EMBO reports

Microbe-induced drought tolerance by ABA-mediated root architecture and epigenetic reprograming

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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.)

Dear Prof. Hirt,

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees indicate that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed as indicated in the reports, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures (up to 8) and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) 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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

9) Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, and remove the author contributions from the manuscript. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In this manuscript Khairiah Alwutayd et.al. describe an SA190-mediated drought tolerance mechanism in plants. While overall the manuscript is of good quality, I find that several points need to be improved.

Major points:

1. In contrast to the initial observation about the effect of SA190 on drought tolerance the molecular mechanism behind is not clear at all. The authors claim the effect is through ABA and aquaporin. But to substantiate the claim they should show the effect in SnrK2 mutants, especially since, those have been shown to target aquaporins.

The involvement of aquaporins is a bit confusing for me. In Fig 5D and E the authors show that AgNO4 and HgCl2 press SA190 affect on fresh weight in 25%PEG. But no effect can be seen in mock-treated (no SA190) plants when comparing mock (no inhibitors) and inhibitors-treated plants. So If I understand it correct;y the authors can not observe any effect on fresh weight in 25%PEG when aquaporins are inhibited? So I am confused - if aquaporins are not in evolved in drought then why are they required for SA190-mediated drought tolerance?

2. The title of the work use the work "epigenetic reprogramming" I understand this in a way that authors suggest that pre-incubation of the plants with SA190 induces H3K4me on specific genes that allows the genes to respond stronger/faster to drought. This point has not been proven conclusively as the SA190 bacteria are present throughout the experiment. I think a wash of the bacteria combined with antibiotic treatment after initial exposure to SA190 but before drought would help to prove the point.

3. The experiments with alphaalpha are promising but based on one year trial. As a minimum, the authors should clearly state in the discussion that those are preliminary data and can not be easily extrapolated.

4. I am not a specialist but I do find that several bacterial-mediated droughts tolerance examples have been described. I, therefore, like to ask the authors to discuss the novelty of their finding in light of the published work in a more clear way. It would be highly beneficial for the manuscript to compare side by side the Sa190-induced drought tolerance with at least one of the other bacterial strains published before. Are those also mediated by the root growth as the authors find for SA190?

Mainor point:
ChIPS shown in fig4 seems to be based on 2 bio-rep which is not enough.

Referee #2:

The manuscript by Alwutayd et al deals with the impact of a root endophytic bacterium on plant tolerance to drought stress. In this manuscript, the authors report that inoculation by Pseudomonas argentinensis SA190, an endophytic bacterium isolated from the desert plant Indigofera argentea, leads to improved drought stress tolerance in Arabidopsis in laboratory conditions as well as in alfalfa in field conditions. Colonization of roots is increased in osmotic stress conditions. They then uncover part of the molecular mechanisms responsible for this phenotype. They show that this increased tolerance is due to ABA-dependent changes in epigenetic marks in target genes including aquaporin encoding genes.

This work is original and describe some novel molecular mechanisms by which soil bacteria change plant gene expression and contribute to stress tolerance. The experimental approaches are sound and clearly support the conclusions. The results are presented and discussed in the context of earlier literature. While many papers address the impact of bacteria in model plants in laboratory conditions, this manuscript also reports their impact in realistic conditions (including field trials), and it describes an original molecular mechanism involving changes in epigenetic marks in the plant genome that are responsible for the increased stress tolerance. Besides its scientific interest, this manuscript also provides a potential strategy to improve crop productivity in

drylands particularly from Africa to India (native range of Indigofera argentea) where drought is a major issue and crop productivity is low. Because of this, I believe it will be of interest for a large community of researchers from microbial ecology to plant genetics and epigenetics.

Major points:

1) In the manuscript, the authors report results using "realistic" water stress treatments (in pots in Arabidopsis and in field conditions for alfalfa) and well as PEG-treatments (osmotic stress) as a proxy for drought in Petri dishes experiments in Arabidopsis. While this experimental approach is perfectly fine, the results of these different experiments are all presented as "drought stress". This is confusing because 1) PEG treatment is different from drought and 2) the reader does not know how the results presented were produced (e.g. text on the RNAseq data). To avoid this shortcoming, I recommend replacing throughout the text and in the figure legends "drought stress" by either "PEG treatment" or "osmotic stress" when describing the results of experiments performed with PEG and use drought stress only when using more realistic systems.

2) Pseudomonas argentinensis SA190 was isolated from nodules of a desert Fabaceae plant. Could it fix nitrogen, and could that somehow contribute to its effect? This is particularly critical for the field trials with alfalfa - another nitrogen-fixing plant - as it could explain the beneficial effect of inoculation without having any link with increased water-stress tolerance (and could explain the beneficial impact observed without water stress). Indeed, transfer of nitrogen-fixation from rhizobia to other bacteria has been reported. One quick way to test it would be to inoculate alfalfa grown in sterile conditions without any N source (+ control without inoculation and a control with an alfalfa-infecting rhizobium) and test whether the SA190 strain could provide N to the plant. Testing the effect on a cereal in field conditions would be another way to address this point.

This is a critical point to address to make sure the effect reported in field conditions is really related to increased drought tolerance.

3) Root colonization by SA190 is enhanced by PEG treatment. Bacteria seems to enter the vascular tissues based on Fig 1J. If true, it would be nice to describe this in more details in the text (is there a qualitative change in root colonization in response to PEG).

4) Materials and methods, Line 565, I believe the formula given for the calculation of the "transpiration rate" gives a transpiration ratio instead (transpiration rate would be in transpiration/time).

5) Field trial with alfalfa, did the authors monitor the soil water content (fraction of transpirable soil water - FSTW - for instance) to have an idea of the onset and intensity of the stress faced by plants in both treatments?

Minor points:

6) Abstract, line 26, replace "root morphogenesis" by "root development".
7) Line 59, drought stress is not a "trait".
8) Fig 1, the order to the subpanel in the figure should follow their appearance in the text. This is not the case in this version.
9) Fig 1B, would be nice to change the organization of the pictures to be homogeneous with Fig 1A. It would make it easier to read.
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11) Line 177, rather than showing "a strongly developed root system", SA190-inoculated plants maintained their root development at WT level while mock-treated plants had a strong reduction in root growth.
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15) Line 332, correct "idnuced"
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Referee #3:

The authors described an interesting study "Microbe-induced plant drought tolerance by ABA-mediated root morphogenesis and epigenetic reprogramming of gene expression" The results showed that a PGPB- Pseudomonas argentinensis sp. SA190, enhances drought stress tolerance in Arabidopsis. The authors showed that the drought tolerance induced by SA190 is ABA dependant.

The data also showed that the application of the SA190 enhances the drought resilience of alfalfa in field trials, however the authors only showed yield data in Fig.1 but not pictures of the plants. Was the drought stress applied in the field a real stress? On Fig.1 I no difference is observed between the well-watered and drought stress controls. Most of the studies using single

PGPB show no plant growth promoting effect in the field (where the introduction of a single bacteria to an establish community will not make a difference)! Can the authors comment on this?

Also the authors should show fresh and dry biomass data from the field trials and not only yield harvest data! It is well known that drought stress in plants is ABA dependant, I do not see something new that is shown in this study to explain the molecular mechanism of drought stress resilience when plants are inoculated with PGPB-SA190.

I have several comments :
Line 54 - at the end missing reference.
Line 132- The authors described the screen with several dessert bacteria but did not show the data only for SA190!
Line 141- Fig. not Figs
Fig. 1B - The seedlings were grown for 16 days (the primary roots will overgrown the length of the plate as well as the lateral roots are overlapping so how did the authors quantify the roots properly?. I also do not see any bacteria around the root! I wonder why?
Fig.1 J- It is not very clear from the picture where the colonization is (I think the authors should show a higher magnification. It is even not clear if this is the root elongation zone, because at the root elongation zone there are no root hairs yet.
Line 173- Root morphology should be root architecture.
Line 240- root structure should be changed to root architecture under drought stress
Line 274- To me it looks like TIP1-2, PIP1-5 AND TIP2-2 are significantly increased upon SA190 inoculation in non-stress conditions, but this is not indicated in the plots!
Fig.3- I think something is not correct with the statistics again. For instance in the last plot- Fig.3C (for TIP2-2) there is a clear significant difference between col-0 Mock and Col-0 + SA190 in 1/2 MS, but this is not indicated with asterisks?
Line 303- Fig.4 Again something is wrong with the statistics. For example the first plot (PIP1-1), the asterisks of R2 are not correct.
Line 348- replace massive with observed.

Dear Achim,

We hereby submit the revised manuscript: "**Microbe-induced drought tolerance by ABA-mediated root architecture and epigenetic reprograming**" for publication in your esteemed journal.

As shown by the point-by-point discussion below, we have answered to and corrected all issues raised by the reviewers. Thanks to the very precise and helpful criticism of the reviewers, we hope that you agree that the revised manuscript could be substantially improved to meet the expectations of EMBO Reports.

Best regards
Heribert

Referee #1:

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Major points:

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We agree with the reviewer that the SnrK2s should be involved in the regulation of the beneficial effect of SA190 and the aquaporin genes. However, we think this is clearly proven by the experimental evidence provided in this work. The role of ABA is supported by the use of two individual mutants, the *aba2* biosynthesis mutant and the quadruple *pyr/pyl* ABA signaling complex mutant, which compromises the function of the SnrK2s. Both mutants completely suppress the beneficial effect of SA190 with respect to PEG-induced drought stress (Fig. 2) and both mutants fully suppress the SA190-mediated regulation of the aquaporin genes both at the level of expression (Fig. 3) and its epigenetic regulation (Fig. 4). To test multiple *snrK2* knock out mutants will be time consuming and, in our view, will not provide further insight into the function of SA190.

The involvement of aquaporins is a bit confusing for me. In Fig 5D and E the authors show that AgNO4 and HgCl2 press SA190 affect on fresh weight in 25%PEG. But no effect can be seen in mock-treated (no SA190) plants when comparing mock (no inhibitors) and inhibitors-treated plants. So If I understand it correct;y the authors can not observe any effect on fresh weight in 25%PEG when aquaporins are inhibited? So I am confused - if aquaporins are not in evolved in drought then why are they required for SA190-mediated drought tolerance?
We agree that the role of aquaporins as the sole mechanism for SA190-mediated drought stress is not clear. Our genetic experiments do not support an essential role for aquaporins. However, since aquaporins are a large gene family in Arabidopsis, and we only tested a restricted number of mutants, it is possible that higher order mutants would be necessary to clarify this issue with certainty. On the other hand, the chemical inhibitor studies show a clear effect on the SA190 mediated drought tolerance,

but these inhibitors are likely not very specific and could have other targets as well. In conclusion, we interpreted these data carefully to omit overstatements.
For the specific question: When the plants were treated with PEG, a very strong growth inhibition was observed which was not enhanced upon treatment with AgNO4 or HgCL2. This can be explained as under these conditions, there is no induction of aquaporins as SA190 was absent in this experiment. However, when SA190 was present, the SA190-enhanced growth under PEG stress was completely inhibited by both inhibitors, suggesting that aquaproins might play a role in SA190-inudced drought stress tolerance.

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We thank the reviewer for pointing out that the experimental details were not sufficiently clear in the text. We included now two sentences to describe the procedure in more detail indicating that the SA190-teated plants were transferred to PEG medium containing no SA190. We also described how the mock-treated control was analyzed in parallel.
“As described in Figure 1, plants were germinated on SA190 containing 1/2MS media for 5 days before transfer onto ½ MS+25%PEG but containing no bacteria. As control, we used plants that were germinated in parallel but in the absence of SA190 (mock).”

3. The experiments with alphaalpha are promising but based on one year trial. As a minimum, the authors should clearly state in the discussion that those are preliminary data and can not be easily extrapolated.
We agree with the comment of the reviewer and changed the sentence in the discussion accordingly.

4. I am not a specialist but I do find that several bacterial-mediated droughts tolerance examples have been described. I, therefore, like to ask the authors to discuss the novelty of their finding in light of the published work in a more clear way. It would be highly beneficial for the manuscript to compare side by side the Sa190-induced drought tolerance with at least one of the other bacterial strains published before. Are those also mediated by the root growth as the authors find for SA190?
We agree with the reviewer that reporting drought tolerance of plants by a bacterial strain is absolutely not novel but so far, no publication unraveled the underlying molecular mechanisms which is the novelty of this work.

Mainor point:
ChIPS shown in fig4 seems to be based on 2 bio-rep which is not enough.
The experiment was repeated with 3 biological replicates. However the ChIP values for one of the replicates were out of the range compared with the other two replicates, so we decided to represent only 2 replicates here. As ChIP assays contain a long and complicated experimental procedure, it is normal to obtain different ChIP qPCR values between different replicates. However a similar trend was observed between all 3 replicates.

Referee #2:

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argentinensis SA190, an endophytic bacterium isolated from the desert plant Indigofera argentea, leads to improved drought stress tolerance in Arabidopsis in laboratory conditions as well as in alfalfa in field conditions. Colonization of roots is increased in osmotic stress conditions. They then uncover part of the molecular mechanisms responsible for this phenotype. They show that this increased tolerance is due to ABA-dependent changes in epigenetic marks in target genes including aquaporin encoding genes.

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We agree with the reviewer that PEG and drought are not the same conditions. So we changed the text where necessary to clarify the experimental conditions used.

2) Pseudomonas argentinensis SA190 was isolated from nodules of a desert Fabaceae plant. Could it fix nitrogen, and could that somehow contribute to its effect? This is particularly critical for the field trials with alfalfa - another nitrogen-fixing plant - as it could explain the beneficial effect of inoculation without having any link with increased water-stress tolerance (and could explain the beneficial impact observed without water stress). Indeed, transfer of nitrogen-fixation from rhizobia to other bacteria has been reported. One quick way to test it would be to inoculate alfalfa grown in sterile conditions without any N source (+ control without inoculation and a control with an alfalfa-infecting rhizobium) and test whether the SA190 strain could provide N to the plant. Testing the effect on a cereal in field conditions would be another way to address this point.
We thank the reviewer for this aspect. We discuss this point as follows in the manuscript discussion section.
“Analysis of the complete SA190 genome did not reveal any of the known genes involved in nitrogen fixation. Moreover, we did not observe that SA190 could enhance growth of Arabidopsis on N-free or N-limiting conditions. We therefore think that N-fixation is not part of the mechanism how SA190 induces drought tolerance in plants.”
This is a critical point to address to make sure the effect reported in field conditions is really related to

increased drought tolerance.

3) Root colonization by SA190 is enhanced by PEG treatment. Bacteria seems to enter the vascular tissues based on Fig 1J. If true, it would be nice to describe this in more details in the text (is there a qualitative change in root colonization in response to PEG).
Maybe the reviewer missed this paragraph, but these experiments and observations were described in detail:
"Since SA190 was isolated from roots of the desert plant *Indigofera argentea*, we characterized the interaction between SA190 and Arabidopsis in more detail. To this end, SA190 was stably transformed with a GFP expressing cassette (SA190:GFP). Confocal microscopy revealed that SA190:GFP mainly colonized roots (Fig. 1J), preferentially epidermal cells of the root elongation zone (Fig. 1J). Interestingly, the roots of plants grown on 25% PEG showed enhanced colonization when compared to plants grown only on ½ MS agar (Fig. 1J). These findings were confirmed by determining the SA190 colony forming units (CFU) of root and shoot tissues (Fig. 1K).

We next investigated the root morphology of SA190-colonized plants. After 16 days on normal or 25% PEG medium, root length and lateral root density were determined. Our results show that SA190 did not significantly influence the morphology or development of Arabidopsis roots under non-stress conditions (Fig. 1B, EV1B, EV1D). However, on 25% PEG medium and in contrast to non-colonized plants, SA190-colonized plants showed a strongly developed root system (Fig. 1B), with a massive increase in root fresh and dry weight (EV1B, EV1D) mostly due to enhanced growth of the primary root and development of the lateral root system (Fig. 1E-F)."

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corrected.

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This was not done but we agree this would have been useful.

Minor points:

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Thank you for this idea. We changed the Figure accordingly.
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The data also showed that the application of the SA190 enhances the drought resilience of alfalfa in field trials, however the authors only showed yield data in Fig.1 but not pictures of the plants. Was the drought stress applied in the field a real stress?
In contrast to the Arabidopsis soil drought assays, where irrigation was completely stopped for 3 weeks before resumption, the alfalfa field trials were performed with alfalfa seeds coated with SA190 or no bacteria (Mock) and irrigation of the plants with the usual irrigation system or 25 % less water. Interestingly, and in contrast to the optimal growth conditions for Arabidopsis in growth chambers, we observed a 21 % increase in alfalfa growth and biomass yield under typical desert agriculture conditions. On Fig.1 I no difference is observed between the well-watered and drought stress controls.
Under 25 % reduced irrigation conditions, slightly slower growth and yield was observed, but SA190 still enhanced biomass yield by 14 %. We interprete these data not so much as drought assays, but as a trial to perform agriculture under reduced irrigation as water is the most limiting factor on arid and hyperarid lands.
Most of the studies using single PGPB show no plant growth promoting effect in the field (where the introduction of a single bacteria to an establish community will not make a difference)! Can the authors comment on this?
The reviewer is correct that very often PGPB effects obversed in the lab are often not translatable to the field. We think that this is mostly true for rhizospere PGPB strains as these have to compete with the natural soil microbiome. In our approach, we systematically isolated endosphere root microbial strains which enter the root apoplast upon seed germination and are therefore protected from competition with the surrounding soil microbiome.

Also the authors should show fresh and dry biomass data from the field trials and not only yield harvest data! It is well known that drought stress in plants is ABA dependant, I do not see something new that is shown in this study to explain the molecular mechanism of drought stress resilience when plants are inoculated with PGPB-SA190.

We agree that ABA has been shown to be the key regulator for drought stress tolerance of plants. However, despite a number of reports showing that PGPBs can protect plants against drought, the mechanism at the plant level has not been revealed. Our work shows that SA190 enhances drought tolerance via modifying the epigenetic status of genes and that this mechanism is based on manipulating the plant ABA pathway. Together with our previous work on SA187-induced salt and heat toleranc by an epigenetic mechanism mediated by maniplutaing the plant ethylene pathway, we think these findings set the stage for a first molecular understanding how PGPBs confer stress tolerance in plants.

I have several comments :

Line 54 - at the end missing reference.

corrected

Line 132- The authors described the screen with several dessert bacteria but did not show the data only for SA190!

We intend to publish a manuscript of the in depth taxonomic and genomic comparison of the strains used in the screen. However, this information has enormous size and we do not think this information adds to the current manuscript, except to say that SA190 was always among the top 5 performing strains.

Line 141- Fig. not Figs

corrected.

Fig. 1B - The seedlings were grown for 16 days (the primary roots will overgrown the length of the plate as well as the lateral roots are overlapping so how did the authors quantify the roots properly?. I also do not see any bacteria around the root! I wonder why?

We thank the reviewer for looking into this. The labeling of Fig. 1B was not correct. Photographs of the plants on 1/2MS plates were taken after 14 days, whereas those upon growth under PEG stress after 21 days. This was corrected in the revised figure legend.

Fig.1 J- It is not very clear from the picture where the colonization is (I think the authors should show a higher magnification. It is even not clear if this is the root elongation zone, because at the root elongation zone there are no root hairs yet.

The reviewer is correct. The labeling of elongation zone is not corrected. We therefore corrected the figure and respective text. We chose the low magnification to get a better overview of the colonization.

Line 173- Root morphology should be root architecture.

Corrected.

Line 240- root structure should be changed to root architecture under drought stress

Corrected.

Line 274- To me it looks like TIP1-2, PIP1-5 AND TIP2-2 are significantly increased upon SA190 inoculation in non-stress conditions, but this is not indicated in the plots!

Thank you for looking into this. We have carried out the statistical tests again and confirmed the reviewers finding that there were mistakes in the calculation. We changed the figure with the new calculations.

Fig.3- I think something is not correct with the statistics again. For instance in the last plot- Fig.3C (for TIP2-2) there is a clear significant difference between col-0 Mock and Col-0 + SA190 in 1/2 MS, but this is not indicated with asterisks?

As above, the calculations were not correct and have been rectified in the revised figure.

Line 303- Fig.4 Again something is wrong with the statistics. For example the first plot (PIP1-1), the asterisks of R2 are not correct.

Again, we want to thank the reviewer for his oversight. We agree with the reviewer and replaced the figure with the corrected version.

Line 348- replace massive with observed.

Changed.

Dear Prof. Hirt,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, referee #2 now fully supports the publication of your study in EMBO reports. In contrast, referees #1 and #3 are not fully satisfied with the revisions, have remaining concerns and ask for more clarifications. I thus ask you to address the remaining points in a final revise manuscript. Please also provide a final p-b-p-response addressing these remaining concerns.

Moreover, I have these editorial requests I also ask you to address:

- Please reduce the number of keywords to five and move them below the abstract.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please order the manuscript sections like this (using these names as headings):
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure Legends

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. Presently, it seems there are diagrams or boxplots with partial statistics.

- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. One digit is missing from the grant number in the manuscript BAS/1/1062-01-01 - BAS/01/1062-01-01. Please check.

- Please make sure that all figure panels are called out separately and sequentially (main, and EV figures). Presently, panels E&F of Fig. 1 are called out after 1I, panel EV2 is called out after EV3A and callouts for panels A&C of Fig. EV1 are missing. Please check.

- Figure 1G 'Drought Stress 'looks to have a darkening filter halfway over the figure. The figure looks ok, but this darker shade is a bit odd. Is this necessary? Please check.

- There is one table mentioned in the methods section ('Supplementary Table 1'), but I could not find the actual file. Please upload this as EV table and name it Table EV1. Please also use this name for its callout and remove the legend for 'Supplementary Table 1' from the main manuscript text file.

- As I already indicated in my first decision letter, we now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. It seems you have been contacted already by our source data coordinator, who indicated which figure panels we would need source data for. I attach again the source data checklist and the FAQ. Please make sure that all the requested source data is provided. Please upload all source data for one figure as one pdf per figure or (if there is more than one file) ZIPed together as one folder. Finally, please upload the filled in source data checklist

with your final revised files.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text and comments. Please use the attached file as basis for further revisions and provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

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Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

I am mostly satisfied with the response to my comments. I still think the mechanistic understanding of the mechanism behind the bacteria-induced drought resistance is the weak part of the study. The link to ABA signalling is not explored comprehensively - that is why I asked to analyse SnRK2 involvement.

I also asked if the bacteria pre-exposure before stress can be interpreted as "epigenetic reprogramming" and if the bacteria are not still present in the media during drought induction. The authors clarified that their procedure includes exposing plants to bacteria and then the transfer of plants to MS with no bacteria.
But at the same time the authors responded to a question by a different reviewer: "In our approach, we systematically isolated endosphere root microbial strains which enter the root apoplast upon seed germination"

In my understanding, this suggests that the bacteria cells are likely still present in/on the roots once the plants are moved to MS. I think it is OK for the manuscript but if I am right the authors should discuss this point in the manuscript.

Referee #2:

This new version of the manuscript addresses most of my major concerns.

Referee #3:

The authors did not answer convincingly to my questions. The statistics was corrected after my observations, but the authors did not comment on changing the conclusions based on the new statistical analysis used. Also the authors do not explain why the statistical analysis was not done properly in the first place.

I am not convinced about the field experiment and the conclusions as well, since there was no drought stress! The authors explained it in the comments as less water availability, but is it then stress for the plants if there is no biomass reduction compared to the control? In this case the conclusions should not be misleading!

The authors also described the screen with several dessert bacteria but did not show the data in the revised manuscript, except for SA190! In this case the authors can also remove the description of the screen, because they do not show the data.

Referee #1:

I am mostly satisfied with the response to my comments. I still think the mechanistic understanding of the mechanism behind the bacteria-induced drought resistance is the weak part of the study. The link to ABA signalling is not explored comprehensively - that is why I asked to analyse SnRK2 involvement.
In this current manuscript, we show that the SA190-inudced drought tolerance of Arabidopsis is mediated by an epigenetic mechanism that depends on the plant ABA biosynthesis and signaling pathways. Despite many publications showing bacterially induced drought tolerance, no molecular mechanism has so far been identified to our knowledge. However, we agree with the reviewer that our work can only be the beginning of further investigations which will require many years of work and be part of several future publications hopefully from many labs.
Just to illustrate the complexity of the system, we want to share some preliminary results that are part of different ongoing works: In collaboration with Salim Al-Babili's lab, a specialist on the carotenoid pathway, we analyzed whether SA190 can produce ABA itself and thereby influence Arabidopsis drought tolerance. Targeted metabolomic analysis showed that SA190 cannot produce ABA but a number of carotenoids that might potentially contribute to drought tolerance. We are currently testing various carotenoid pathway mutants in this direction. We also test the possibility that SA190 might induce ABA in Arabidopsis. Our analysis showed only a small increase in ABA levels in total root samples that were, however, not statistically significant to non-colonized roots. We then used an ABA-reporter and found specific induction of a particular region upon SA190 colonization. However, this region is not colonized by SA190, suggesting that SA190 induces a signal that is transported from the colonization site to the region where ABA is locally produced. So in collaboration with Ora Hazak's lab, we now test the hypothesis that some CLE-like peptides and their receptors might be involved in a systemic signal to induce ABA synthesis, which according to mutant analysis might actually be the case. All these data require a lot more work to clarify the way how a seemingly simple bacterial interaction can modify the response of Arabidopsis to water stress.

I also asked if the bacteria pre-exposure before stress can be interpreted as "epigenetic reprogramming" and if the bacteria are not still present in the media during drought induction. The authors clarified that their procedure includes exposing plants to bacteria and then the transfer of plants to MS with no bacteria. But at the same time the authors responded to a question by a different reviewer: "In our approach, we systematically isolated endosphere root microbial strains which enter the root apoplast upon seed germination" In my understanding, this suggests that the bacteria cells are likely still present in/on the roots once the plants are moved to MS. I think it is OK for the manuscript but if I am right the authors should discuss this point in the manuscript.
We thank the reviewer to ask for clarification of this point. We describe the endophytic nature of SA190 at several places in the manuscript, but clearly the most convincing experimental proof is the confocal analysis with the GFP labeled strain (Fig. 1J) and the CFU determination of the thoroughly washed plants (Fig. 1K). In response to the reviewer, we highlight the endophytic localization in the following sentences:
"We next investigated the root colonization by SA190.To this end, SA190 was stably transformed with a GFP expressing cassette (SA190:GFP). Confocal microscopy revealed that SA190:GFP mainly colonized roots (Fig. 1J), preferentially epidermal cells of the root differentiation zone (Fig. 1J). These findings were confirmed by determining the SA190 colony forming units (CFU) of root and shoot tissues (Fig. 1K). Interestingly, the roots of plants grown on 25% PEG showed enhanced colonization when compared to plants grown only on ½ MS agar (Fig. 1J). At later stages of colonization, SA190:GFP was also found inside of root tissues revealing the endophytic nature of SA190 (Fig. 1J). The preferential colonization of roots was also revealed by determining the SA190 colony forming units (CFU) of the colonized plants

after intensive washing. However, both shoots and roots showed enhanced colonization by SA190 upon PEG stress treatment (Fig. 1K).

Referee #2:

This new version of the manuscript addresses most of my major concerns.

Referee #3:

The authors did not answer convincingly to my questions. The statistics was corrected after my observations, but the authors did not comment on changing the conclusions based on the new statistical analysis used. Also the authors do not explain why the statistical analysis was not done properly in the first place.
We explained and apologized for this oversight which was duly corrected.

I am not convinced about the field experiment and the conclusions as well, since there was no drought stress! The authors explained it in the comments as less water availability, but is it then stress for the plants if there is no biomass reduction compared to the control? In this case the conclusions should not be misleading!
We agree with the reviewer. The field data cannot represent a drought stress experiment. We therefore removed these data and replaced them with drought experiments where alfalfa plants were grown in climate chambers. One set of plants obtained continuous watering for 52 days (Control) whereas in the other set of plants irrigation was stopped at day 42. Both sets of plants were analysed for biomass at day 52. In three independent sets of experiments, the SA190-colonized alfalfa showed an overall 37 % enhanced performance compared to the non-colonized plants.

The authors also described the screen with several dessert bacteria but did not show the data in the revised manuscript, except for SA190! In this case the authors can also remove the description of the screen, because they do not show the data.
In agreement with the reviewer, we removed the screening part in the manuscript and only describe the behavior of SA190.

Prof. Heribert Hirt
King Abdullah University of Science and Technology
Center for Desert Agriculture
4700 King Abdullah University of Science and Technology
Thuwal, orcid||||||| 23955-6900
Saudi Arabia

Dear Prof. Hirt,

Thank you for the submission of your revised manuscript to our editorial offices. I now went through your final p-b-p-response and consider the remaining points by the referees as adequately addressed.

I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Achim Breiling
Editor
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****

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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Supplemnetary Table 1

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Figures
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	No data points were excluded
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Stats were performed according to sample sizes and data points (see Figures).

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
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Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Yes	no permit required for alfalfa field studies

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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
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