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Rhizobacterium-derived diacetyl modulates plant immunity in a phosphate-dependent manner

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1st Editorial Decision 30th Jul 2019

Thank you for submitting your manuscript for consideration by the EMBO Journal. I sincerely apologise for the unusual delay in the assessment of your work due to belated submission of referee reports. We have now received two referee reports on your manuscript, which are included below for your information.

As you will see from the comments, both reviewers appreciate the described interplay between Bacillus amyloliquefaciens-produced diacetyl and plant growth in a phosphate-dependent manner. However, they also raise a number of concerns that need to be addressed before they can support publication here. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of both reviewers.

REFEREE REPORTS:

Referee #1:

In this comprehensive study Morcillo et al. describe the effects of volatiles of the bacterium Bacillus amyloliquefaciens GB03 on plant growth under sufficient Pi and low Pi conditions. The authors demonstrated that volatiles produced by GB03 are able to increase the growth of Arabidopsis under nutrient sufficient conditions but the same volatiles decrease plant growth under nutrient deficient conditions. They could show convincingly that phosphate availability determines Arabidopsis response to the volatiles. This is per se already a very interesting observation (Figure 1 and 2). The authors further analysed the response of Arabidopsis to 7 of the 30 previously identified GB03 volatiles. Of these 7, one induced anthocyanin hyperaccumulation in plants with Pi deficiency, this was DA. This effect was impaired in the phr1phl1 mutant, similar to the natural volatiles (Figure 3). The authors could produce several lines of evidence that the volatiles-triggered plant

hypersensitivity to Pi deficiency is indeed mediated by DA and that DA exacerbates plant sensitivity to Pi deficiency via activation of immunity/accumulation of SA and JA (Figure 4). Until this point the paper is robust and the data shown are well documented.
What remains unclear from the data shown is if DA is also involved in the beneficial effects to the plants during growth in Pi sufficient medium. In particular figure 5 and the data shown here are not convincing. This unfortunately is the basis for the discussion. The authors could show that incubation for 48hrs with DA enhances root colonization in Pi sufficient conditions. Here no controls are shown on the effects of DA to bacterial growth. They also did not test the effects of DA on colonization in Pi limiting conditions. It remains obscure if the improved colonization correlates with growth promotion under Pi sufficient and limiting conditions. The effects to the pathogens DC3000 is not well documented/described. Measurements of the inoculum at time 0 is missing and how the experiment was done is not described. The effects to ROS production are minimal and we know that this read out is not particular robust, here significance must be calculated. Are the other volatiles with a similar structure affecting the ROS burst too? The experiment shown in figure 5 G is also less convincing. Why are the effects to GB03 set to 1? Is there a negative effect of DA to GB03?
Finally the discussion is not focused really on the data shown. The authors did not test the colonization of the bacterium under Pi sufficient or limiting conditions and thus they cannot comment on the strategies used by the plants to respond to fungi and to bacteria. The authors convincingly showed that volatiles effect the plant growth and that these effects are Pi dependent. They also demonstrated that DA is involved in the negative effects under Pi starvation but they did not convincingly show the role of DA during Pi sufficient conditions. I still think that by downplaying some of their conclusions and by refocussing the discussion on the data that seems to be robust this would be an excellent paper. This also means that the abstract needs to be adjusted. I do not think that the authors demonstrated that a bacterial volatile integrally modulates the immune system and the PSR and determines the relationship between the bacterium and the plants. They show that natural volatiles effects growth and that DA effect colonization under Pi sufficient conditions but has a negative effect on the plant during Pi starvation. The relationship was not fully analysed and the response of the plant to the colonization by the bacterium under these two conditions (or I missed it). Are the used amount of DA biologically relevant? Can the authors comments on the concentration measured in the free space of growing bacterial colonies and in soil?
Minor comments:
I would suggest to harmonize all the figures by using a similar style and by calculating significances using ANOVA or t-test (e.g. Fig. 1C; 2A; 2D; 3C; 3E; 4A; 4B; 4E; 5B; 5C). All the figures showing transcriptional data are not clearly labelled, Are these fold changes or relative expression? To which genes the data are normalized (e.g. AtUBI)? (e.g. 2D; 3C; 3E; 4A....). The legends are also not really informative. Information about the age of the plants is often missing and when present is not consistent with the information given in the M&M (e.g. trypan blue 11 DAT or 10DAT?). Number of replicates, plants used, incubation and preincubation times.....
Sometimes DA and sometimes BTDN is used in the figures. Sometimes letter are used and sometimes asterisks (e.g. figure 5 F could be analysed by ANOVA).
Figure 5G should be Ba not Bs.
Figure 1E cannot be read and so the corresponding supplementary figures. Why no figure was shown for the 0.005T vs. 0.005C as this would be to me the most obvious comparison. Also this is not significance of the expression as written in the legend of figure 1E, this is enrichment analysis of GO terms.
It would be good to introduce the organism earlier in the paper. It is only mentioned at the end of the introduction.

Referee #2:

The manuscript by Morcillo et al presents how the plant growth-promoting rhizobacteria Bacillus amyloliquefaciens GB03 facilitates its association with Arabidopsis through phosphate-dependent modulation of plant immunity. They show that volatile molecules produced by B. amyloliquefaciens promotes plant growth under P sufficient conditions, while inhibiting growth under Pi deficiency. Such suppression of plant growth under deficient conditions is through the enhancement of SA and JA-mediated immunity, resulting in anthocyanin accumulation and enhanced cell death. They further identify diacetyl produced by GB03 that suppresses immunity under Pi-sufficieny and

promotes immunity under Pi-sufficiency.

The paper represents a significant advance, that is interesting and novel, with broad interest for those working in microbial associations. However, the results are really perplexing, because the authors appear to be claiming completely opposing effects of DA under Pi-sufficient and deficient conditions: how can the same molecule both suppress or induce immunity? Furthermore, the results shown in this paper are in conflict with Hacquard et al (2016 Nature Communications) that demonstrated beneficial responses were prioritized in root endophytic fungi-colonized roots under phosphate-deficient conditions, whereas defense responses were activated under phosphate-sufficient conditions. The authors need to justify why these studies are inconsistent.

Comments:
1. The results under PI-sufficient and deficient conditions are completely opposing, DA promotes immunity under Pi-deficiency and suppresses immunity under Pi-sufficiency. I find it hard to see how the same molecule can do both of these things, simply based on a change in the Pi-status. This confusion is confounded by the fact that different assays are presented for DA treatment under Pi-sufficient and deficient conditions. I think there needs to be consistency in the assays performed under Pi-sufficient and deficient conditions, ie between figure 4 and 5. Only with equivalent assays can we assess whether the effects are really indeed opposing or whether infact the molecules are having alternative effects at different Pi concentrations. I would like to see the effects of DA on immunity performed in figure 5B, C, D and F under Pi sufficient and deficient conditions.
2. There is no introduction for Bacillus amyloliquefaciens GB03 and its produced volatile chemicals. Is there any report how GB03 promotion of plant growth without transfer of nutrients from soil? How important are the volatiles for plant-bacterial interactions?
3. Line 91: 'induced by nutrient-deficiency were enriched in immune response', how do you define these genes are immunity-related? Also how do these immunity genes compare to the overall transcriptional changes, what percentage is this of the total transcriptional changes?
4. The text in Fig. 1E and Fig. 5A and Supp Figs.S1D, S1E, S1F and S2A is illegible at this magnification.
5. Fig.1: why was plant fresh weight not measured? I think this is a more direct test for plant growth. Fig1C, there is an obvious difference between GB0.5MS and control 0.5MS in panel A, but why do you see no difference in panel C? Please include statistical analysis to panel C. Fig.1D needs quantification. Fig.1E or supplementary needs to include the comparison between 0.05T vs 0.05C.
6. Fig.2: There is a very clear P starvation response in Figure 1 between control 0.5MS and control 0.05MS, but the starvation genes in 2A show little difference. Why this inconsistency? Again you need the statistical analysis in this panel. Fig.2B is not so clear to see the root blue color. Fig.2C, the same issue as I mentioned above, from the pictures in Fig.1a and Fig.2B, the controls definitely show the differences in anthocyanin accumulation, but Fig.2C shows all the controls and even GB03 0.5MS are pretty same, can you explain this? Fig.2D shows GB03-induced IPS expression can be completely blocked by supplement of Pi, however, Fig.2C just shows partially dependent on Pi supplement, could you comment on this? Fig.2E-2F, define what cm2 is.
7. I find it confusing the Diacetyl is refered to as DA in the text and in figure legnds, but BTDN in figures. You need to be consistent.
8. Fig.3: in fig.3B, there is a significant difference for mock treatments between +P and -P, why you cannot see in fig.2C? Since the controls are quite variable, I am not convinced by the conclusions.
9. Fig.4: Fig.4C shows BTDN treatment cannot inhibit plant growth under +P, but SA does, suggesting that BTDN and SA have different functions. This is not what is stated in the text. Fig.4F shows the NahG plants just partially required for BTDN-induced anthocyanin under low Pi, how about other SA-deficient mutants like sid2? It would be useful to see quantification of Fig. 4F
10. Fig.5. Is the BTDN suppression of flg22-induced ROS significant?
11. Fig.2B and Fig.S6B show BTDN can induce massive SA and JA synthesis under low Pi, but very surprisingly the author could not see any pathogen resistance phenotype in fig.5E, how could you explain this? If you mix inoculation of GB03 and PstD3000 or Ralstonia GMI1000 under high P and low P conditions, can you see any pathogen growth effect?
12. Fig.6: Is DA only produced by B. amyloliquefaciens? What promotes plant growth under B. amyloliquefaciens colonization under high P conditions?
13. The authors present the model as if the plant wishes to be colonized under high Pi conditions. For what purpose? Rather I think the authors could consider DA as a chemical effector, that facilitates colonization under high Pi conditions. Other reports have shown that immunity is maximized under high Pi, this would make sense. However, in this paper the authors are arguing the

opposite. They need to exlain this discrepancy.
14. Fig.S8 panel A is just repeating the main Fig.6, I think it is not required.
15. All the qRT-PCR figures need to add statistical analysis.
16. The authors need to be careful in the use of the term 'symbiosis' In my view this reflects a very tight association between plant and microbe, such as that seen in legumes with rhizobia and during mycorrhizal associations. Arabidopsis lacks symbiosis signaling and lacks these closely associated intracellular symbionts. I think it is inaccurate to call the association described in this manuscript a symbiosis. Rather a commensal association would be a more appropriate term.
17. In the abstract and line 261-263, the authors claim that DA modulates the PSR system. I see no evidence for this. Rather the DA response is dependent on the PSR system, this is very different to demonstrating that DA modulates PSR. Please be more careful in your wording
18. Lines 63-64 and 271-273. The authors are making very broad claims here about differences between bacterial and fungal associations. The system they describe appears to be quite specific to this particular bacteria, or do they have evidence that all mutualistic bacteria produce DA? Please remove such broad claims and be more careful in your wording. The work you have demonstrated is describing one bacterial association and unless there is evidence that this is representative of all bacteria, then modify the breadth of the claims.
19. Xiao et al 2018 ref is missing
20. Line 302: show the data or don't make the statement.

1st Revision - authors' response 8th Oct 2019

Point-to-point response to the reviewer comments:

Referee #1:

In this comprehensive study Morcillo et al. describe the effects of volatiles of the bacterium Bacillus amyloliquefaciens GB03 on plant growth under sufficient Pi and low Pi conditions. The authors demonstrated that volatiles produced by GB03 are able to increase the growth of Arabidopsis under nutrient sufficient conditions but the same volatiles decrease plant growth under nutrient deficient conditions. They could show convincingly that phosphate availability determines Arabidopsis response to the volatiles. This is per se already a very interesting observation (Figure 1 and 2). The authors further analysed the response of Arabidopsis to 7 of the 30 previously identified GB03 volatiles. Of these 7, one induced anthocyanin hyperaccumulation in plants with Pi deficiency, this was DA. This effect was impaired in the phr1phl1 mutant, similar to the natural volatiles (Figure 3). The authors could produce several lines of evidence that the volatiles-triggered plant hypersensitivity to Pi deficiency is indeed mediated by DA and that DA exacerbates plant sensitivity to Pi deficiency via activation of immunity/accumulation of SA and JA (Figure 4). Until this point the paper is robust and the data shown are well documented.

What remains unclear from the data shown is if DA is also involved in the beneficial effects to the plants during growth in Pi sufficient medium. In particular figure 5 and the data shown here are not convincing. This unfortunately is the basis for the discussion.

Answer: We have performed additional experiments as suggested to improve Figure 5. Although DA alone does not induce plant growth-promotion, DA can be considered as beneficial to plants grown in Pi-sufficient medium, because it clearly increases GB03 colonization to roots. This beneficial effect can be attributed to the observations that DA specifically and partially suppresses ROS production in plants exposed to microbial elicitors. Our findings are consistent with recent reports where suppression of ROS production help the establishment of rhizobia symbiosis with plants (for details, please see the Discussion, Paragraph 2).

The authors could show that incubation for 48hrs with DA enhances root colonization in Pi sufficient conditions. Here no controls are shown on the effects of DA to bacterial growth.

Answer: We have checked the effects of DA on GB03 growth. The results show that the growth rate of GB03 is not altered by DA. Please see Appendix Figure S5C-S5D for the results.

They also did not test the effects of DA on colonization in Pi limiting conditions.

Answer: We have performed this experiment as suggested. The results show that DA failed to increase GB03 colonization in Pi-deficient plants (Appendix Figure S5E), possibly due to the strongly activated SA/JA pathway.

It remains obscure if the improved colonization correlates with growth promotion under Pi sufficient and limiting conditions.

Answer: Because GB03 is a PGPR strain for Pi-sufficient plants, it is deduced that better colonization will contribute to more efficient plant growth promotion. In fact, in our routine lab work, if no or poor growth promotion was observed in PGPR-inoculated plants, mostly it was correlated with poor PGPR colonization.

The effects to the pathogens DC3000 is not well documented/described. Measurements of the inoculum at time 0 is missing and how the experiment was done is not described.

Answer: As shown in the revised Figure 5E, CFU counts of the inoculum at time 0 are similar among the samples. Experimental details is described in Material and Methods, section Pathogen inoculation and quantification.

The effects to ROS production are minimal and we know that this read out is not particular robust, here significance must be calculated.

Answer: In addition to Figure 5B (Figure EV4 C in revised manuscript) that shows the dynamics of ROS production, Figure S7C (Figure 5B in revised manuscript) shows that ROS production (quantified as the total RLU of 60 minutes) was clearly suppressed by DA with statistical significance. To make this point clear, we have switched the positions of the two figures in the revised manuscript.

Are the other volatiles with a similar structure affecting the ROS burst too?

Answer: 2,3-butanediol (BTDL) and acetoin (ATN) are two MVs released by GB03 and they are structurally similar to DA. These two MVs were tested with DA in the same assay, but they did not show suppression on ROS burst as DA did (Figure EV4D in the revised version).

The experiment shown in figure 5 G is also less convincing. Why are the effects to GB03 set to 1? Is there a negative effect of DA to GB03?

Answer: In order to see whether DA has differential effects on GB03 and pathogens, chemotaxis assays were performed and the effects on GB03 were set as 1. In addition, multiple assays were performed using different dosages of DA. In order to show which dosage causes the greatest difference between GB03 and the pathogens, the effects on GB03 were set as 1 in all assays.

At low concentrations (0.001 mM, 0.005 mM and 0.05 mM), DA attracts GB03 more than the pathogens. Meanwhile, when applied at high concentrations (1 mM, 10 mM and 50 mM as tested), DA became deterrent to all three tested bacteria; however, GB03 was less deterred compared to the pathogens. Therefore the chemotaxis assays collectively indicate that DA increases the competitiveness of GB03 over pathogens in terms of bacteria motility (Fig 5 G).

Finally the discussion is not focused really on the data shown. The authors did not test the colonization of the bacterium under Pi sufficient or limiting conditions and thus they cannot comment on the strategies used by the plants to respond to fungi and to bacteria.

Answer: The discussion, as depicted in Figure EV4; Appendix Figure S5, contemplates the effect of P availability on plant interactions with bacteria or fungi, as judged by whether the plant-microbe association reflects mutualism or immunity. Therefore, prior to the revision, we did not examine GB03 colonization to Pi-deficient plants, because our results had demonstrated that Pi-deficient plants are severely stressed by GB03-produced volatiles or by DA alone. In addition, because the stress was clear at both the phenotypic level and the molecular level, we assumed that GB03 colonization would be unfavourable to Pi-deficient plants. This assumption is now confirmed by our new results, which demonstrate that GB03 colonization is less in Pi-deficient plants compared to Pi-sufficient plants.

The authors convincingly showed that volatiles effect the plant growth and that these effects are Pi dependent. They also demonstrated that DA is involved in the negative effects under Pi starvation but they did not convincingly show the role of DA during Pi sufficient conditions. I still think that by downplaying some of their conclusions and by refocussing the discussion on the data that seems to be robust this would be an excellent paper. This also means that the abstract needs to be adjusted. I do not think that the authors demonstrated that a bacterial volatile integrally modulates the immune system and the PSR and determines the relationship between the bacterium and the plants. They show that natural volatiles effects growth and that DA effect colonization under Pi sufficient conditions but has a negative effect on the plant during Pi starvation. The relationship was not fully analysed and the response of the plant to the colonization by the bacterium under these two conditions (or I missed it).

Answer: We are thankful for these helpful suggestions and have revised the manuscript accordingly.

Are the used amount of DA biologically relevant? Can the authors comments on the concentration measured in the free space of growing bacterial colonies and in soil?

Answer: GB03 produces DA at a rate of 5.13 µg per mL free space per 24 hr, as measured from the free space of growing bacteria colonies (Farag et al., 2006). No parallel data of GB03-produced DA is available in soil, because quantification of a volatile compound from a particular microbe species in the soil is technically challenging. However, although the average concentration of DA from GB03 in a given volume of soil probably would be lower than that from medium-grown bacterial colonies, it is possible that the porous soil may provide micro-environments where the bacterial volatiles can accumulate to a significantly high level and affect the root in vicinity.

In this study, we used DA-containing solid agar droplets for the volatile treatment, in order to mimic the bacterial way of continuous releasing of DA. Even if the solid droplets release DA completely all at once, the volatile concentration would be just 9.7 µg per mL free space in the petri dish. Thus we consider the slowly released DA as biologically relevant. Details of the treatments are described in Materials and Methods under the subtitle “Natural GMV and chemical treatments”.

Minor comments:
I would suggest to harmonize all the figures by using a similar style and by calculating significances using ANOVA or t-test (e.g. Fig. 1C; 2A; 2D; 3C; 3E; 4A; 4B; 4E; 5B; 5C).

Answer: We have made these revisions as suggested.

All the figures showing transcriptional data are not clearly labelled, Are these fold changes or relative expression? To which genes the data are normalized (e.g. AtUBI)? (e.g. 2D; 3C; 3E; 4A....).

Answer: In all RT-qPCR measurements, gene expression levels are normalized by the house-keeping gene ACTIN 2 and then presented as values relative to the expression level of the corresponding mock samples. In the revised version, we have made clear labels.

The legends are also not really informative. Information about the age of the plants is often missing and when present is not consistent with the information given in the M&M (e.g. trypan blue 11 DAT or 10DAT?). Number of replicates, plants used, incubation and preincubation times.....

Answer: Revisions have been made as suggested.

Sometimes DA and sometimes BTDN is used in the figures. Sometimes letter are used and sometimes asterisks (e.g. figure 5 F could be analysed by ANOVA). Figure 5G should be Ba not Bs.

Answer: Revisions have been made as suggested.

Figure 1E cannot be read and so the corresponding supplementary figures.

Answer: The mission of Figure 1E is to highlight the differences between "0.05T vs 0.5C" and "0.05C vs 0.5C". Because of the limited space for the figure, only the highlighted GO categories are made easily readable. Similarly, we emphasize the overall pattern and have to sacrifice the legibility of DEG AGI numbers in Figure 5A. However, lists of the corresponding DEGs (differentially expressed genes) are provided as supplementary tables. In the revision, we have added reminders of DEG lists to the legends of Figure 1E and of the other figures wherever applicable.

Why no figure was shown for the 0.005T vs. 0.005C as this would be to me the most obvious comparison. Also this is not significance of the expression as written in the legend of figure 1E, this is enrichment analysis of GO terms.

Answer: Compared to the control samples (0.5C), the effects of 0.05T are contributed by both of the nutrient deficiency (0.05 MS) and the bacteria exposure (T). So we present the transcriptome patterns that are solely caused by nutrient deficiency (0.05C vs 0.5C) in parallel to the patterns of "0.05T vs 0.5C", in order to distinguish bacteria effects from the effects of nutrient deficiency while providing the whole profiling of 0.05T.

These suggested modifications have been made, including a new supplementary figure Appendix Figure S1C-D showing the comparison of 0.05T vs 0.05C.

It would be good to introduce the organism earlier in the paper. It is only mentioned at the end of the introduction.

Answer: Revision made.

Referee #2:

The manuscript by Morcillo et al presents how the plant growth-promoting rhizobacteria Bacillus amyloliquefaciens GB03 facilitates its association with Arabidopsis through phosphate-dependent modulation of plant immunity. They show that volatile molecules produced by B. amyloliquefaciens promotes plant growth under P sufficient conditions, while inhibiting growth under Pi deficiency. Such suppression of plant growth under deficient conditions is through the enhancement of SA and JA-mediated immunity, resulting in anthocyanin accumulation and enhanced cell death. They further identify diacetyl produced by

GB03 that suppresses immunity under Pi-sufficieny and promotes immunity under Pi-sufficiency.

The paper represents a significant advance, that is interesting and novel, with broad interest for those working in microbial associations. However, the results are really perplexing, because the authors appear to be claiming completely opposing effects of DA under Pi-sufficient and deficient conditions: how can the same molecule both suppress or induce immunity?

Answer: DA shows differential, instead of "completely opposing", effects on P-sufficient and P-deficient plants. Briefly, in P-deficient plants, DA elevates plant SA and JA levels as well as the SA- and JA-dependent immune responses; in P-sufficient plants, the effects of DA on plant immunity is demonstrated only by a partial suppression of microbe-induced ROS production and of the expression of some immune response-related genes, meanwhile the other examined PTI responses such as MAPK activation are not affected by DA, and the levels of SA and JA are not affected by DA either. In fact, that DA partially suppresses microbe-induced ROS production was also observed in P-deficient plants (new results, shown as Figure EV4E in the revised version).

Why does DA show differential effects on plant immunity under different P conditions? This is a primary question about the molecular mechanism of DA, and we are still pursuing the answer (please also see the last paragraph of Discussion), because it is also the most difficult question that requires identifying the plant sensor of DA. In the past of a few years, evidence are accumulating that point to a significant role of P in plant immunity (e.g., Hiruma et al., Cell, 2016; Castrillo et al., Nature, 2017). DA partially suppresses microbial induction of ROS but not disease resistance in P-sufficient plants, whereas it strongly induces SA- and JA-mediated immunity and hyper PSR (phosphate starvation responses) in P-deficiency plants. These observations not only reveal the bi-faceted role of DA in mediating plant-microbe interaction, but also highlight the importance of the interplay between plant immunity and PSR for future discoveries of DA's molecular action.

Furthermore, the results shown in this paper are in conflict with Hacquard et al (2016 Nature Communications) that demonstrated beneficial responses were prioritized in root endophytic fungi-colonized roots under phosphate-deficient conditions, whereas defense responses were activated under phosphate-sufficient conditions. The authors need to justify why these studies are inconsistent.

Answer: In addition to Hacquard et al. (2016), previously it was also reported that plant Pi deficiency is required for the establishment of Arabidopsis symbiosis with *Colletotrichum tofieldiae*, an endophytic fungus that can transfer phosphorus to its host (Hiruma et al., Cell, 2016). It is thus intriguing whether plant mutualistic associations with beneficial soil microbes commonly prefer Pi deficiency. We discovered that P-sufficient plants benefit from the bacteria *Bacillus amyloliquefaciens* strain GB03, whereas P-deficient plants suffer from the same bacteria. Thus our findings and those reports on plant-fungi interactions together demonstrate that plants use different strategies for bacteria and fungi when determining mutualism or immunity.

We emphasized this point of view in the first paragraph of Discussion and hypothesized a reason for it in the legend of Figure EV4; Appendix Figure S5. "Pi-deficient plants allow symbiosis with certain fungi because endophytic fungi can transfer phosphorus to plants, whereas Pi-sufficient plants need no fungi-assisted Pi uptake and so they deploys Trp-derived secondary metabolites to defend against fungi invasion (Hiruma et al., Cell, 2016). In contrast, because rhizobacteria do not transfer phosphorus to plants, plants allow mutualistic association with these rhizobacteria only under Pi-sufficient condition, whereas Pi-deficient plants deploy phytohormone-mediated immunity to ward off the bacteria competitors for Pi uptake".

Comments:

1. The results under PI-sufficient and deficient conditions are completely opposing, DA promotes immunity under Pi-deficiency and suppresses immunity under Pi-sufficiency. I find it hard to see how the same molecule can do both of these things, simply based on a change in the Pi-status. This confusion is confounded by the fact that different assays are presented for DA treatment under Pi-sufficient and deficient conditions. I think there needs to be consistency in the assays performed under Pi-sufficient and deficient conditions, ie between figure 4 and 5. Only with equivalent assays can we assess whether the effects are really indeed opposing or whether infact the molecules are having alternative effects at different Pi concentrations. I would like to see the effects of DA on immunity performed in figure 5B, C, D and F under Pi sufficient and deficient conditions.

Answer: DA shows differential, instead of "completely opposing", effects on P-sufficient and P-deficient plants. Briefly, in P-deficient plants, DA elevates plant SA and JA levels as well as the SA- and JA-dependent immune responses; in P-sufficient plants, the effects of DA on plant immunity is demonstrated only by a partial suppression of microbe-induced ROS production and of the expression of some immune response-related genes, meanwhile the other examined PTI responses such as MAPK activation are not affected by DA, and the levels of SA and JA are not affected by DA either. In fact, that DA partially suppresses microbe-induced ROS production was also observed in P-deficient plants (new results, shown as Figure EV4; Appendix Figure S5 in the revised version).

Before the revision, we did not examine the effects of DA on bacteria colonization and plant PTI responses under P-deficient condition, because under this condition we focused on the reasons for DA-induced plant hypersensitivity to P deficiency. During the revision, we performed these assays with P-deficient plants. The results (Figure EV4; Appendix Figure S5 in the revised version) show that DA has similar effects on plant PTI responses in P-deficient plants and P-sufficient plants; however, DA does not increase GB03 colonization to P-deficient plants. The latter observation probably is due to the strongly activated SA- and JA-mediated immunity.

2. There is no introduction for Bacillus amyloliquefaciens GB03 and its produced volatile chemicals. Is there any report how GB03 promotion of plant growth without transfer of nutrients from soil? How important are the volatiles for plant-bacterial interactions?

Answer: A brief introduction of *Bacillus amyloliquefaciens* GB03 and its volatiles has been added to the revised manuscript. GB03 colonizes roots and is capable of stimulating plant vigor through production of microbial volatiles (MVs), which modulate plant hormone homeostasis and nutrient uptake (Ryu et al., 2003; Zhang et al., 2007; Zhang et al., 2009; Paré et al., 2011; Beauregard et al., 2013).

Bacteria release volatile emissions along with other secretions. Like some known non-volatile bacteria factors, certain bacteria volatiles can play important roles in affecting plant growth and/or stress responses (e.g, reviewed in Liu and Zhang, *Frontiers in Plant Science*, 2015). For instance, dimethyl disulfite can improve plant sulphur nutrient assimilation (Meldau et al., Plant Cell., 2013), while indole stimulates plant lateral root formation (Bailly et al., Plant J., 2014). In soil, the average concentration of bacteria volatile in a given volume of soil probably would be lower than that from medium-grown bacterial colonies; however, it is possible that the porous soil may provide micro-environments where the bacterial volatiles can accumulate to a significantly high level and affect roots in vicinity.

3. Line 91: 'induced by nutrient-deficiency were enriched in immune response', how do you define these genes are immunity-related? Also how do these immunity genes compare to the overall transcriptional changes, what percentage is this of the total transcriptional changes?

Answer: We used BioMaps Function Analysis of Virtual Plants 1.3 platform for Gene Ontology (GO) enrich analysis, using the annotation/classification established by the platform as source for the analysis. According with this platform, the percentage (expected frequency) of

the annotated "immune response" genes in nutrient-deficiency conditions is 4.3% (69 out of 1606 genes), while in normal conditions the expected frequency is 1% (260 out of 24961 genes).

4. The text in Fig. 1E and Fig. 5A and Supp Figs.S1D, S1E, S1F and S2A is illegible at this magnification.

Answer: The mission of Figure 1E is to highlight the differences between "0.05T vs 0.5C" and "0.05C vs 0.5C". Because of the limited space for the figure, only the highlighted GO categories are made easily readable. Similarly, we emphasize the overall pattern and have to sacrifice the legibility of DEG AGI numbers in Figure 5A. In order to compensate this, lists of the corresponding DEGs (differentially expressed genes) are provided as supplementary tables. In the revision, we have added reminders of DEG list to the legends of Figure 1E and of the other figures wherever applicable.

5. Fig.1: why was plant fresh weight not measured? I think this is a more direct test for plant growth.

Answer: The results of plant fresh weight were provided as a supplementary data in Figure EV1B.

Fig1C, there is an obvious difference between GB0.5MS and control 0.5MS in panel A, but why do you see no difference in panel C?

Answer: In Figure 1, Panel A shows plant images taken at 11 days after treatment (DAT); Panel C shows plant photosynthesis efficiency over a course of time (from 3 to 11 DAT). Although in Panel A leaf colour of the "GB03—0.5MS" plants looked greener than the "control—0.5MS" plants, the average Fv/Fm value (the indicator of photosynthesis efficiency) of "GB03—0.5MS" plants is not statistically different from that of the "control—0.5MS" plants, even though the former is slightly higher than the latter.

Please include statistical analysis to panel C.

Answer: Statistical analysis has been included in all the graphs.

Fig.1D needs quantification.

Answer: In addition to Fig. 1D that shows cell death visualization by trypan blue staining, the inhibitory effects of GB03 volatiles on plant growth were also demonstrated by the other results in Fig. 1 and Figure EV1, including plant size as measured by leaf area and fresh weight, photosynthesis efficiency, and the expression of stress indicator genes including cell death-related genes. For this reason, we proceeded to investigate the mechanisms without further quantification of Fig. 1D.

Fig.1E or supplementary needs to include the comparison between 0.05T vs 0.05C.

Answer: This data has been included in the revised version as Appendix Figure S1C-D.

6. Fig.2: There is a very clear P starvation response in Figure 1 between control 0.5MS and control 0.05MS, but the starvation genes in 2A show little difference. Why this inconsistency? Again you need the statistical analysis in this panel.

Answer: In Fig 2 A, there are statistically significant differences between "control 0.5MS" and "control 0.05MS", especially in roots. But these are not eye-catching because the gene expression levels of these two samples are markedly lower than that of "GB03—0.05MS". In the revised version, we have included the statistical analysis to all data sets so as to indicate those differences.

Fig.2B is not so clear to see the root blue color.

Answer: We have adjusted the image contrast for improved visualization of root blue colour.

Fig.2C, the same issue as I mentioned above, from the pictures in Fig.1a and Fig.2B, the controls definitely show the differences in anthocyanin accumulation, but Fig.2C shows all the controls and even GB03 0.5MS are pretty same, can you explain this?

Answer: The anthocyanin levels of "control 0.5MS" and "control 0.05MS" were quantified in many experiments in this study, and we are confident that their anthocyanin levels are similar as measured, even though their leaf colours sometimes may show variation. The difference in leaf colours, in terms of the levels of darkness, reflects differences in not only anthocyanin levels but also chlorophyll contents. Under 0.5 MS condition, GB03 volatiles increase plant chlorophyll contents (Zhang et al., The Plant Journal, 2008). This can make the "GB03—0.5MS" plants look darker than the "control—0.5MS" plants.

Fig.2D shows GB03-induced IPS expression can be completely blocked by supplement of Pi, however, Fig.2C just shows partially dependent on Pi supplement, could you comment on this?

Answer: Fig. 2C shows measurements of anthocyanin accumulation, which could be induced in the 0.05MS by certain other stress conditions in addition to P deficiency, especially after a long time exposure to the stress condition in combination with DA. Thus it is reasonable that the supplementation of P to the 0.05MS medium does not completely reset the DA-induced anthocyanin level. This is different from the case of *IPS1* (Fig. 2D), which is a marker gene of P deficiency response.

Fig.2E-2F, define what cm2 is.

Answer: cm^2 is the abbreviation of square centimetre. This definition has been added in the revised version.

7. I find it confusing the Diacetyl is refered to as DA in the text and in figure legnds, but BTDN in figures. You need to be consistent.

Answer: We are thankful for this and the other careful corrections. We have made the modifications as pointed out.

8. Fig.3: in fig.3B, there is a significant difference for mock treatments between +P and -P, why you cannot see in fig.2C? Since the controls are quite variable, I am not convinced by the conclusions.

Answer: After identifying P deficiency as the key factor for GB03-induced plant stress, we further studied P-deficiency stress by transferring seedlings to the –P (phosphorus depletion) growth medium instead of using 0.05MS. Fig. 3B used –P for the stress condition, while Fig. 2C used 0.05 MS that still contains P at the level of 1/20 of MS medium. Because the stress of P deficiency is severer in the –P medium than in 0.05MS, the stress-induced anthocyanin accumulation is relatively more obvious with –P (Fig. 3B) than 0.05MS (Fig. 2C).

9. Fig.4: Fig.4C shows BTDN treatment cannot inhibit plant growth under +P, but SA does, suggesting that BTDN and SA have different functions. This is not what is stated in the text.

Answer: We proposed that DA-induced hyper PSR is mediated through SA-/JA-dependent pathway. This is not equal to a conclusion that DA and SA/JA have the same functions in every way. Functional overlap does not necessarily mean the two compounds function exactly in the same way. In addition, compound dosages can be another factor that may possibly lead to variations in the outcomes.

Fig.4F shows the NahG plants just partially required for BTDN-induced anthocyanin under low Pi, how about other SA-deficient mutants like sid2? It would be useful to see quantification of Fig. 4F

Answer: We have examined sid2 as suggested, and we observed a reduction in DA-induced anthocyanin accumulation in sid2, but the degree of reduction is less than that in the NahG plants (data not shown). This is consistent with the reports that NahG degrades SA from all sources within the plant, whereas *sid2/ics1* suppresses only ICS1-dependent SA accumulation (Wang et al., *Plant cell* 2013).

Quantification of Fig. 4F is shown in Fig. 4G.

10. Fig.5. Is the BTDN suppression of flg22-induced ROS significant?

Answer: Yes it is. In addition to Figure 5B (Figure EV4 C in revised manuscript) that shows the dynamics of ROS production, Figure S7C (Figure 5B in revised manuscript) shows that ROS production (quantified as the total RLU of 60 minutes) was clearly suppressed by DA with statistical significance. To make this point clear, we have switched the positions of the two figures in the revised manuscript.

11. Fig.2B and Fig.S6B show BTDN can induce massive SA and JA synthesis under low Pi, but very surprisingly the author could not see any pathogen resistance phenotype in fig.5E, how could you explain this?

Answer: During the revision, we have performed independent experiments and have confirmed these results. That DA does not increase pathogen resistance in P-deficient plants can be explained by the findings in Mammarella et al., (*Phytochemistry*, 2015). In their study, ROS play an important role in SA-mediated defense against *Pseudomonas syringae*. In our study, DA suppresses ROS production in P-deficient plants (Figure EV4E in the revised version); this may counteract the elevation in SA and JA levels.

If you mix inoculation of GB03 and PstD3000 or Ralstonia GMI1000 under high P and low P conditions, can you see any pathogen growth effect?

Answer: In addition to the differential microbial effects on plants, direct microbe-microbe interactions can be another factor that can make plant responses complicated and different from those in response to individual microbes. Currently we prefer to focus on investigating the early signalling events of plant perception of DA.

12. Fig.6: Is DA only produced by B. amyloliquefaciens? What promotes plant growth under B. amyloliquefaciens colonization under high P conditions?

Answer: Some other bacteria may also produce DA. Interestingly, DA was not detected in the volatile emissions released from *R. solanacearum* strains GMI1000 and phcA (Spraker et al., *J Chem Ecol.*, 2014). Ryu et al. (*PNAS*, 2003) reported that 2,3-butanediol in GB03 volatiles can promote plant growth. In our study, the suppression of ROS production and the induction of hyper PSR are specific effects of DA and not of 2,3-butanediol or several other examined volatile components.

13. The authors present the model as if the plant wishes to be colonized under high Pi conditions. For what purpose? Rather I think the authors could consider DA as a chemical effector, that facilitates colonization under high Pi conditions.

Answer: We agree with the point that DA is a chemical effector that facilitates colonization to P-sufficient plants. Indeed, in the legend of Figure 6 that shows the proposed model, we objectively described the effects of DA under P sufficient and deficient conditions. "*In*

phosphate (Pi)-sufficient plants, Diacetyl (DA) partially suppresses .... resulting in enhanced symbiont colonization without compromised disease resistance".

The model is also shown in Figure EV5 (in the revised version), which is meant to highlight the difference between our findings of plant-bacteria interactions and the findings of plant-fungi interactions by Hiruma et al. (Cell, 2016). In the legend of Figure EV5, we have toned down by modifying the statement from "…..the different strategies underlying plant decisions on immunity or mutualism with bacteria and fungi" to "…. the different strategies underlying plant responses to bacteria and fungi in terms of immunity or mutualism".

Other reports have shown that immunity is maximized under high Pi, this would make sense. However, in this paper the authors are arguing the opposite. They need to explain this discrepancy.

Answer: As exemplified in Hiruma et al. (Cell, 2016), P-sufficient plants accumulate Trp-derived secondary metabolites (but not phytohormone-mediated immunity) for defence against fungi, whereas such defence activity is lowered in P-deficient plants to allow symbiosis. In our study, phytohormone-mediated immunity is activated by DA in P-deficient plants but not in P-sufficient plants. These findings are not arguing the opposite on the same issue; instead, these findings collectively suggest that plants use different strategies in response to bacteria and fungi to determine mutualism or immunity (Figure EV5 in the revised version).

We have also hypothesized a reason for the differential plant-microbe interactions in legend of Figure EV5, "*Pi-deficient plants allow symbiosis with fungi because endophytic fungi can transfer phosphorus to plants, whereas Pi-sufficient plants need no fungi-assisted Pi uptake and so they deploys Trp-derived secondary metabolites to defend against fungi invasion (Hiruma et al., Cell, 2016). In contrast, because rhizobacteria do not transfer phosphorus to plants, plants allow mutualistic association with these rhizobacteria only under Pi-sufficient condition, whereas Pi-deficient plants deploy phytohormone-mediated immunity to ward off the bacteria competitors for Pi uptake*".

14. Fig.S8 panel A is just repeating the main Fig.6, I think it is not required.

Answer: Figure S8 (now Figure EV5 in the revised version) is meant to highlight the difference between plant-bacteria interactions and plant-fungi interactions. Usually supplementary figures are shown as online files separated from the main manuscript. Thus by showing the Panel A together with the Panel B in the same figure, it would be more convenient for the comparison between the two panels.

15. All the qRT-PCR figures need to add statistical analysis.

Answer: Revision has been made as suggested.

16. The authors need to be careful in the use of the term 'symbiosis' In my view this reflects a very tight association between plant and microbe, such as that seen in legumes with rhizobia and during mycorrhizal associations. Arabidopsis lacks symbiosis signaling and lacks these closely associated intracellular symbionts. I think it is inaccurate to call the association described in this manuscript a symbiosis. Rather a commensal association would be a more appropriate term.

Answer: We agree that "symbiosis" can be misleading and have replaced it with "mutualistic association".

17. In the abstract and line 261-263, the authors claim that DA modulates the PSR system. I see no evidence for this. Rather the DA response is dependent on the PSR system, this is very different to demonstrating that DA modulates PSR. Please be more careful in your wording.

Answer: In both the abstract and lines 261-263, we have revised "*DA integrally modulates the PSR system and phytohormone-mediated immunity in Arabidopsis to determine the types of relation between plants and rhizobacteria*" to "*DA affects the types of relation between plants and certain rhizobacteria in a way that depends on plant PSR system and phytohormone-mediated immunity*".

18. Lines 63-64 and 271-273. The authors are making very broad claims here about differences between bacterial and fungal associations. The system they describe appears to be quite specific to this particular bacteria, or do they have evidence that all mutualistic bacteria produce DA? Please remove such broad claims and be more careful in your wording. The work you have demonstrated is describing one bacterial association and unless there is evidence that this is representative of all bacteria, then modify the breadth of the claims.

Answer: Thanks for pointing this out. We have revised "…. *reveal that plants use different strategies for bacteria and fungi in determining mutualism or immunity*" to "…. *provide an example where plants use different strategies for bacteria and fungi in determining mutualism or immunity*". In addition, we also added the term "certain" in front of the terms "rhizobacteria" and "fungi" wherever appropriate (e.g., see the answer to Question #17) to avoid claiming broadly.

19. Xiao et al 2018 ref is missing

Answer: Corrected.

20. Line 302: show the data or don't make the statement.

Answer: DA has a strong buttery flavor and aroma. As a result, it is a common food flavoring ingredient. Despite being listed by the US FDA to be 'generally regarded as safe' (GRAS), multiple lines of evidence suggest that exposure to high concentrations of DA vapor causes long-term impairments in lung function (Brass and Palmer, Toxicology, 2017). In this part of Discussion, we meant to draw attention on possible effects of DA on human immunity and endophytic microbes. We consider this as an important opinion and is worth of mentioning as a perspective in the Discussion, even though it may turn out to be wrong in the future.

But with this review opinion, we realized that the sentence "DA is found in a variety of beverages and dairy foods, and is emerging as an important factor in some human diseases" can be misleading. So we have revised it to "DA is found in a variety of beverages and dairy foods".

Again, we thank both reviewers for the critical reading and the helpful comments.

2nd Editorial Decision 6th Nov 2019

Thank you for submitting a revised version of your manuscript. It has now been seen by the original referees, who find that their main concerns have been addressed and are now broadly in favour of publication of the manuscript. There remain only a few mainly editorial issues that have to be dealt with before I can extend formal acceptance of the manuscript:
1. Please address the remaining minor comments from the reviewer #2 and include appropriate discussion and tone down the statements as requested.

REFEREE REPORTS:

Referee #1:

The paper is improved and all my concerns were addressed with either new experiments or by better stating their conclusions. I am fully satisfied. It is an interesting thought to have bacteria or fungal associations depending on Pi requirements.

Referee #2:

The revised manuscript has addressed the previous major concerns. However, there are still some conclusions proposed by authors that need to be corrected to reflect accuracy of what is demonstrated.
In lines 257-258, 'DA suppresses microbe-induced ROS production and certain other defense responses in P-sufficient plants, without sacrificing defense to pathogens'.
Lines 293-294, 'DA can facilitate mutualistic association between plants and beneficial bacteria without sacrificing plant defense against pathogens'

The data presented here is not sufficiently convincing to support these conclusions. Since the authors focus on plant-root bacterial interactions, I am quite surprised that most of assays have been done in Arabidopsis leaves or whole seedlings such as ROS production, MAPKs activation and all the gene expression data. I assume that treatment of DA in roots could have different effect for root pathogen infection. I was wondering why the authors did not inoculate Ralstonia on the roots instead of spray inoculation of DC3000 on the leaves for the DA treatment. Please be more circumspect in the above statements, since these statements are not fully accurate for what has been demonstrated

There are some minor comments
1. Fig.1A, the 'S' is missing for '0.05 M'. Fig.1B, align the letters with corresponding columns. Fig.1D should be added the bar.
2. Fig.1E. There is still some blank space in figure 1, I think authors should zoom figure.1E to make the text legible.
3. Fig.3C. The columns for '+Pi' in the graph look like grey not yellow.
4. Fig.4G. NahG should be italic.
5. Fig.6. The model should use dash line to show DA inhibition of pathogens as there is no data to support this in the manuscript.
6. Arabidopsis and strain names should be italicised in the main text.

2nd Revision - authors' response 8th Nov 2019

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the gene expression data. I assume that treatment of DA in roots could have different effect for root pathogen infection. I was wondering why the authors did not inoculate Ralstonia on the roots instead of spray inoculation of DC3000 on the leaves for the DA treatment. Please be more circumspect in the above statements, since these statements are not fully accurate for what has been demonstrated

Answer: We agree that the statement is over broad and have revised it to "…. without sacrificing defense to the examined pathogen *Pst* DC3000".

For these experiments, we did not use Ralstonia because it is so devastating that it easily kills Arabidopsis and also because we wanted to reduce any potential risk of spreading this devastating pathogen into the environment. Alternatively, we used DC3000, which is a model pathogen for studying bacteria pathogen-triggered plant immune responses. Although DC3000 can also affect plants by root infection (e.g., Bias et al., *Plant Physiol*. 2004), its effects on plants are typically investigated in shoots or in whole plants especially for Arabidopsis. Unlike large size plants such as maize, Arabidopsis has small roots that are difficult to accurately quantify for the ROS measuring assays. In contrast, leaf disks of the same size can be easily reproduced from Arabidopsis. Thus we followed standard protocols to investigate flg22-triggered PTI responses and the disease resistance to DC3000 in aerial portions or in whole plants.

In addition, DC3000 is also a model pathogen for studying ISR (induced systemic resistance) that can be triggered by some beneficial rhizobacteria (Pieterse et al., *Annu Rev Phytopathol.*, 2014). This is another reason that we used DC3000 for studying plant immune responses in this work.

There are some minor comments
1. Fig.1A, the 'S' is missing for '0.05 M'. Fig.1B, align the letters with corresponding columns. Fig.1D should be added the bar.
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5. Fig.6. The model should use dash line to show DA inhibition of pathogens as there is no data to support this in the manuscript.
6. Arabidopsis and strain names should be italicised in the main text.

Answer: Corrections were made. We sincerely thank the reviewers for the critical reading.

3rd Editorial Decision 14th Nov 2019

Thank you for submitting the revised version of your manuscript. The main issues have now been addressed and I am pleased to inform you that your manuscript has been accepted for publication.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Huiming Zhang
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-102602

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:

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- 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.

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

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- 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.
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  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?	Data from each experiment were collected from 3 or more biological replicates. For each biological replicate, a minimum of five individual plants were used.
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?	All samples from experiments without any technical error were analyzed.
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.	Experiments were repeated at least three times independently. Attentions were paid to potential technical errors or systemic variances among independent experiments, in order to avoid any false positive or false negative.
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, we used standard softwares such as IBM SPSS

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Is there an estimate of variation within each group of data?	Standard Errors within each group were analyzed by using Microsoft Excel and Prism.
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).	NA
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

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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
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GSE138478
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
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

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