. For example, is the p value for cfu between HP and LP? And standard deviation bars overlap quite a bit between HP and LP, therefore, this result indicates the difference of Pto DC3000 colonization is not statistically significant.

4. Authors tried to explain the logic how they develop this manuscript regarding the role of FER in Pi-stress signaling. They addressed the reviewer's question with "Based on current research on the molecular function of FER, this receptor is involved in many biological processes such as immunity and nutrition stress responses (Stegmann et al, 2017, Guo et al, 2018; Zhang et al, 2020 and Xu et al, 2019)". If they add this explanation after "Recent studies have highlighted the role of FER in plant immunity regulation (Stegmann et al, 2017; Guo et al, 2018; Zhang et 131 al, 2020)", it will be much logical for readers to understand their story in this manuscript.
5. Line 203-206: Authors dealt with the role of RALF23 in colonization of rhizosphere bacteria that help plants resist LP stress. I suggest this part can move into the text of the section at " LP and RALF23 treatment promotes the colonization of rhizosphere bacteria that help plants resist LP stress". And remove "(e.g., RALF23)" in "Collectively, we identified several RALFs (e.g., RALF23) as direct targets of PHR1."
6. Authors should revise the legend of Fig S9; it is not clear to explain this result. I presume that this result shows the role of RALF23 in bacteria colonization under Pi-sufficient conditions.

Thank you and the reviewers for constructive comments that have greatly improved our manuscript during this revision. We have studied reviewer's comments carefully and revised our manuscript according to those comments. Additional experiments were performed according to requirements and we made our point-to-point response as below with our response highlighted in blue. The figure shown in the response to reviewers were named as Fig R + number. The corresponding modifications in the manuscript are marked by yellow highlight. Here we would like to submit our revised manuscript and hope this version will be suitable for publication in *The EMBO Journal* now.

Response to Comments of Reviewer #2

In this revised manuscript, the authors address and clarify the most concerns raised. The current manuscript is comprehensive enough to describe the role of RALF-FER module in Pi-deficiency induced immunity response.

Response: Thank you very much for your positive comments on our revised manuscript.

There are some further comments:

Line 126: Castrillo *et al*. reported that PHR1 is a negative regulator for SA-responsive genes expression under the combined effect of Pi-deficiency and bacterial elicitation. However, Pi starvation alone showed a minimal effect.

Response: Thanks for the suggestion. We have deleted the citation in line 126 and corrected the sentence. We would like to explain that under our experimental conditions, we do see the inhibition of immune response under Pi starvation. In most of our assays, we either used flg22 treatment, which mimics the bacterial elicitation, or used bacterial inoculation.

1. Fig. 1A: What is the pMAPK profile in *fer-4* without flg22 treatment? This might

be relevant in clarifying whether LP-mediated inhibition via FER is a specific process or just due to pleiotropic effect/constitutive pMAPK activation.

Response: We have performed the MAPK phosphorylation assay in *fer-4* without flg22 treatment. The result showed that activation of phosphorylated MAPK proteins is suppressed under LP condition in WT but not in *fer-4* mutants (Fig R1), indicating that FER is involved in LP-mediated MAPK suppression. We have updated Appendix Fig S1G and the figure citation in line 138.

Figure for referees removed

2.Please explain how the 738 defense responsive genes were selected in Fig. 1D and E? In Fig. 1D, it is unclear how the color correlates with the degree of "significance"?

Response: We are sorry for the word “detected” which causes misunderstanding. We have selected the 738 defense responsive genes from the website g:Profiler (Raudvere

et al., 2019) and analyzed them based on the differential expression between HP and LP treatment, or RALF23 and mock treatment. We revised our text and cited this work in line 148.

For the correlation between the color and the degree of "significance", the whiter color means more significant. That means, if the *p*-value is closer to 0, which represents highly significance, the color will be whiter. If the *p*-value is closer to 0.05, then the color will be darker. That is why the "sig." figure is somehow "opposite" with the fold-induction figure. In order to avoid readers from having the same misunderstanding, we removed the "significance" and please see updated Fig 1D, thanks.

3. Fig. S2: please indicate the ID for each GO term. The genes involved in "Immune response" and "Defense response" are enriched in both up-regulated and down-regulated genes after RALF23 treatment. However, the genes involved in "Immune response" and "Defense response" are only enriched in down-regulated genes after Pi starvation. This seems contradictory to the result shown in Fig. 1E.

Response: We have added the ID of each GO term and please see (Fig R2) the updated Appendix Fig S2. We only showed the top 10 of GO terms in Figure S2. We checked other GO terms that are not shown and found there are also "Immune response" genes up-regulated after Pi starvation. The difference between RALF23 and LP regulated GO terms could be that RALF23 treatment was performed with a relatively high dose, while under Pi starvation, it is likely that RALF23 accumulation will be lower. We hope this is fine.

Figure for referees removed

4. Fig. 4. The figure legends stated that samples are technical replicates. How reproducible is the data?

Response: Thanks for the question. We performed the 16S sequencing by collecting three independent rhizosphere samples. We would believe our sequencing data should be reproducible. Yet, we did not repeat the 16S sequencing because of high cost. We hope this is fine. In addition, the recent publication by Song *et al*. also revealed that the *fer* mutant reshaped a new microbiota, confirming that FER is indeed involved in microbiome regulation (Song *et al*, 2021).

5. The natural microbiome population is an interesting resource. May the authors elaborate on the rationale for site selection, preparation method, characterization and propagation procedure? It may be helpful to indicate the geographic coordinate (latitude and longitude).

Response: We collected soil samples from the local region (Tianma Road 19, Changsha, Hunan, China (+112°94’54, +28°17’57)), because this field is free of pesticide and heavy mental contamination for many years. We consider the microbiome population from such fields is real natural. For soil harvesting, we first carefully removed the surface and then collected the soil from the top 8 cm, which is rich for microbiota colonization. We diluted the soil with sterile water in a 1:1 (w/v) ratio and shook for 10 mins. Finally, the soil solution was watered onto the roots of the *Arabidopsis* seedlings. We have updated this information in lines 647-651.

6. Line 335-337: The authors state that is induced in *fer-4* mutants. Please show the data or cite the reference.

Response: We have updated the expression of genes involved in synthesis of the secondary metabolites glucosinolates in Appendix Fig S11(Fig R3) and please see line 334.

Figure for referees removed

7.=Line 424-427: The authors claim that *Pto* DC3000 inoculation significantly

promoted LR growth in WT plants and the *phr1* mutants under LP conditions. The single image in Fig. S10 is not convincing enough.

Response: Thanks for pointing this out. We agree with you that a single image is not convincing enough. We have calculated the average number of LR after bacterial inoculation under LP condition and performed statistical analysis. The result is updated in Appendix Fig S10 B (Fig R4)

Figure for referees removed

8.Fig. 5, the authors state no effect on *fer-4* mutant after inoculation of *Pto* DC3000 or

B. subtilis inoculation. However, when checking the seedlings in Fig. 5A, the anthocyanin accumulation in the *fer-4* mutant was alleviated by *Pto* DC3000 or *B. subtilis* inoculation under LP. Please explain.

Response: Thanks. Previous studies have reported that *Pto* DC3000 inoculation affects anthocyanin accumulation (Romero-Pérez *et al*, 2021). Therefore, the anthocyanin we saw in Fig. 5A was probably because of this reason. Besides, the anthocyanin was just one of the indicators of PSR. According to the shoot fresh weight data we measured, there is no statistical difference on the shoot fresh weight of *fer-4* mutants after inoculation with water, *Pto* DC3000 or *B subtilis*. We hope this explains the issue.

9. It is curious why the plant growth phenotypes of the "Mock" controls in Fig. 5A and 6A are quite different.

Response: We performed the plant growth phenotypes assays in Fig. 5A and 6A with different soils. In Fig. 5A, we only used the vermiculite for plant cultivation. While, in Fig. 6A, we used the vermiculite and a small proportion of natural soil, which may cause the different phenotypes. When we performed the experiments in Fig. 5A, we thought that sole vermiculite would be the best for plant growth and treatment. However, later on we optimized the soil and found the combination of vermiculite and natural soil was the best. That is why the phenotype of mock are different.

10. In the model of Fig. 7, the arrows and lines are a bit confusing. Sometimes, it indicates action or route, but sometimes, it shows a consequence. Pi should also be transported into the cell under HP (left side). Not sure what Pi inside the bacterium means.

Response: We appreciate your professional suggestions. We have rephrased this figure based on your comments, and please see updated Fig 7 (Fig R5).

Figure for referees removed

Minor concerns

1. Line 126: "Reposted" should be "reported".

Response: We have deleted the part containing “reposted”.

2. Line 331: "*Pseudomonads*" should be "*Pseudomonas*" (a genus name, italic and first letter capitalized) or pseudomonads (a bacteria family, non-italic, non-capitalized).

Response: Thanks for the comment. We have used “*Pseudomonas*” instead of “*Pseudomonads*” and please see line 328.

3. Title: "...under PSR" should be replaced by "...under Pi deficiency/starvation".

Response: We have used “under Pi starvation” instead of “under PSR” in the title.

Response to Comments of Reviewer #3

Authors revised and resubmitted their manuscript to propose combined mechanisms of plant defense and Pi-starvation stress responses. They addressed reviewer's questions and here additional comments below.

Response: Thank you very much for your positive comments on our revised manuscript.

Major concerns

1. In Fig 1D and 1E, authors suggested that "Pi starvation and RALF23 regulate similar type of plant defense response at the transcriptional level". However, authors did not include the expression pattern in HP. How about these gene expression in HP?

Response: Thanks for your good comments.

We showed the heatmap data by using the log foldchange value of LP vs HP or RALF23-treat vs Mock to indicate that LP treatment or RALF23 treatment cause the similar trend of gene differential expression. In this analysis, the gene expression in Pi-sufficient (HP) or No RALF23 treatment were used for normalization. That means for Pi starvation regulated genes, the expression of All genes in HP were used as controls for normalization. Therefore, we did not show the gene expression pattern in these two conditions. We hope the explanation is fine.

2. Authors showed comparison of *FRK1* expression in WT and *RALF23-OE* with flg22 treatment under HP and LP conditions at Fig 1F. However, in Fig S1A, *FRK1* expression with flg22 treatment is much higher in WT, compared in that in Fig 1F, even though authors seem to use the same condition in these figures. I know it is not reality to show the exact same qRT-PCR results at this experiment, but it seems to show too much inconsistency between these figures.

Response: The qRT-PCR data in Fig S1A have been normalized to the data without

flg22 treatment under HP condition, which better reflects the fold change. While, in Fig 1F we used the raw data. We have updated Fig 1F as what we have did for Fig S1A (Fig R6B). Fig R6 indicates the *FRK1* expression from the same dataset. In Fig R6A, it is not normalized while in Fig R6B it is normalized. In order to avoid readers from having the same misunderstanding, we replaced the Fig 1F with Fig R6B and please see updated Fig 1F, thanks.

Figure for referees removed

3.=Authors used RALF promoter-luciferase system to test PHR1-mediated RALF= expression in Fig 2F. In Fig 1B and 1E, PHR1 appears not to be involved in RALF1 expression, even though RALF1 promoter has a P1BS motif. This result (Fig 2F) needs a negative control (e.g. promoter without P1BS) to confirm PHR1-mediated expression of the luciferase reporter.

Response: We have performed new experiments with a promoter without P1BS (i.e., *RALF24* as a negative control) and similar results were observed (Fig R7). We have updated the Figure in the revised manuscript (Fig 2F) and lines 198-199.

Figure for referees removed

4.=Authors described reduction of the inhibitory effect of flg22-induced MAPK= activation in *phr1* mutant (Fig 3A). However, even though authors provide quantified MAPK level in this result, it is hard to conclude "The PSR inhibits flg22-induced MAPK activation in a PHR1-dependent manner".

Response: We appreciate this professional comment. We have changed the word "dependent" to "is involved in" and revised the sentence to "To clarify whether PHR1 is involved in mediating immune repression", and please see lines 214-218.

5.=Line 298: "significantly alleviated Pi-starvation phenotypic characteristics" - what= phenotypes did authors examine? In authors' response, "Firstly, the *Pto* DC3000 and *B.*

subtilis inoculation will up-regulated the phosphate transporter *PHT1;1* and *PHT1;4* expression (Fig 5C) (Fig R15 C) to alleviate Pi-starvation phenotypic. This could be a character of Pi-stress phenotype. Secondly, leaf size, leaf color as well as plant size are characters of Pi starvation phenotype. We have updated the main text and added such explanations (lines 291-297 and 303-309)." - However, I cannot find out their updated explanation regarding what alleviated Pi-starvation phenotypic characteristics they observed. I am pretty sure that most readers will get confused on this explanation. Clearly indicate it.

Response: We appreciate this professional comment. We have revised the conclusion based on your comments, and please see lines 293-301.

Minor concerns

1. "ROS assays also confirmed a role of FER in Pi starvation mediated immunity suppression in roots (Fig 1B and 1C)" - As the description of these results are after explanation of Fig 1G, authors can move these figures after Fig 1G. It would be better to support this result if authors provide ROS assay results for *RALF23-OE* as supplementary information.

Response: We appreciate your professional suggestions. We have descripted part of the results of ROS assays (Fig 1B and 1C) before Fig. 1G in line 140. We have performed ROS assay in *RALF-OE* plants and updated the data in Appendix Figure S3B and S3C (Fig R8).

Figure for referees removed

2.=Authors showed flg22-induced MAPK activation in WT and *fer-4* mutant roots= under HP and LP conditions. However, I cannot see a control experiment regarding MAPK activation in *fer-4* mutant roots without flg22.

Response: We have performed the MAPK phosphorylation assay in *fer-4* without flg22 treatment (Fig R1) and we have updated Appendix Fig S1G.

3.=In Fig S1D, authors concluded that "*Pto* DC3000 had more colonization in roots= under LP conditions than under HP conditions". I am not sure how authors performed statistical analysis on this data; however, I presume readers, including me, would not agree with this data with authors statement due to unclear description in the legend.

Asterisks indicate *p* values in this figure, but it is not clear what the *p* value is for. For example, is the *p* value for cfu between HP and LP? And standard deviation bars overlap quite a bit between HP and LP, therefore, this result indicates the difference of *Pto* DC3000 colonization is not statistically significant.

Response: The *p* value in Fig S1D is for the statistical difference of the log(cfu/root) between HP and LP and we have updated it in Appendix Fig S1D legend. We carried out the gradient dilution of the fully ground root and spread 0.25 μl of the dilution on the LB solid medium. After 2 days of culture at 28°C, the number of single colonies were counted. We listed the raw data below and performed the statistical analysis by using SPSS 23.0 software (SPSS). We do find it is statistically significant.

Col-0							
3 dpi				5 dpi			
HP		LP		HP		LP	
CFU/root	Log(CFU/root)	CFU/root	Log(CFU/root)	CFU/root	Log(CFU/root)	CFU/root	Log(CFU/root)
1200	3.079181	1600	3.20412	48000	4.681241	92000	4.963788
1600	3.20412	2000	3.30103	40000	4.60206	80000	4.90309
400	2.60206	2000	3.30103	52000	4.716003	108000	5.033424
400	2.60206	1600	3.20412	52000	4.716003	112000	5.049218
800	2.90309	1200	3.079181	72000	4.857332	84000	4.924279
800	2.90309	2000	3.30103	76000	4.880814	116000	5.064458
400	2.60206	2400	3.380211	76000	4.880814	112000	5.049218
400	2.60206	2000	3.30103	68000	4.832509	108000	5.033424
400	2.60206	1200	3.079181	56000	4.748188	108000	5.033424
400	2.60206	1200	3.079181	40000	4.60206	92000	4.963788
400	2.60206	1200	3.079181	60000	4.778151	84000	4.924279
400	2.60206	1200	3.079181	60000	4.778151	104000	5.017033
400	2.60206	1600	3.20412	56000	4.748188	100000	5
400	2.60206	2400	3.380211	42000	4.716003	84000	4.924279
800	2.90309	1600	3.20412	56000	4.748188	100000	5
1600	3.20412	1600	3.20412	64000	4.80618	88000	4.944483
800	2.90309	2000	3.30103	44000	4.643453	84000	4.924279
2000	3.30103	1600	3.20412	48000	4.681241	96000	4.982271

	3 dpi	5 dpi

	HP	LP	HP	LP
n	18	18	18	18
Mean	2.8012	3.2159	4.7454	4.9853
Std. Deviation	0.05968	0.02456	0.02021	0.0124

Student's *t* test		**Significant**
3dpi LP	3dpi HP	0.000048
5dpi LP	5dpi HP	0.014

4. Authors tried to explain the logic how they develop this manuscript regarding the role of FER in Pi-stress signaling. They addressed the reviewer's question with "Based on current research on the molecular function of FER, this receptor is involved in many biological processes such as immunity and nutrition stress responses (Stegmann et al, 2017, Guo et al, 2018; Zhang et al, 2020 and Xu et al, 2019)". If they add this explanation after "Recent studies have highlighted the role of FER in plant immunity regulation (Stegmann et al, 2017; Guo et al, 2018; Zhang et 131 al, 2020)", it will be much logical for readers to understand their story in this manuscript.

Response: We appreciate your professional suggestions. We have rephrased this paragraph based on your comments and please see lines 129-130. In the updated text, we first described the role of FER in plant immunity regulation, and then we mentioned that FER is also involved in nutrient stress response, and therefore we connect the FER pathway with Pi starvation mediated immune regulation. We hope the updated logic is easy for reader to follow.

5. Line 203-206: Authors dealt with the role of RALF23 in colonization of rhizosphere bacteria that help plants resist LP stress. I suggest this part can move into the text of the section at " LP and RALF23 treatment promotes the colonization of rhizosphere bacteria that help plants resist LP stress". And remove "(e.g., RALF23)" in "Collectively, we identified several RALFs (e.g., RALF23) as direct targets of PHR1."

Response: We appreciate your professional suggestions. We are very sorry we did not understand the first question here; in lines 203-206, we did not deal with the role of

RALF23 in colonization of rhizosphere bacteria. We have removed “(e.g., RALF23)” in “Collectively, we identified several RALFs (e.g., RALF23) as direct targets of PHR1”, and please see lines 206-207.

6. Authors should revise the legend of Fig S9; it is not clear to explain this result. I presume that this result shows the role of RALF23 in bacteria colonization under Pi-sufficient conditions.

Response: Thanks for pointing out this. We have updated the legend of Appendix Fig S9.

References:

Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H & Vilo J (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists. *Nucleic Acids Research* 47: W191-W198.

Romero-Pérez A, Ameye M, Audenaert K & Van Damme EJM (2021) Overexpression of F-Box nictaba promotes defense and anthocyanin accumulation in Arabidopsis thaliana after pseudomonas syringae infection. *Frontiers in Plant Science* 12: 692606.

Song Y, Wilson AJ, Zhang XC, Thoms D, Sohrabi R, Song S, Geissmann Q, Liu Y, Walgren L, He SY, *et al* (2021) FERONIA restricts Pseudomonas in the rhizosphere microbiome via regulation of reactive oxygen species. *Nature Plants* 5: 644-654.

Dear Prof. Yu,

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Before we can transfer your study to the production team, however, you need to provide the paper's synopsis composed of:

- a short 'blurb' text summarizing in two sentences the study (max. 250 characters). Add as well three to four 'bullet points' highlighting the main findings. Bullet points and standfirst text should be submitted as a separate manuscript file in LaTeX, RTF or MS Word format.
- A "synopsis image", which can be used as a "visual title" for the synopsis section of your paper. The image should be PNG or JPG format with pixel dimensions of 550 x 300-600 (width x height).

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Corresponding Author Name: Feng Yu
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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?	According to related references
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?	NA
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.	NA
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?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, student's t test and ONE-Way ANOVA were used for statistical analysis
Is there an estimate of variation within each group of data?	No

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http://1degreebio.org
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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).	MAPK： Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101 Cell Signaling Technology, Boston, USA ACTIN： M20009, Abmart, Shanghai, China GFP: MA9023 Abmart, Shanghai, China FLS2: AS12 1857 Agrisera BAK1: AS12 1858 Agrisera
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
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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	Yes, we have provided a "Data Availability" section at the end of the Materials and Methods section. RNA-seq data: PRJCA005011 Microbiome sequencing data: PRJCA005003
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).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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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