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Peer Review File

A root system architecture regulator modulates OsPIN2 polar localization in rice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Li et al. report on the identification of GLS1 when identifying the lesion responsible for the gls1 mutant in rice. In the manuscript, gls1 is reported to exhibit a shallower, but more extensive root system and increased nutrient uptake and grain yield compared to wild type. The authors report the cloning and confirm that other alleles of gls1 exhibit similar root phenotypes as well as complementation after insertion of a GLS1proGLS1-GFP transgene.

The authors took the extra step of identifying gls1 loss- of function mutants generated via CRISPR in the Heijing2 background. This work appears to be quite sound and well documented.

A GUS fusion demonstrated that GLS1 is ubiquitously expressed, although more highly expressed in root tips and stem bases of 7 d seedlings. Direct examination of the GFP fusion and immunolocalizations using anti-GFP sera indicates a basal PM localization of the GLS1 signal. I find the characterization of the GLS1 as basally localized to be somewhat unconvincing. The appears to be considerable signal present on the lateral walls. At the resolution sampled, it would be difficult to distinguish higher intensity for a thicker basal plasma membrane from a true increase in abundance. The authors could clarify how they assured that such an artifact is not involved.

Subsequent mutational analysis shown by the authors supports the basal localization and the dependence of that localization on phosphorylation of Ser30.

The authors then established genetic interaction between PIN2 and GLS1 and showed that pin2 was epistatic to gls1. Analysis with DII Venus demonstrated altered auxin localization with loss of GLS1. In an effort to show that PIN2 is the sole target of GLS1, the authors analyzed AUX1 and PIN gene expression as well as polar localization of PIN1. A 50% loss of polar localization of PIN2 in the gls1 background supports a direct function in PIN2 localization. One might argue that analysis of ABCB4 interaction would be appropriate, but ABCB4 does not exhibit polar localization and is likely to transport substrates such as BR in addition to auxin.

The authors demonstrate direct interaction of GLS1 and PIN1 with pulldown assays in protoplasts. There is some concern about these results, as PIN2 is not normally localized to the PM in protoplasts. The secondary confirmation with BiFC helps, but requires more detail regarding controls to be sufficient.

The analysis of PIN2 ubiquitination that can be attributed to GLS1 is convincing.

Overall, the authors present a convincing analysis of GLS1 function and present those results in an appropriate manner.

Reviewer #2 (Remarks to the Author):

The paper by Li et al. describes the identification of GLS1, the rice ortholog of Arabidopsis thaliana WAVE3 proteins. Work conducted in Arabidopsis thaliana had previously shown that WAVE3, together with two homologues, regulates the polar distribution of the auxin transporter PIN2 (Konstantinova et al., Nature Communications 2022). GLS1, WAVE3 as well as PIN2 share roles in root gravitropism and auxin transport regulation.

Previous work published on WAVE3 proteins already showed a role in PIN2 polarity regulation in Arabidopsis (Konstantinova et al., Nature Communications 2022). From this point of view, the paper here is not novel and also, qualitatively inferior when compared to the methods and tools available in Arabidopsis. The rice work on GLS1 presented here is strong on the physiological side and in that it identifies phosphoregulation of GLS1, as well as a direct interaction between GLS1 as an E3 ligase of PIN2. Unfortunately, neither the phosphoregulation is/can be explained in more detail (identity of upstream kinase?) nor the observed protein degradation of PIN2 via GLS1 can be used to explain PIN2 polarity regulation. From that point of view, this paper falls behind the already published one on the WAVE proteins.

Response to reviewers' comments and suggestions

Comments

Reviewer #1

1. This work appears to be quite sound and well documented.

Response: Thanks for your positive comments.

2. A GUS fusion demonstrated that GLS1 is ubiquitously expressed, although more highly expressed in root tips and stem bases of 7 d seedlings. Direct examination of the GFP fusion and immunolocalizations using anti-GFP sera indicates a basal PM localization of the GLS1 signal. I find the characterization of the GLS1 as basally localized to be somewhat unconvincing. The appears to be considerable signal present on the lateral walls. At the resolution sampled, it would be difficult to distinguish higher intensity for a thicker basal plasma membrane from a true increase in abundance. The authors could clarify how they assured that such an artifact is not involved.

Response: Thank you for your comments. We agree that OsGLS1 also localizes to the lateral PM (especially the lower lateral PM connecting the basal PM) except the basal PM (Fig. 2a, b; Supplementary Fig. 9). We have added new experimental data to confirm the OsGLS1 localization using FM4-64 staining (a lipophilic styryl dye stains the PM) as a control because that the signal intensity of FM4-64 at the whole lateral PM is uniform (Fig. 2a, b). We quantified the fluorescence intensity of GFP and FM4-64 at different PM sites, including upper, middle, lower and basal PM of root epidermal cells, and found that the ratios of GFP to FM4-64 of lower and basal PM were significantly higher than that of the upper and middle PM, indicating higher protein abundance of OsGLS1 at the lower and basal PM (Supplementary Fig. 9b,c). We have added these descriptions in the revised manuscript (Line 224 – 228).

3. The authors then established genetic interaction between PIN2 and GLS1 and showed that pin2 was epistatic to gls1. Analysis with DII Venus demonstrated altered

auxin localization with loss of GLS1. In an effort to show that PIN2 is the sole target of GLS1, the authors analyzed AUX1 and PIN gene expression as well as polar localization of PIN1. A 50% loss of polar localization of PIN2 in the gls1 background supports a direct function in PIN2 localization. One might argue that analysis of ABCB4 interaction would be appropriate, but ABCB4 does not exhibit polar localization and is likely to transport substrates such as BR in addition to auxin.

Response: Thank you for your suggestion. We have examined the possible protein-protein interaction of OsGLS1 with rice ABCB1, ABCB14, ABCB16 (the three closest homologs of AtABCB4 in rice) and OsAUX1 using yeast two-hybrid assay and found that OsGLS1 did not interact with OsABCB1, OsABCB14, OsABCB16 or OsAUX1, we have added this data and descriptions in the revised manuscript (Supplementary Fig. 21d and line 472 – 476).

4. The authors demonstrate direct interaction of GLS1 and PIN2 with pulldown assays in protoplasts. There is some concern about these results, as PIN2 is not normally localized to the PM in protoplasts.

Response: Thank you for your comments. Our pervious results have shown that OsPIN2 is localized to the PM in rice protoplasts (Wang et al., 2018) which is consistent with the results in this study. We have performed additional experiment to further confirm the interaction between OsGLS1 and OsPIN2 by BiFC in rice protoplasts (Supplementary Fig. 22). Additionally, we also checked the subcellular localization and co-localization of OsPIN2 and OsGLS1 in rice protoplasts, the results showed that OsPIN2 is localized at PM and cytoplasm, however, the abundance of the PM localized OsPIN2 was significantly decreased when OsPIN2 was co-expressed with OsGLS1 in rice protoplasts (Supplementary Fig. 23b). These results further confirmed that OsPIN2 interacts with OsGLS1 and is degraded through OsGLS1 mediated pathway. These results were added in the revised manuscript (Line 384 – 386).

5. The secondary confirmation with BiFC helps, but requires more detail regarding controls to be sufficient.

Response: Thank you for your suggestion. We have added FM4-64 as the PM maker and redone the BiFC experiment in rice protoplasts which showed that OsGLS1 interacts with OsPIN2 (Supplementary Fig. 22).

Reviewer #2

Previous work published on WAVE3 proteins already showed a role in PIN2 polarity regulation in Arabidopsis (Konstantinova et al., Nature Communications 2022). From this point of view, the paper here is not novel and also, qualitatively inferior when compared to the methods and tools available in Arabidopsis. The rice work on GLS1 presented here is strong on the physiological side and in that it identifies phosphoregulation of GLS1, as well as a direct interaction between GLS1 as an E3 ligase of PIN2. Unfortunately, neither the phosphoregulation is/can be explained in more detail (identity of upstream kinase?) nor the observed protein degradation of PIN2 via GLS1 can be used to explain PIN2 polarity regulation. From that point of view, this paper falls behind the already published one on the WAVE proteins.

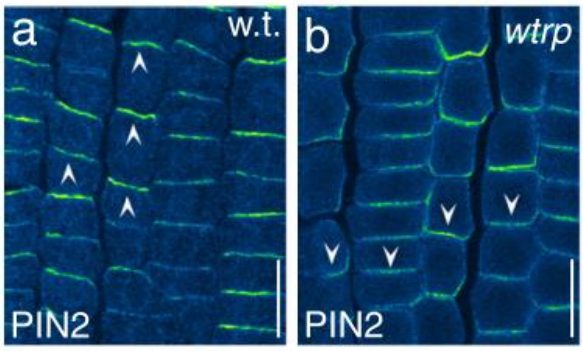
Response: Thanks for your comments. We agree that the *gls1* homologous proteins in Arabidopsis WAVE3 proteins already showed a role in PIN2 polarity regulation (Konstantinova et al., Nature Communications 2022). The work of Konstantinova et al. is great which indicated that the AtWAV3/WAVH are involved in regulating the polarity localization of AtPIN proteins via preventing PIN sorting into basal, ARF GEF-mediated trafficking using genetic and cell biological analyses. However, our work proved that in rice, OsGLS1 regulates OsPIN2 polarization by ubiquitination mediated degradation of OsPIN2 in the lower PM using genetic, physiological and biochemical evidences. The mechanism of OsGLS1 functioning on OsPIN2 differs with the one WAV3 functioning on AtPIN2 in Arabidopsis based on the following aspects:

1) As reported by Konstantinova et al., the growth and development of roots and shoots of Arabidopsis *wav* triple mutants are significantly inhibited compare to wild type (as shown below); however, our results showed that the root and shoot biomass of rice *gls1* mutant are significantly higher compare to that of wild type at different growth stages (Supplementary Fig. 1). And the nutrient uptake efficiency and grain yield of

gls1 mutant also increased significantly under soil culture conditions (Fig. 1; Supplementary Fig. 2, 3), suggesting that *AtWAV3/WAVH* and *OsGLS1* function differently on plant growth between Arabidopsis and rice.

(Fig. 1a and Fig. 4i, j from Konstantinova et al., Nature Communications, 2022)

2) Konstantinova et al. reported that AtPIN2 is basal polarity in root epidermis cells of *wav* triple mutants, but is apical polarity in that of wild type (as shown below); however, our result show that the polarity of OsPIN2 is lost in *gls1* mutant rather than a reversed polarity compared to the wild type (Fig. 3), suggesting that the underlying molecular regulatory mechanisms are also different between Arabidopsis and rice.



(Fig. 2a, b from Konstantinova et al., Nature Communications, 2022)

3) Konstantinova et al. suggested that the AtWAV3/WAVH is necessary for affecting apical PIN proteins sorting decisions in Arabidopsis, although the molecular mechanism of AtWAV3/WAVH impact on polar PIN sorting decisions is unclear. Whether AtWAV3/WAVH interact with AtPIN2 or not is not reported, authors also suggest that ubiquitin ligase activity of WAV3/WAVH is not directly involved affected PIN2 polarity in Arabidopsis (Nature Communications, 2022). While in rice, we proved

that OsGLS1 localized more on basal PM which interacts directly with, ubiquitinates and promotes the degradation of basally localized OsPIN2 (Fig. 4, 5), thus establishing the apical PM localization of OsPIN2 in rice (Fig. 6), a mechanism different from that reported in Arabidopsis. In addition, we have analyzed the subcellular localization of OsPIN2 and OsGLS1 by transient expression in rice protoplasts and found that the abundance of OsPIN2 on the PM was significantly decreased when OsGLS1 was co-expressed, confirming that OsGLS1 should directly degrade OsPIN2 in vivo (Supplementary Fig. 23b).

Based on the above reasons, our work is innovative which shows that OsGLS1 functions on OsPIN2 polar localization in a mechanism different from the one reported in Arabidopsis. Furthermore, our work identified OsGLS1 as the key root architecture regulator for nutrient uptake and yield in crop which will benefit nutrient efficient crop breeding. Therefore, we think that our work advanced our understanding on the root architecture improvement for nutrient efficient crops and elucidated new mechanism on PIN2 polar localizations.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript provided by the authors largely resolves my concerns and almost ready for publication. The test for direct interactions between Os GLS1 and OsABCB1 , 6, and 14 via split U year 2 hybrid would be sufficient to discount interaction with the ABCBs discussed if ABCB functionality, or at least, membrane localisation is confirmed. Although most ABCB1-like transporters can be functionally expressed in S. cerevisea, Arabidopsis ABCB4, 6, 19, and 20 are not. The authors will need to demonstrate that their Y2H is actually testing GLS1 interactions with functional, or, at least, properly localised protein. It is also puzzling that the authors specify 6 and 14 as most similar to ABCB4. They are most similar to ABCB1 and 19. As noted by the authors, OsABCB1 is most similar to ABCB4 with OsABCB10 next in similarity. While the authors are certainly not expected to spend a great deal of energy chasing down a negative interaction, the test that they have implemented is somewhat cursory and unconvincing. The choice is to either state that the possibility is not fully explored or test for interaction by another method.

Reviewer #3 (Remarks to the Author):

In this study, the authors demonstrated that improving the RSA using the gls1 gene leads to improvements in shoot growth and yield through improved nutrient absorption capacity, and the obtained knowledge will be extremely useful for future rice breeding. In addition, very careful and thorough analysis revealed that OsGLS1 controls root morphology through the degradation of OsPIN2, and there are no problems with the quality of the experiment and the interpretation of the results. Overall, it can be said that excellent research results have been obtained. However, as shown below, there are some parts that are insufficient in the explanation, and I request that these be reconsidered.

Comment 1

In the comment from reviewer 2, the novelty of this research was pointed out, and the authors responded by stating the difference in the control mechanism from Arabidopsis. This point is very important in demonstrating the novelty of this research, so I believe that the summarised contents of Responses 2) and 3) in particular should be described in the paper.

Comment 2

In this study, the authors conclude that the superior shoot growth and yield of the gls1 mutant is due to its high nutrient absorption ability. However, since the distribution patters of auxin also directly affects the shoot organ growth, this point needs to be considered more carefully. In this regard, it showed that when grown hydroponically, SDW of gls1 mutant is significantly higher than that of the wild type after 5 weeks from sowing. Doesn't this indicate that the gls1 gene itself directly controls the growth of the above ground? In addition, auxin is thought to control the number of tillers in association with cytokinin, but was there a tendency for the increased number of tillers in the gls1 mutant 5 or more weeks after sowing in hydroponic culture? If such a tendency exists, it is reasonable to conclude that the superior shoot growth and yield of the gls1 mutant is due to its high nutrient absorption ability, as well as the direct positive control of the shoot growth by the gls1 gene itself.

Comment 3

On line 327, it says the following:

“In situ immunostaining of OsPIN2 in longitudinal sections of XS63 and gls1 root tips using an anti-OsPIN2 antibody confirmed the polar localization of OsPIN2 to the apical PM in most root epidermis, exodermis and sclerenchyma cells in the meristem and elongation zones of XS63 (about 74.8%). Notably, OsPIN2 was evenly distributed between the apical and basal PM in most (about 74.9%) root epidermal cells in gls1. OsPIN2 showed polar localization to the basal PM in root cortex cells of both gls1 and XS63 (Fig. 3g,h and Supplementary Fig. 20). These results suggest that OsGLS1 is important for the normal abundance and polar localization of OsPIN2 to the PM in root epidermis, exodermis and sclerenchyma cells.”

In this case, which tissues were measured in Fig. 3h? Also, from the statement "Notably, OsPIN2 was evenly distributed between the apical and basal PM in most (about 74.9%) root epidermal cells in gls1," it is assumed that OsGLS1 functions more in epidermal cells than in exodermis and sclerenchyma cells. By showing the data in Fig. 3h for each different tissue, the tissue in which OsGLS1 functions can be more clearly understood.

Comment 4

On line 420, it says, "In addition, downregulating OsGLS1 expression by editing its promoter or producing a weak allele may help identify a line with nutrient-efficient RSA and high yield for use in

the field." It is unclear what the statement is trying to mean. Is it aimed at further improving the changes in RSA caused by gls1 used in this study, or is it intended to produce similar RSA to gls1 mutant in other varieties? I think this is an important part that describes the effectiveness of the OsGLS1 locus in breeding, so please describe it in more detail.

Response to reviewers' comments and suggestions

Comments

Reviewer #1

1. The test for direct interactions between OsGLS1 and OsABCB1, 6, and 14 via split U year 2 hybrid would be sufficient to discount interaction with the ABCBs discussed if ABCB functionality, or at least, membrane localisation is confirmed. Although most ABCB1-like transporters can be functionally expressed in S. cerevisea, Arabidopsis ABCB4, 6, 19, and 20 are not. The authors will need to demonstrate that their Y2H is actually testing GLS1 interactions with functional, or, at least, properly localised protein.

Response: Thank you for your comments. We have done western bolting experiment to detect the fusion proteins of HA-ABCBs-NubG in related yeast cells, and confirmed that the OsABCB1, 6 and 14 were successfully expressed in yeast (Supplementary Fig. 21e).

2. It is also puzzling that the authors specify 6 and 14 as most similar to ABCB4. They are most similar to ABCB1 and 19. As noted by the authors, OsABCB1 is most similar to ABCB4 with OsABCB10 next in similarity. While the authors are certainly not expected to spend a great deal of energy chasing down a negative interaction, the test that they have implemented is somewhat cursory and unconvincing. The choice is to either state that the possibility is not fully explored or test for interaction by another method.

Response: Thank you for your comment and suggestion. We agree with you that the possibility of OsGLS1 interaction with these OsABCB proteins is not fully explored, we have added this statement in the revised manuscript (Line 482 – 483).

Reviewer #3:

1. In the comment from reviewer 2, the novelty of this research was pointed out, and

the authors responded by stating the difference in the control mechanism from Arabidopsis. This point is very important in demonstrating the novelty of this research, so I believe that the summarised contents of Responses 2) and 3) in particular should be described in the paper.

Response: Thank you for your suggestions. We have added the relevant description in the revised manuscript (Line 497 – 506). "**Konstantinova *et al*. reported that AtPIN2 is basal polarity in root epidermis cells of *wavh1 wavh2 wav3* triple mutants, but is apical polarity in wild type. Furthermore, the ubiquitin ligase activity of WAV3/WAVHs is not directly involved affected PIN2 polarity in Arabidopsis. However, the molecular mechanism of AtWAV3/WAVHs impact on PIN polarity is unclear. In this study, our results show that the polarity of OsPIN2 is lost in *gls1* mutant rather than a reversed polarity compared to the wild type (Fig. 3). We proved that OsGLS1 localized more on basal PM which interacts directly with, ubiquitinates and promotes the degradation of basally localized OsPIN2 (Fig. 4, 5; Supplementary Fig. 23b), thus establishing the apical PM localization of OsPIN2 in rice (Fig. 6), a mechanism different from Arabidopsis**".

2. In this study, the authors conclude that the superior shoot growth and yield of the gls1 mutant is due to its high nutrient absorption ability. However, since the distribution patters of auxin also directly affects the shoot organ growth, this point needs to be considered more carefully. In this regard, it showed that when grown hydroponically, SDW of gls1 mutant is significantly higher than that of the wild type after 5 weeks from sowing. Doesn't this indicate that the gls1 gene itself directly controls the growth of the above ground? In addition, auxin is thought to control the number of tillers in association with cytokinin, but was there a tendency for the increased number of tillers in the gls1 mutant 5 or more weeks after sowing in hydroponic culture? If such a tendency exists, it is reasonable to conclude that the superior shoot growth and yield of the gls1 mutant is due to its high nutrient absorption ability, as well as the direct positive control of the shoot growth by the gls1 gene itself.

Response: Thank you for your suggestions. We agree that *OsGLS1* itself may directly regulates shoot development in rice. We have added the relevant description in the revised manuscript (Line 418 – 420). "**Meanwhile, the shoot dry weight of *gls1* mutant is significantly higher than that of the wild type after 5 weeks grown in solution culture, suggesting that the *OsGLS1* may directly control shoot growth in rice**".

3. On line 327, it says the following:

"In situ immunostaining of OsPIN2 in longitudinal sections of XS63 and gls1 root tips using an anti-OsPIN2 antibody confirmed the polar localization of OsPIN2 to the apical PM in most root epidermis, exodermis and sclerenchyma cells in the meristem and elongation zones of XS63 (about 74.8%). Notably, OsPIN2 was evenly distributed between the apical and basal PM in most (about 74.9%) root epidermal cells in gls1. OsPIN2 showed polar localization to the basal PM in root cortex cells of both gls1 and XS63 (Fig. 3g,h and Supplementary Fig. 20). These results suggest that OsGLS1 is important for the normal abundance and polar localization of OsPIN2 to the PM in root epidermis, exodermis and sclerenchyma cells." In this case, which tissues were measured in Fig. 3h? Also, from the statement "Notably, OsPIN2 was evenly distributed between the apical and basal PM in most (about 74.9%) root epidermal cells in gls1," it is assumed that OsGLS1 functions more in epidermal cells than in exodermis and sclerenchyma cells. By showing the data in Fig. 3h for each different tissue, the tissue in which OsGLS1 functions can be more clearly understood.

Response: Thank you for your comments. The cells measured in Fig. 3h are the outer cell layers of rice root including epidermis, exodermis and sclerenchyma cells (The out cell layers was named in Huang et al., 2009, Plant Cell Physiol), as the polar localization pattern of OsPIN2 are same among these cell types. We have also replaced "epidermal cells" with "outer cell layers" in the revised manuscript (Line 121, 208, 223, 234, 303, 306, 332, 403 and 492). We agree that OsGLS1 functions more in epidermis cells than that in exodermis and sclerenchyma cells as both OsGLS1 and OsPIN2 are accumulated

more in epidermis cells than in exodermis and sclerenchyma cells. We have described the tissues measured in Fig. 3h and discussed in the discussion (Line 457 – 461). "**There is no significant difference in the polarity orientation of OsGLS1 among the epidermis, exodermis and sclerenchyma cells, however, OsGLS1 accumulated more in epidermis cells than that in exodermis and sclerenchyma cells in root tip (Supplementary Fig. 9), same as OsPIN2 (Fig. 3g)**".

4. On line 420, it says, "In addition, downregulating OsGLS1 expression by editing its promoter or producing a weak allele may help identify a line with nutrient-efficient RSA and high yield for use in the field." It is unclear what the statement is trying to mean. Is it aimed at further improving the changes in RSA caused by gls1 used in this study, or is it intended to produce similar RSA to gls1 mutant in other varieties? I think this is an important part that describes the effectiveness of the OsGLS1 locus in breeding, so please describe it in more detail.

Response: Thank you for your comments. We are sorry for the ambiguous description of this sentence. We were trying to say that we can produce plants with suitable root growth angle which is nutrient-efficient and not drought-sensitive through genome editing in promoter of *OsGLS1* in future. We have revised the description in the revised manuscript (Line 424 – 426). "**Therefore, downregulating *OsGLS1* expression by editing its promoter or producing a weak allele may help produce a line with suitable RGA for nutrient-efficiency, drought tolerance and high yield for use in the field in future**".

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors responded to reviewer comments properly. This revised manuscript improves upon ambiguities in the presentation and interpretation of experimental results and asserts the novelty of the findings. As I judged previously, the authors demonstrated that improving the RSA using the gls1 gene leads to improvements in shoot growth and yield through improved nutrient absorption capacity, and the obtained knowledge will be extremely useful for future rice breeding. In addition, very careful and thorough analysis revealed that OsGLS1 controls root morphology through the degradation of OsPIN2, and there are no problems with the quality of the experiment and the interpretation of the results. Thus, I think this manuscript is at the level of being accepted.