nature portfolio

Peer Review File

oot hair developmental regulators orchestrate drought triggered microbiome changes and the interaction with beneficial Rhizobiaceae

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Wang et al studied 2 mutants affected in the formation of root hairs and found that mutant plants with more root hairs were more drought tolerant, but only in the presence of a microbiome. They continue to study the root microbiome and show that root hairs are more abundantly colonized by a number Rhizobiaceae ASVs. Moreover, a number of Rhizobiaceae spp isolated from drought stressed plants can confer PEG tolerance. A particular Rhizobium confers PEG tolerance in a way that is independent of several know hormonal signaling pathways, suggesting that the tolerance is not dependent of plant signalling, but the direct result of something produced by bacteria. From root metagenome analysis it is deduced that proline metabolism is more active in the microbiome of root hair overproducing mutants, and metabolome analysis shows that indeed more proline is detected on those roots. Based on literature the authors suggest proline functions as an osmolyte and that Rhizobium produced proline confers drought tolerance.

I think this a rather impressive paper combining many techniques to build a nice story that fits well together. There are, however, some limitations to the paper:

• Mutants with altered root hair densities exhibit distinct drought tolerant phenotypes in non-sterilized soil only. However sterilization does more than kill microbes; the heating strongly affects the physics and chemistry of the soil. The author could or should have inoculated the autoclaved soil with e.g. 1% of the non-sterilized soil, to see if the restoration of the microbiome also restores the drought resistance. Moreover I wonder whether the gl2 mutant is also more drought tolerant in other soils, or is this (presumed) microbiome-associated phenotype specific for this soil?

• Rhizobiaceae are more abundant on the gl2 mutant in the one natural soil that was tested and can induce PEG tolerance in vitro. However, PEG tolerance is something else than drought tolerance. I think the authors should show that the Rhizobiaceae spp also confer drought tolerance. Moreover I wonder whether the PEG tolerance is conferred specifically by Rhizobiaceae. If you would test a random number of isolated from other genera in this in vitro test, would not quite some others also confer PEG tolerance?

• The metagenome and metabolome data suggests that microbiome-produced proline could be responsible for the stress tolerance, but the data is far from conclusive (this is also recognized by the authors). I do wonder if there is any evidence to suggest that Rhizobiaceae specifically produce proline. To demonstrate the role of proline, it would have been great to test rhizobial knock-outs defective in the synthesis of proline. A quick google search teaches me that such mutants exist and could easily be tested. Regardless, the metabolome and metagenome data are not mentioned in the discussion, and I think the authors should add some discussion on these findings

• Personally, I think it is a good thing that the field shows less and less co-occurrence network analysis, but clearly the authors don't agree as they used a number of (supplementary) figures and

quite some text for their network analysis. Personally I don't think Figure 4 and lines 199-228 do not offer any useful insights and could be omitted without losing value.

• In general the English of the manuscript was not bad. Nonetheless, there were many small grammatical mistakes and imprecisions. The text good do with a thorough revision.

Minor comments:

line 13: rephrase, suggestion: orchestrate the dynamic assembly of the microbiome in response to drought stress

Line 16 dry-hot valley: do you mean desert valley?

Line 18 Rhizobiaceae strains were detected as key biomarker species: Rhizobiaceae is not a species

Line 26 mediated

Line 52 reference 13 is not about "which might be related to pathogen-triggered recruitment of beneficial Pseudomonads."

Line 54 replace "disrupting several" with " disrupted in distinct'

Line 65 unclear what is meant by ' plant effects', please rephrase

Line 72 rephrase the sentence is grammatically incorrect

Line 76 tree seedlings? What tree species?

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Line 102 dampen is vague -> eradicate large parts of soil microbiomes

Fig 1a is a bit pixelated and the quality/resolution should be improved

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Line 112 Supplementary Fig. 1 Does not clearly show me that root hair related mutants do not affect primary and lateral root growth on plates. I see really big plants with loads of roots in all cases but the number of lateral roots and length of primary roots formed can be easily measured and statistically tested, right? Or are these mutants well characterized else where?

Line 122. Strongly supports that that an intact soil microbiome community is essential for protecting plants from drought stress ->it suggests that, but there are also severe physical and chemical changes that a soil undergoes when autoclaved. The author could have inoculated the autoclaved soil with 1% of the non-sterilized soil, to see if the restoration of the microbiome also restores the drought resistance

Line 134 Mutants altering root hair densities shifted microbiome composition under drought stress -> Mutants with altered root hair densities harbor distinct microbiomes under drought stress

Line 145 We found that gl2 show enhanced alpha diversity in root microbiomes under well-watered conditions (Fig. 2a), suggesting a positive role of root hairs in maintaining microbial diversity. ->is this relevant?

Line 169 This suggests that gl2 mutant has strong deterministic influence on root microbiome assembly.--> if you want claim that the βNTI is distinct between your plant genotypes, then you should apply a statistical analysis to figure 2c.

171-179 the reader would benefit from a bit more explanation of RCbray and a definition of homogenous selection.

Line 186-188 We found that the abundance of Rhizobiaceae was significantly higher in gl2 than Col-0 and rsl2 rsl4 under drought stress, which is correlated with a decrease of root hair densities (Supplementary Fig. 3b, c, d). -> yes Rhizobiaceae appears to be significant more abundant in ANOVA with bonferoni correction sFig 3d, but it does not stand out in the triplots of fig s3C. It's quite central in the triangle.

Line 188-191 Okay, so this the reason for focusing on Rhizobiaceae? That makers more sense than Fig S3. Please describe the finding of fig 3 a and b first. Perhaps move lines 186-188 to the end of this paragraph (line 196)? A sentence describing what Linear discriminant analysis Effect Size does would help readers.

Line 199-214 co-occurrence networks and network connectivity IMHO do not offer any useful in sights, I consider lines 199-214 page filling that should be left out. Co-occurrences do not per se reflect interactions between microbes. Also, what is the story here? Does a specific taxon (Rhizobiaceae) increase on root hairs and help with drought tolerance ? Or do root hairs increase 'network connectivity' and this is what helps the plant against drought.

Line 216- 236 This is some more network topology. Not really

necessary.

Line 228 Hub nodes (ASVs)-> remove brackets: Hub node ASVs, also 'usually' doesn't make sense in this sentence

Line 230 were these the same ASVs that were enriched on gl2 following drought? Please mention in text.

Line 240 The ASV don't have effect on the plant but the microbes represented by the ASVs

Line 241 and 242 together don't make sense. First you obtain 1500 isolates and then you obtain 265 isolates. Are the 256 isolates what remains after dereplications of isogenic/sibling isolates?

So 256 unique isolates? Please describe the dereplication step in main text. I'm guessing 16S amplicon sequencing…

Line 245-246 Are Ensifer spp part of Rhizobiaceae? Please mention

Line 247: delete " The" before "ASV 17" line 248 remove brackets hube node (ASV) -> hube node ASV. Please check the entire text for this.

Line 254: change into :Four of the five test Rhizobiaceae strains are significantly enriched on gl2 mutant plants

Line 254: justy above you describe 10 (4+6) Rhizobiaceae strains. Now 5 were tested?

Fig 5b is not readable.

Line 270 and usually also has more robust phenotypes critical for agricultural application. I don't agree, the combination of strains can also antagonistic effects. Perhaps: "and can have more robust phenotypes"

Line 296 "mutants could block" change into "mutants were impaired in" (also rephrase line 290; the mutant is impaired it doesn't block)

Line 355-370 could easily be skipped. If the discussions starts at line 372, it would still be fine. Moreover that would leave more space to discuss the final metagenome and metabolome work (fig 5) which is currently not, but should be, touched upon in the discussion.

Reviewer #2 (Remarks to the Author):

The paper requires significant English language editing including context focus (not just grammar) before it is suable for publication.

My review is focused specifically on the metabolomics aspect of the manuscript.

The authors specify that “16 plants were pooled per replicate”. It can be inferred from the results that the treatments included the wild type genotype and two mutants. Were these sampled for both the control (well watered) and drought stress condition? Please clarify how many bioreplicates were generated per treatment and which treatments were included.

The results suggest that the original goal of the metabolomics analysis was to to confirm suggested variation in proline concentrations. The authors should justify their choice to use a nontargeted metabolomics approach rather than a targeted analysis for proline.

No information is provided about how samples were prepared/extracted for analysis and how much sample was injected. No details are provided about instrument operation (liquid chromatography and mass spectrometry). This section requires extensive improvement to enable replication of the analysis.

Likewise, there is no information at all provided about how the MS data was processed. How were the metabolite annotations determined? What tools were used? How confident are the annotations?

Was any quality control performed? Were the samples randomized for analysis? Please describe.

Were data normalized prior to statistical analysis?

Was a multiple testing correction performed as part of the statistical analysis? (i.e. are value adjusted for multiple testing?).

As per the requirements of the journal and as a general standard of the field the raw metabolomics data should accessible (via a public repository). Additionally, the processed output used for statistical analysis should also be included as supplemental data.

Supplemental Figure 7 – The PCA plot only shows data from drought stressed plants, were control plants analyzed? If not, why? The metabolite names are likely too specific for the output of a nontargeted application. (although this is hard to assess since there are no methods). As an example, PA(18:1(9Z)/18:1(9Z)) should be reported as PA(36:2). Standard nontargeted metabolomics methods do not provide sufficient evidence to assign more specific lipid annotation. The pathway enrichment analysis is not clearly described in the methods. How is the differential abundance score determined and what does it mean? All the circles are the same size although the caption implies that different size circles indicate the number of metabolites. Why are some of the pathways bolded?

As written, the paper is clearly focused on the genomic analysis. There is no mention of the metabolomics data in the discussion and the only figure relating to this data is a supplemental figure. I would like to see the justification for the metabolomics data more clearly described and improved integration of the results into the discussion and overall conclusions of the paper.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Wang et al studied 2 mutants affected in the formation of root hairs and found that mutant plants with more root hairs were more drought tolerant, but only in the presence of a microbiome. They continue to study the root microbiome and show that root hairs are more abundantly colonized by a number Rhizobiaceae ASVs. Moreover, a number of Rhizobiaceae spp isolated from drought stressed plants can confer PEG tolerance. A particular Rhizobium confers PEG tolerance in a way that is independent of several know hormonal signaling pathways, suggesting that the tolerance is not dependent of plant signalling, but the direct result of something produced by bacteria. From root metagenome analysis it is deduced that proline metabolism is more active in the microbiome of root hair overproducing mutants, and metabolome analysis shows that indeed more proline is detected on those roots. Based on literature the authors suggest proline functions as an osmolyte and that Rhizobium produced proline confers drought tolerance. I think this a rather impressive paper combining many techniques to build a nice story that fits well together.

Reply: We appreciate for R1's careful and professional comments and edits. We also tried to further improve the manuscript in several aspects:
1) We conducted more experiments to show that the drought alleviating activity of our strains also exist in the soil system rather than PEG plate system;
2) We conducted genetic knock-out experiment in *Rhizobium* sp. 4F10, and provided solid genetic evidence that non-conventional proline biosynthesis gene *OCD* is a genetic determinant of 4F10 mediated drought protecting;
3) We conducted RNAseq analysis to reveal the molecular basis underlying *Rhizobium* sp. 4F10 mediated stress protection in roots.

There are, however, some limitations to the paper:
• Mutants with altered root hair densities exhibit distinct drought tolerant phenotypes in non-sterilized soil only. However sterilization does more than kill microbes; the heating strongly affects the physics and chemistry of the soil. The author could or should have inoculated the autoclaved soil with e.g. 1% of the non-sterilized soil, to see if the restoration of the microbiome also restores the drought resistance. Moreover I wonder whether the gl2 mutant is also more drought tolerant in other soils, or is this (presumed) microbiome-associated phenotype specific for this soil?
Reply: Thanks for the two great suggestions.
[1]We agree that a full factorial replicate of our natural soil and soil sterlization experiments in a different soil type would strongly strengthen our core conclusion. As suggested, we provided factorial replicate of this experiment in a different natural soil. To make the tested soil as different as possible from our previous soil (rainforest and dry hot valley from southern China), we harvested new soil from a farm from in Guangzhou, China (about 1200 km away from the natural soil site used before). We basically observed very similar trend that the hairless mutants have lower microbiome mediated drought protecting effect [Sup. Fig 4], which strongly suggested that the effects of our mutants on root microbiome is robust among different rhizosphere ecosystems.

[2] We understand that natural soil amendment experiment is a canonic, however, preliminarily approach (we added “preliminarily” in line 104) to test the involvement of soil microbiota on plant-soil feedback phenotypes. It seems really challenging to use only 1% soil amendment system for comparing the difference among many tested genotypes. We understand that it is essential for the early stage plant-microbiome interactions studies (like disease suppressive soil studies in 1976: [1]), because there was no microbiome sequencing and reductionist based high-throughput culturomics approaches to validate the microbiome composition and function changes. It seems not the golden standard for microbiome studies and not necessary for some high-impact microbiome studies recently (ref. [2, 3]). Importantly, in our project, we did not just draw the conclusion solely based on the soil sterilization experiment. We conducted systematic microbial ecological analyses (composition, biomarker, network features) and reductionist based strain isolation and function validating experiments. Our data collectively confirmed the involvement of root hair development regulators in regulating stress alleviating root microbiome upon drought stress;

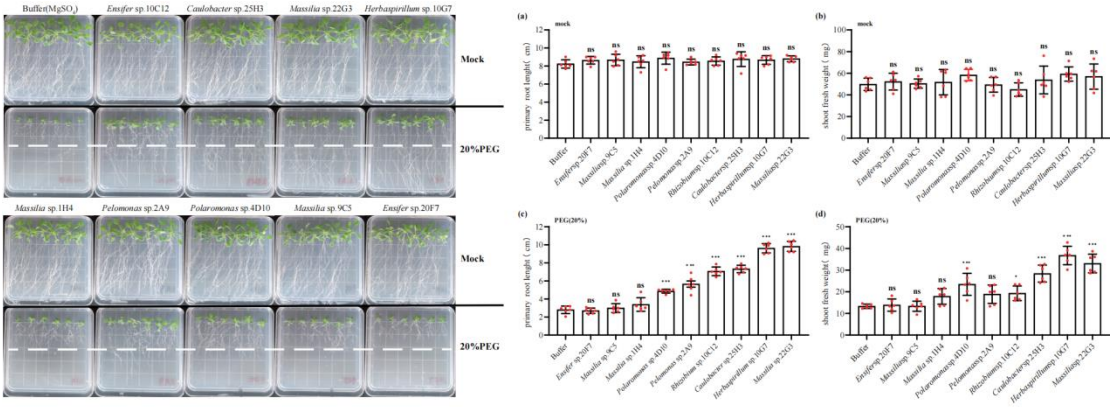
• Rhizobiaceae are more abundant on the gl2 mutant in the one natural soil that was tested and can induce PEG tolerance in vitro. However, PEG tolerance is something else than drought tolerance. I think the authors should show that the Rhizobiaceae spp also confer drought tolerance. Moreover I wonder whether the PEG tolerance is conferred specifically by Rhizobiaceae. If you would test a random number of isolated from other genera in this in vitro test, would not quite some others also confer PEG tolerance?

Reply: Thanks for this suggestion.

We totally agree that PEG stress tolerance is different from drought tolerance. As suggested, we inoculated plants grown in soil (Sup Fig. 9), and we were able to confirm the significant drought alleviating abilities of those tested Rhizobiaceae strains. This further confirmed the drought alleviating functions of drought responsive microbiome in *gl2*.

For the second comment,

1. Not all tested Rhizobiaceae strains can protect roots from osmotic stress, which suggests that not all strains can protect roots from PEG stress (Fig 4b,c).
2. Our other ongoing other projects tested a few more strains from different families, and only three (25H3, 10G7, 22G3) of nine exhibit strong shoot growth promotion effect under osmotic stress (Fig shown below，d).



• The metagenome and metabolome data suggests that microbiome-produced proline could be

responsible for the stress tolerance, but the data is far from conclusive (this is also recognized by the authors). I do wonder if there is any evidence to suggest that Rhizobiaceae specifically produce proline. To demonstrate the role of proline, it would have been great to test rhizobial knock-outs defective in the synthesis of proline. A quick google search teaches me that such mutants exist and could easily be tested. Regardless, the metabolome and metagenome data are not mentioned in the discussion, and I think the authors should add some discussion on these findings

Reply: Thanks for this really helpful comment. We agree that it seems unlikely that Rhizobiaceae will export proline outside to protect roots under drought stress. We thoroughly removed discussions about "microbiome-produced proline could be responsible for the stress tolerance".

After extensive exploration, we successfully established a gene knock-out system for the wild isolated strain *Rhizobium* sp. 4F10. This enabled us to test the effect of proline metabolic related genes using solid genetic approaches. Our metagenomic results indicates that the arginine and proline metabolism related function is enriched in *gl2* root microbiome (Fig. 5d), which might be correlated with drought protection. After a careful examination of metagenome results (Fig. 5c), we focused on the proline metabolic gene K01750 which is highly enriched in *gl2* root microbiome upon drought stress. This gene encodes a ornithine cyclodeaminase (*OCD* gene) which catabolize L-ornithine to proline. We found that 4F10*Δocd* knocked out strain significantly dampens the PEG stress protecting effects. Our genetic evidence further confirms that proline metabolic genes in *Rhizobium* sp. 4F10 mediated drought protecting for plants, and probably through enhancing bacterial fitness under stressed conditions.

Thanks for the comments about our discussion part, we tried to thoroughly discuss the potential implications of our metabolome and metagenome data, and added independent paragraph in the discussion part.

• Personally, I think it is a good thing that the field shows less and less co-occurrence network analysis, but clearly the authors don't agree as they used a number of (supplementary) figures and quite some text for their network analysis. Personally I don't think Figure 4 and lines 199-228 do not offer any useful insights and could be omitted without losing value.

Reply: Thanks for this professional comments. We understand that pure and superficial co-occurrence network description without key findings has no novelty at all. We had substantionally deleted most of our network analysis related paragraph, and removed the previous main Figgure 4 into supplementary Fig. 6.

There are at least two potentially interesting findings from our network analysis: a) the network complexity of *gl2* is highest among mutants under drought; b) Among the top 7 abundant families, we found that only the network degree centrality of Rhizoblaceae is positively correlated with the root hair densities of different mutants. Those results are briefly introduced in the revisied manuscript (Line 214-224).

• In general the English of the manuscript was not bad. Nonetheless, there were many small grammatical mistakes and imprecisions. The text good do with a thorough revision.

Reply: We had careful checked the manuscript, and we had also sent our manuscript to the Springer

Nature Research Editing Service (edited by a language editor who specializes in the subject area). We hope we had largely improved the manuscript.

Minor comments:

line 13: rephrase, suggestion: orchestrate the dynamic assembly of the microbiome in response to drought stress

Reply: Thanks, We have updated as suggested. We also sent our manuscript to the commercial language service (Nature publishing group).

*Line 16 dry-hot valley: do you mean desert valley?

Reply: Thanks for reminding this. We added a new Supplementary fig 1 to illustrate the ecosystems of natural soil harvesting sites. We carefully compared dry-hot valley with desert valley, and it seems not exactly the same. Both of the two ecosystem are famous for extremely high temperature. The desert valley seems more widely used in Eastern California (Death Valley), however, the dry-hot valley region (DHV) ecosystem is more widely used in tropical and subtropical areas of southwestern China (which is also characterized by high temperatures, low levels of rainfall, and high rates of evaporation; https://doi.org/10.1007/s12665-016-5986-6). The key difference seems to be that desert valley or death valley has much less plant species than our dry hot valley (new Sup Fig. 1).

*Line 18 Rhizobiaceae strains were detected as key biomarker species: Rhizobiaceae is not a species

Reply: Thanks for careful checking. This sentence has been rephrased to "Rhizobiaceae was detected as a key biomarker taxa……". [Line 20]

Line 26 mediated

Reply: Thanks for catching this. We have corrected. [Line 27]

Line 52 reference 13 is not about "which might be related to pathogen-triggered recruitment of beneficial Pseudomonads."

Reply: Thanks for the comments. We have deleted the inaccurate statement. [Line 49]

Line 54 replace "disrupting several" with " disrupted in distinct'

Reply: We have updated accordingly. [Line 50]

Line 65 unclear what is meant by ' plant effects', please rephrase

Reply: We changed that sentence to "Numerous studies indicate that plant host might be involved in orchestrating drought-induced root microbiome changes". [Line 61-62]

*Line 72 rephrase the sentence is grammatically incorrect

Reply: This sentence had been updated to:

"Integrated multi-omics approaches have suggested that glycerol 3-phosphate (G3P) and iron are potential metabolic cues affecting drought induced dynamic assembly of root microbiome [22, 23]." [Line 68]

*Line 76 tree seedlings? What tree species?

Reply: We are sorry about the confusion. The original *Science* paper [https://www.science.org/doi/10.1126/science.adf2027] studied a total of 21 different tree species, and conducted different host-microbiome interaction analyses. To provide more accurate information, we changed the sentence to: "A recent study inoculated 21 different tree species with different soil microbiomes, and found that inoculation of soil microbiomes sourced from drier, warmer, or colder sites can promote plant survival when faced with drought, heat, or cold stress, respectively." [Line 71-73]

*Line 91 Please also introduce the function of RSL2-5 genes. Are they transcription factors that regulate root hair development?

Reply: Thanks for the suggestion. We have updated as suggested: "In contrast, a group of upstream basic helix-loop-helix (bHLH) transcription factors including *ROOT HAIR DEFECTIVE 6* (*RHD6*) and its homolog gene *RHD6-LIKE 1* (*RSL1*), which positively regulate the expression of two closely related downstream bHLH transcriptional factors (*RSL2* and *RSL4*) genes to promote root hair development [31]. The *rsl2 rsl4* double mutant show completely loss of root hairs[32]". [86-90]

*Line 100 Mutants with altered root hair densities exhibit distinct drought tolerant phenotypes in sterilized and non-sterilized soil

Reply: This sub-title had be updated to "Mutants with altered root hair densities affect microbiome mediated drought protection in natural soil". [Line 101]

*Line 102 dampen is vague -> eradicate large parts of soil microbiomes

Reply: Thanks for the professional correction. Had been corrected accordingly. [Line 103]

*Fig 1a is a bit pixelated and the quality/resolution should be improved

Reply: Thanks for catching this. We improved all figure qualities in the revised manuscript. We will make sure our figure quality fit journal requirements. [Fig. 1]

*Line 109 it would be good to indicate what are the phenotypes of each mutant with reference to fig 1b at this point

Reply: Thanks for really careful checking! We updated the sentence to: "We chose to use the *gl2* (hairy mutant) and *rsl2 rsl4* double mutant (hairless mutant) as genetic tools to manipulate root hair densities (Fig. 1b, c)". [Line 110-112]

*Line 112 Supplementary Fig. 1 Does not clearly show me that root hair related mutants do not affect primary and lateral root growth on plates. I see really big plants with loads of roots in all cases but the number of lateral roots and length of primary roots formed can be easily measured and statistically tested, right? Or are these mutants well characterized else where?

Reply: Thanks for the suggestion. We have updated a new Supplementary Fig. 2 to better illustrate the root phenotypes of each genotype. We quantified the primary root length, lateral root number and calculated lateral root densities in different genotypes. We did not observerd significant side effects of these root hair regulators on primary and lateral root development [new Supplementary Fig. 2].

Line 122. Strongly supports that that an intact soil microbiome community is essential for protecting plants from drought stress ->it suggests that, but there are also severe physical and chemical changes that a soil undergoes when autoclaved. The author could have inoculated the autoclaved soil with 1% of the non-sterilized soil, to see if the restoration of the microbiome also restores the drought resistance

Reply: Thank you for your suggestions. We agreed that autoclave affects the physical and chemical properties of the soil substrate, but this effect is same for all tested genotypes. Moreover, we set a well-watered control group which also used the auto-claved soil, and we used the relative fresh weight (shoot fresh weight in drought group/shoot fresh weight in well-watered group under same soil condition) to reflect drought tolerance in sterlized soil. This internal control group also helps eliminates the difference in sterlized/ nonsterlized soil.

Line 134 Mutants altering root hair densities shifted microbiome composition under drought stress -> Mutants with altered root hair densities harbor distinct microbiomes under drought stress
Reply: Thank for the really nice edit here. We also sent our manuscript to Nature Research Editing Service, which was edited by a language editor who specializes in the subject area.

Line 145 We found that gl2 show enhanced alpha diversity in root microbiomes under well-watered conditions (Fig. 2a), suggesting a positive role of root hairs in maintaining microbial diversity. ->is this relevant?
Reply: We deleted "suggesting a positive role of root hairs in maintaining microbial diversity". [Line 155]

Line 169 This suggests that *gl2* mutant has strong deterministic influence on root microbiome assembly.--> if you want claim that the βNTI is distinct between your plant genotypes, then you should apply a statistical analysis to figure 2c. (betaNTI)
Reply: Thanks for the suggestion, we conducted Students' T test to compare the significance in the revised manuscript. [Fig. 2c]

171-179 the reader would benefit from a bit more explanation of RCbray and a definition of homogenous selection.
Reply: Thanks for the great suggestion, we discussed with ecologists to further improve our description.
[1] "Homogeneous selection means that the overall environmental effects on community assembly is non-selective for all (or most) microbes, thus competitive exclusion within microbial community (between different microbes) would mainly drive community assembly. This indicates gl2 mutant (hairy) might have overall homogeneous effects on root microbiome through root exduates or other physiological effects. " [Line 186]

[2] Line 174 "The Bray-Curtis-based Raup-Crick index (RCbray) is used to classify the contribution of different stochastic (2-<βNTI<2) community assembly cues in iCAMP framework. This approach creates a re-scaled probability metric ranging from-1 to 1, indicating whether local communities are more dissimilar (approaching 1), as dissimilar (approaching 0), or less dissimilar (approaching - 1),

than expected by random chance." This index helps provide some information on the degree to which pairwise communities are more different (or more similar) than expected by chance.

Line 186-188 We found that the abundance of Rhizobiaceae was significantly higher in gl2 than Col-0 and rsl2 rsl4 under drought stress, which is correlated with a decrease of root hair densities (Supplementary Fig. 3b, c, d). -> yes Rhizobiaceae appears to be significant more abundant in ANOVA with bonferoni correction sFig 3d, but it does not stand out in the triplots of fig s3C. It's quite central in the triangle.

Reply: Thanks for the comments! As we can see from the Supplementary Fig. 3d, the abundance of Rhizobiaceae was significant but only weakly higher in *gl2* relative to wildtype and *rsl2 rsl4*. That is probably the reason why Rhizobiaceae is "quite central" in the triplots graph. To make our expression more accurate, we changed the expression to "Rhizobiaceae was slightly but significantly higher in……"[Line 206]

Line 188-191 Okay, so this the reason for focusing on Rhizobiaceae? That makers more sense than Fig S3. Please describe the finding of fig 3 a and b first. Perhaps move lines 186-188 to the end of this paragraph (line 196)? A sentence describing what Linear discriminant analysis Effect Size does would help readers.

Reply: Thanks for helping us improve the logic! That totally make sense and we have updated accordingly.

We also added more information about the LEfse assaya according to the original research paper:

"Linear discriminant analysis Effect Size (LEfSe analysis) is widely used for biomarker characterization, which firstly utilizing non-parametric test to identify differential abundant species among groups, and then estimate the effect size of each differentially abundant species based on a LDA model (averaging the differences between class means with the differences between class means along the first linear discriminant axis) [4]". [Line 198-206]

Line 199-214 co-occurrence networks and network connectivity do not offer any useful in sights, I consider lines 199-214 page filling that should be left out. Co-occurrences do not per se reflect interactions between microbes. Also, what is the story here? Does a specific taxon (Rhizobiaceae) increase on root hairs and help with drought tolerance ? Or do root hairs increase 'network connectivity' and this is what helps the plant against drought.

Reply: Thanks for the suggestion. Since we had substantially improved our manuscript with genetic knock-out strain and root RNA-seq analyses, we also want to decrease the size of our previous manuscript to accommodate new data. We had deleted previous lines 199-214.

For the previous story here, we briefly described some overall network indexes among different mutants, including network size and connectivity (line 214-224). We agree it is hard to use this microbial ecological method to draw solid conclusions like "specific taxon (Rhizobiaceae) increase on root hairs and help with drought tolerance". We thus largely deleted this part to save space for our new data.

*Line 216- 236 This is some more network topology. Not really necessary.

Reply: Thanks for the suggestion. We largely deleted this part, and only kept very brief introductions in Line 214-224.

Line 228 Hub nodes (ASVs)-> remove brackets: Hub node ASVs, also 'usually' doesn't make sense in this sentence

Reply: This part had been deleted in the revision manuscript as suggested.

Line 230 were these the same ASVs that were enriched on gl2 following drought? Please mention in text.

Reply: Thanks for the comments, there is no direct link between differentially abundant-ASVs and network hub node ASVs. In our case, those two hubs are not DA-ASVs.

Line 240 The ASV don't have effect on the plant but the microbes represented by the ASVs

Reply: "To verify whether microbes corresponding to *gl2* regulated ASVs……". [Line 227]

Line 241 and 242 together don't make sense. First you obtain 1500 isolates and then you obtain 265 isolates. Are the 256 isolates what remains after dereplications of isogenic/sibling isolates?

Reply: We are sorry about the confusion. We added new method description to address this comment:

".Paired-end reads of 1479 isolates were merged to obtain clean sequences. Sequence deduplication was performed using VSEARCH v2.15.2. 526 unique sequences were clustered into 265 operational taxonomic units (OTUs) at 97% similarity using UPARSE implemented with USEARCH v11.0.667. Representative OTU sequences were taxonomically annotated using the 16S Silva v132 database with USEARCH11. For each OTU, we only select one sequence for phylogenetic tree construction."

So 256 unique isolates? Please describe the dereplication step in main text. I'm guessing 16S amplicon sequencing…

Reply: We are sorry about the confusion. We added new method description to address this comment. Indeed, the unmber of unique sequences (OTUs) not equal to number of isolates, because a unique sequence encompasses a set of identical V3-V4 sequences coming from a single or multiple isolates. We have deleted the this inaccurate descriptions. [Line 571-581]

Line 245-246 Are Ensifer spp part of Rhizobiaceae? Please mention

Reply: *Ensifer* is also often referred to its synonym *Sinorhizobium*. To avoid confusion in our case, we used *Sinorhizobium* instead of *Ensifer* in the revision. [Line 231]

Line 247: delete " The" before "ASV 17" line 248 remove brackets hube node (ASV) -> hube node ASV. Please check the entire text for this.

Reply: Thanks for the editing, we had also sent our manuscript to NPG professional language editing service before re-submission.

Line 254: change into : Four of the five test Rhizobiaceae strains are significantly enriched on gl2 mutant plants

Reply: To avoid confusion (in the next comment), we deleted this part.

Line 254: justy above you describe 10 (4+6) Rhizobiaceae strains. Now 5 were tested?
Reply: Sorry about confusion. In this part, we actually meant five tested strains in a 48 well plate based root CFU calculating result. However, that is a mono-association assay which we just inoculate roots with one microbe strain per group, and seems add little novelty to the current microbiome story. To avoid confusion here, we also deleted this part to save space for our new data and analysis.

Fig 5b is not readable.
Reply: Thanks for the comment. We updated the previous Fig 5b to a new style (new Fig. 4) to make it look clearer.

Line 270 and usually also has more robust phenotypes critical for agricultural application. I don't agree, the combination of strains can also antagonistic effects. Perhaps: "and can have more robust phenotypes"
Reply: Thanks for the nice suggestion. We have updated accordingly. [Line 252]

Line 296 "mutants could block" change into "mutants were impaired in" (also rephrase line 290; the mutant is impaired it doesn't block)
Reply: Thanks for the nice suggestion. We have updated accordingly.
Line 355-370 could easily be skipped. If the discussions starts at line 372, it would still be fine. Moreover that would leave more space to discuss the final metagenome and metabolome work (fig 5) which is currently not, but should be, touched upon in the discussion.
Reply: Thanks for the suggestion, we deleted the whole paragraph to save space for our new discussion paragraph for integrated multi-omics analysis. After careful re-checking our manuscript, we agree that in many places the expression/discussion is not clean and clear. We tried to deleted several parts of our previous manuscript (in tracking change model) to save space for new data and discussion.

Reviewer #2 (Remarks to the Author):

The paper requires significant English language editing including context focus (not just grammar) before it is suable for publication.
Reply: We had careful checked the manuscript, and we had also sent our manuscript to the Springer Nature Research Editing Service (edited by a language editor who specializes in the subject area). We hope we had largely improved the manuscript.

My review is focused specifically on the metabolomics aspect of the manuscript.
Reply: Thanks for the very professional comments, which had helped us to improve the manuscript.

The authors specify that "16 plants were pooled per replicate". It can be inferred from the results that the treatments included the wild type genotype and two mutants. Were these sampled for both the control (well watered) and drought stress condition? Please clarify how many bioreplicates were

generated per treatment and which treatments were included.

Reply: Thank you for your comments. From our microbiome results, we found that the differences between different mutants mainly exist under drought conditions, such as changes in Rhizobiaceae abundance and the deterministic community assembly process in the root microbiome of *gl2*. So we only conducted metabolome profiling for the roots of all genotypes under drought treatrment. We mixed 16 plants from each genotype as one bioreplicate, and a total of 3 bioreplicates were sampled for metabolome analysis. We have clarified this in the context in the method part in tracking change model.

The results suggest that the original goal of the metabolomics analysis was to to confirm suggested variation in proline concentrations. The authors should justify their choice to use a nontargeted metabolomics approach rather than a targeted analysis for proline.

Reply: Thank you for the professional comment. We updated this part. Our original goal was to use metabolomics approach to investigate the potential metabolic cues reshaping microbiome changes among different genotypes. In the revised manuscript, we updated the expression:

"To further investigate potential microbiome functional changes that might be related to drought protection, we conducted a metagenomic analysis of the root associated microbiomes of different genotypes under drought conditions.

" [Line 305-307]

No information is provided about how samples were prepared/extracted for analysis and how much sample was injected.

Reply: Thanks for careful checking! We have added these detail in the Method part:

For metabolome analysis, the root samples were firstly stored at -80℃, and then was thawed on ice before analysis. A 400 μL solution (methanol:water, 7:3 v/v) containing internal standard samples was added to around 20 mg root samples. Then vortex for 3 minutes. The sample was subsequently sonicated in an ice bath for 10 minutes, vortex again for 1 minute, and then placed in -20 °C for 30 minutes. Afterward, the sample was centrifuged at 12,000 rpm for 10 minutes at 4 °C, and the sediment was discarded. The supernatant was then centrifuged again at 12,000 rpm for 3 minutes at 4 °C. Finally, 200 μL of the supernatant was used for liquid chromatography-mass spectrometry (LC-MS) analysis. [Line 590-597]

No details are provided about instrument operation (liquid chromatography and mass spectrometry). This section requires extensive improvement to enable replication of the analysis.

Reply: Thanks for reminding this! We had added extensive new description in the method part:

The LC-MS analysis were carried on a Shimadzu LC-30A system (paired with TripleTOF 6600+, SCIEX) equipped with a ACQUITY UPLC BEH C18 column (1.8 μm, 2.1 mm * 100 mm, Waters). The analytical conditions were as follow, mobile phases used were Milli-Q water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), with all solvents being LC-MS grade. The injection volume was 2 μL. The flow rate was set at 0.4 ml/min with column temperature at 40 ℃. The capillary temperature was set at 550 ℃(ESI+) or 450 ℃(ESI-).

The mass spectrometry (MS) data acquisition were operated using the information-dependent acquisition (IDA) mode using Analyst TF 1.17.1 Software (Sciex, Concord, ON, Canada). MS

analysis was conducted in the negative and positive ion modes with electrospray ionization. The source parameters used for MS were as follows : temperature (TEM) at 550 °C; ion source gas 1 (GAS1) at 50 psi; ion source gas 2 (GAS2) at 50 psi; curtain gas (CUR) at 35 psi; ion spray voltage floating (ISVF) at 5000 V and 4000 V for positive and negative modes, respectively; declustering potential (DP) at 60 V or −60 V for positive and negative modes, respectively. [Line 599-611]

Likewise, there is no information at all provided about how the MS data was processed （**Q1**）. How were the metabolite annotations determined（Q2）? What tools were used? How confident are the annotations? (MS data，Q3)

Reply: Thanks for the professional suggestion! We had updated our method part accordingly.

Q1: The original data file obtained by LC-MS was converted into mzML format by ProteoWizard software. Peak extraction, peak alignment and retention time correction were respectively performed by XCMS program. The "SVR" method was used to correct the peak area. The peaks with detention rate lower than 50 % in each group of samples were discarded.

Q2: For metabolite annotations,the metabolic identification information was obtained by query into databases including the database MWDB (by comparing to local standards); DB-all public database, including Metlin (http://metlin.scripps.edu/index.php), HMDB（https://hmdb.ca/），KEGG（https://www.kegg.jp/），Mona（https://mona.fiehnlab.ucdavis.edu/），MassBank（http://www.massbank.jp/); AI prediction database (based on competitive fragmentation modeling82) and metDNA.

Q3: The confidient level of identification of metabolites were considered according to database used. For example, the confidience level 1 represents the metoblites were idfentified using the database MWDB, which based on comparision with standards. Additionally, confidience level 2 and 3 corresponding to the identification based on the DB-all public database and AI prediction batabase, respectively. The metabolites we used for validate the effects on the root-microbes interaction, including targenetin, Corymboside and Kaempferol, were under level 2. We will added one more supplementary tables including all this detail information. [Line 613-628]

*Was any quality control performed? Were the samples randomized for analysis? Please describe.

[2.7] Yes. We performed quality control group, and all samples were randomized before measuring. We also added this description in the new method part:

"For the QC samples, we mixed all the tested samples in equal amounts as a QC sample, and we tested a total of three replicates of QC samples. All QC samples are clustered very closely to each other in PCA graph, which confirms the robustness of our testing system.

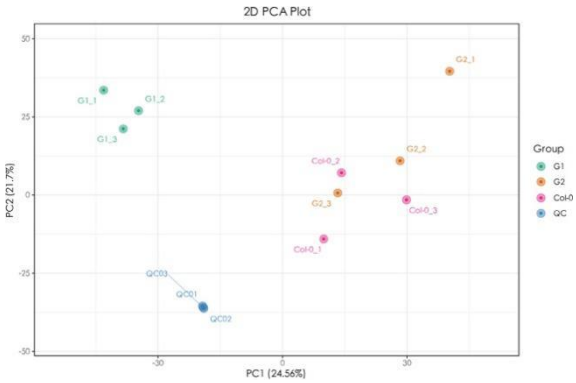


Fig. 1 PCA plot of all tested samples and quality control (QC) samples in our group.

Were data normalized prior to statistical analysis? Was a multiple testing correction performed as part of the statistical analysis? (i.e. are value adjusted for multiple testing?).

Reply: Thanks for the suggestion. We used the Normalization function in MetaboAnalyst R package for normalization step. We used t.test and FDR correction for adjusting multiple testing.

As per the requirements of the journal and as a general standard of the field the raw metabolomics data should accessible (via a public repository). Additionally, the processed output used for statistical analysis should also be included as supplemental data.

Reply: Thank you for the comment. Non-targeted metabolomics data have been deposited in the Metabolights database, with an accession numbers MTBLS10730 (www.ebi.ac.uk/metabolights/MTBLS10730). [Line 754]

Supplemental Figure 7 – The PCA plot only shows data from drought stressed plants, were control plants analyzed? If not, why?

Reply: Thank you for the comment. We mainly focused on the comparasion among genotypes to refelect plant genetic effect on microbiome composition. From our microbiome results, we found that the differences between different mutants mainly exists only under drought conditions, such as changes in Rhizobiaceae abundance and the deterministic community assembly process in the root microbiome of *gl2* (Fig. 2c;Fig. 3a;Sup Fig. 6). So we only conducted metabolome profiling for the roots of all genotypes under drought treatrment.

The metabolite names are likely too specific for the output of a nontargeted application. (although this is hard to assess since there are no methods). As an example, PA(18:1(9Z)/18:1(9Z)) should be reported as PA(36:2). Standard nontargeted metabolomics methods do not provide sufficient evidence to assign more specific lipid annotation.

Reply: Thanks for the professional comment. We deleted the previous fig containing PA(18:1(9Z)/18:1(9Z)) to PA(36:2) in the revised version, because we had re-structured our data.Additionally, We also removed the previous Supplememntary Figure 7c, and changed the Supplememntary Figure 7b into Fig. 5e with a better visualization way to illustrate the differences of relative contents of main metabolites classes among genotypes.

The pathway enrichment analysis is not clearly described in the methods. How is the differential abundance score determined and what does it mean?

Reply: Thanks for the comment, we updated the pathway enrichment analysis in Sup Fig. 11b, which used rich factor represents the ratio of differentially abundant metabolites in a specific pathway relative to the total annotated metabolites in that pathway.

All the circles are the same size although the caption implies that different size circles indicate the number of metabolites. Why are some of the pathways bolded?

Reply: Thanks for catching this, we had corrected the New Sup Fig. 11.

As written, the paper is clearly focused on the genomic analysis. There is no mention of the metabolomics data in the discussion and the only figure relating to this data is a supplemental figure. I would like to see the justification for the metabolomics data more clearly described and improved integration of the results into the discussion and overall conclusions of the paper.

Reply: Thanks for catching this, we totally agree with this comment, and integrated metabolomic and metagenomic data into a new paragraph of discussion. [Line 373-399]

Reference

1. Cook, R.J. and A. Rovira, *The role of bacteria in the biological control of Gaeumannomyces graminis by suppressive soils.* Soil Biology and Biochemistry, 1976. **8**(4): p. 269-273.
2. Yang, K., et al., *RIN enhances plant disease resistance via root exudate-mediated assembly of disease-suppressive rhizosphere microbiota.* Mol Plant, 2023.
3. Zhou, X., et al., *Interspecific plant interaction via root exudates structures the disease suppressiveness of rhizosphere microbiomes.* Molecular plant, 2023. **16**(5): p. 849-864.
4. Segata, N., et al., *Metagenomic biomarker discovery and explanation.* Genome biology, 2011. **12**: p. 1-18.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The paper is much improved. I think the authors have addressed all issues raised by me and I have no further major comments. It would be good to introduce also the concept of microbes alleviating drought stress in the abstract (line 16) already. This is missing currently. The authors jump from drought stress immediately to genetic control of the microbiome.

Reviewer #2 (Remarks to the Author):

The authors have done an excellent job with their revision of the manuscript and have adequately addressed my comments. The readability of the manuscript (english language grammar) is also greatly improved. My only remaining comment is that the authors add an additional supplementary table with the raw and normalized abundance values for the metabolite data (to complement supplementary tables 10 and 11).

Responses to the reviewers' comments:

Reviewer #1 (Remarks to the Author):

The paper is much improved. I think the authors have addressed all issues raised by me and I have no further major comments. It would be good to introduce also the concept of microbes alleviating drought stress in the abstract (line 16) already. This is missing currently. The authors jump from drought stress immediately to genetic control of the microbiome.

Reply: Thanks for catching the logical gap. We had updated the abstract: “Drought is one of the most serious abiotic stresses, and emerging evidence suggest that plant microbiome affects plant drought tolerance.”

Reviewer #2 (Remarks to the Author):

The authors have done an excellent job with their revision of the manuscript and have adequately addressed my comments. The readability of the manuscript (english language grammar) is also greatly improved. My only remaining comment is that the authors add an additional supplementary table with the raw and normalized abundance values for the metabolite data (to complement supplementary tables 10 and 11).

Reply: Thanks for all the help in improving the manuscript. We put the raw and normalized abundance values for the metabolite data into our new Supplementary Data 6-8.