Rice Homeodomain Protein WOX11 Recruits a Histone Acetyltransferase Complex to Establish Programs of Cell Proliferation of Crown Root Meristem

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Review timeline:

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	1st Decision:	January 6, 2017 *revision requested*
TPC2016-00908-RAR1	1st Revision received:	February 23, 2107
	2nd Decision:	March 22, 2017 *revision requested*
TPC2016-00908-RAR2	2nd Revision received:	April 11, 2017
	3rd Decision:	April 18, 2017 *accept with minor revision*
TPC2016-00908-RAR3	3rd Revision received:	April 19, 2017
	4th Decision:	April 21, 2017 *acceptance pending, sent to science editor*
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00908-RA 1st Editorial decision – *revision requested* **January 6, 2017**

The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision and re-review.

In preparing your revision, we ask you to address all comments/concerns from the reviewers. In addition, we ask you to address the following points raised by the editors:

1) BiFC data needs to be quantified. Please see Xing et al., 2016 Plant Physiol. 171, 727-58 for details.

2) In situ hybridization is not a quantitative technique. In Fig. 6C, please address whether the pattern of expression for OsPIN9 and OsCLF6 are changed in the different mutants. Likewise, the resolution of the in situ hybridization images shown in Fig 2A are not sufficient. In which cells in the root apex are OsGCN5 and OsADA2 expressed, and how does this compare to the pattern of WOX11 expression.

3) How many genes are differentially expressed in wox11 roots, and what fraction of genes downregulated in wox11 is also downregulated in the OsGCN5 RNAi line? Given the proposed model, the overlap should be substantial. As pointed out by Reviewer 2, please cite the manuscript by Jiang et al. in your resubmission.

4) For the ChIP analyses, in addition to the statistical analysis requested by the reviewers, these data require additional negative controls. The reported enrichment levels for H3Ac at the genes co-regulated by OsGCN5 and WOX11 are very low. To ensure these values are biologically relevant, negative control genes need to be included. These could be low copy transposons or genes shown to lack H3Ac in genome-wide ChIP analyses. Levels of H3Ac enrichment in the actual IP should be significantly higher than observed for negative control loci. Likewise for ChIP analysis of WOX11, OsGCN5, and OsADA2.

5) The sampling and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly

what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-00908-RAR1 1st Revision received February 23, 2017

Reviewer comments and **author responses**:

RESPONSE TO EDITOR: Thank you for giving us the chance to revise our manuscript.
After carefully reading your decision letter and the comments of the reviewers, we extensively revised the manuscript by including additional experimental data and analysis and by improving data interpretation and discussion in the text.

RESPONSE TO EDITOR Point 1: Quantification of the BiFC data is shown in Fig. 1C.

RESPONSE TO EDITOR Point 2: We added a description of the in situ hybridization data in the text.

RESPONSE TO EDITOR Point 3: There are 434 down-regulated genes in *wox11* mutant root tip (accession N° GSE84933), about 30% (128) of them were also found to be down-regulated in OsGCN5 RNAi. This is now described in the text and the data are now presented in Figure S7B. The manuscript Jiang et al is now accepted in J. Exp. Bot and is cited.

RESPONSE TO EDITOR Point 4: We included Ehd1 as an additional negative control for ChIP-qPCR in Fig. 7. *Ehd1* was not expressed in roots by our RNA-seq data. No acetylation or WOX11-, OsADA2-, or OsGCN5- binding signals could be detected (Fig. 7).

RESPONSE TO EDITOR Point 5: We have revised thoroughly the MS by providing precise information of repeats and root materials used in the experiments. Statistical analysis was added in Figure 7C to E.

Reviewer #1:

In this manuscript, the authors showed that WOX11 recruits the ADA2-GCN5 histone acetyltransferase (HAT) module to activate downstream target genes in crown root meristem, such as energy metabolism-, cell wall synthesis-, and hormone response-related genes. This finding is very important to understand the developmental mechanisms regulating root formation in plants. However, descriptions of some results are not clear and appropriate. I request the authors to revise the following points.

Point 1. In "Introduction", the authors cited their previous papers, as "WOX11 is expressed in the whole root meristem region and stimulates crown root emergence and elongation by regulating genes of both auxin and cytokinin signaling". On the other hand, they described the WOX11 function as "Our previous and present data showing that the rice WOX11 is required to stimulate crown root initiation and elongation by stimulating cell division in the meristem" in "Discussion" of this manuscript. I could not understand how they know the WOX11 function in initiation of crown root primordium formation. In this manuscript, the authors got the results that both the both the *OsGCN5* and *OsADA2* RNAi lines produced fewer crown roots. In addition, they wrote, "The staining revealed clear delays of crown root primordium formation in *OsGCN5* and *OsADA2* RNAi plants compared to wild type". The phenotypes of the RNAi plants were similar to the *wox11* mutant (Zhao et al., 2009)." However, there is no discussion about the WOX11 function for crown root initiation and I request the authors to discussing this point.

RESPONSE: This is a good point. However, the present and previous data on rice WOX11 do not allow a conclusion that this gene has a similar function as Arabidopsis WOX11/12 to promote the formation of crown root primordia. As you know, developmental functions of WOX family members are not always conserved between rice and Arabidopsis. Our data only allow concluding that WOX11 has a function to stimulate cell proliferation that occurs during crown root initiation and elongation steps. We have discussed this in the revised version.

Point 2. In this case, I think it is important to clarify whether reduced crown root number in *OsGCN5* and *OsADA2* RNAi plants is caused by delayed shoot growth or not, because these transgenic plants showed also reduced shoot growth. Thus, I request the authors to comparing crown root number between wild type and these transgenic plants by using similar growth stage plants that have similar number of leaves, instead of using same age (seven-day-old) plants in Fig. 3.

RESPONSE: We have obtained plants with the same shoot lengths by differing germination date and observed the root phenotypes in the RNAi or mutant plants with the same shoot lengths as wild type. The data are presented in Figure S3. The results are described in the text.

Point 3. It is difficult to understand what kind of root the authors targeted in several parts, such as Fig. 2, Fig. 7, page 10, line 200, etc. Please indicate the kinds of root and/or how to sample those roots there.

RESPONSE: We have added "crown root" in Lines 225, 229, 283, 289, 601, 603, and 644.

Point 4. "negative segregates" is not clear. Please describe about it in more detail and state what kind of transgenic generations did the authors used.

RESPONSE: We used segregates of T3 generation transgenic plants that do not contain the transgene.

Point 5. Please indicate how the authors determined the meristem size.

RESPONSE: The method was added In the Methods section. Root meristem size was determined by measuring the length from the quiescent center to the first elongated cell in the 6th cortex layer. When length of a cortex cell is twice of that of the neighbor cell, the cell is considered as the first elongated cortex cell.

Point 6. Please indicate the reason why the authors chose these 8 genes among 24 genes that were also down-regulated in *wox11* mutant.

RESPONSE: We chose one or more representative genes from representative categories (including metabolism, cell component, hormones).

Reviewer #2:

Previous work has shown that the Wuschel-related homeobox gene *WOX11* is both necessary and sufficient to promote crown root emergence in rice. In this manuscript, the authors demonstrate that two components of a putative SAGA-like histone acetyltransferase complex, OsADA2 and OsGCN5, are recruited to WOX11 targets to activate gene expression in crown root meristems. OsADA2 was shown to interact with both WOX11 and OsGCN5 in vitro and in vivo and all three proteins were found to associate with WOX-binding motifs present within genes that were downregulated in a *wox11* mutant as well as in RNAi lines that have reduced expression of either *OsGCN5* or *OsADA2*. WOX11 binds these genes independent of histone acetylation and is required for recruitment of OsADA2 and OsGCN5. Downregulation was associated with decreased levels of histone acetylation, leading the authors to conclude that histone acetylation is required for gene activation by WOX11.

The manuscript reports a very thorough analysis of the physical and genetic interactions between the WOX11 transcription factor, ADA2 and GCN5 in crown root development in rice. The experimental work is of high quality and the conclusions are adequately supported by the data presented. I have only a few minor criticisms of the manuscript which I've outlined below:

Point 1. The discussion is the least satisfying section of this manuscript as it reads more like a series of discrete bits of information rather than being a unified discussion of the data and how it relates to previously published information. For example we are told quite a bit about GCN2 and ADA2b in Arabidopsis root development without any comparison to what was found in the experiments being reported here.

RESPONSE: We have substantially revised the discussion section by trying to take into account your suggestions.

Point 2. I notice that there is a related manuscript (Jiang et al submitted) cited. I believe that this should be made available to the editor and perhaps also the reviewers so that we can assess any potential overlap in the data being presented in the two manuscripts.

RESPONSE: This MS is accepted now in J. Exp. Bot. and is cited in our MS. We uploaded this MS in our resubmission for your information.

Point 3. Figure 7; there are error bars shown on the histograms for the ChIP data presented here. What do they represent? What is the replication for these experiments - this could be listed in the Materials and Methods as well as the legend?

RESPONSE: The bar=means ±SD in Fig 7A and 7B are from three technical repeats, and in Fig 7C to 7E are from 3 biological repeats. We have performed 3 biological replicates for the histone acetylation ChIP-PCR (Fig 7A and 7B); all show the same trends but with substantial variations of values between the replicates. Therefore, only one replicate with 3 technical repeats is shown for the H3ace ChIP-PCR. This is now mentioned in the figure legend.

Reviewer #3:

This study shows that the rice WOX homeodomain transcription factor WOX11 recruits OsADA2-OsGCN5 histone acetyltransferase complex to activate a set of target genes in crown root meristem cell proliferation. Although no direct interaction between WOX11 and OsGCN5 was detected in Y2H assays, WOX11 interacts with OsADA2, which physically recruits OsGCN5. Expression analysis showed that WOX11, OsADA2 and OsGCN5 share similar expression domain in root meristem. Knock down the expression of *OsADA2* and *OsGCN5* showed similar defects in cell proliferation in crown root meristem, mimicking the *wox11* mutant phenotype. The study further identified and confirmed 8 new common targets for WOX11, OsADA2 and OsGCN5 through transcriptome analysis of *OsGCN5* RNAi roots, WOX11-binding site prediction, gene expression analysis in *wox11* mutant and ChIP assays. In all, this study established a new molecular mechanism for WOX11 action in crown root meristem development.

Modern clade WOX transcription factors have been shown to recruit TOPLESS corepressor-histone deacetylase complex to repress target genes. Although WOX transcription factors are known to act as activators as well, the molecular mechanism for gene activation by WOX proteins is still unknown. This study shows that intermediate clade WOX transcription factor WOX11 directly activate target genes through recruiting the histone acetyltransferase complex, thus it significantly advances our understanding of the action for WOX proteins.

Point 1. Although the physical interactions between WOX11 and OsADA2 (Y2H, pull-down and BiFC), OsADA2 and OsGCN5 (Y2H, pull-down and Co-IP) were clearly documented, the formation of a WOX11-OsADA2-OsGCN5 complex in vivo still requires more evidence. The in vivo interaction between WOX11 and OsADA2 needs to be confirmed by Co-IP assays using roots as materials. Moreover, the Co-IP assays using transient expression system in rice shoot cells lack an important control of the combination of GFP-WOX11 and OsGCN5-HA without OsADA2.

RESPONSE: We performed the control Co-IP assays using a combination of WOX11-GFP, OsGCN5-HA and a construct in which OsADA2 cDNA was put in the reversed orientation to 35S. In this combination, anti-GFP (WOX11-GFP) could not co-immunoprecipitate GCN5-HA. The data are included in Figure 1F.

We also performed experiments to confirm in vivo interaction between WOX11 and OsADA2. The data are present in Figure 1E. We immunoprecipitated protein extracts of a *WOX11* overexpression line and the *wox11* mutant with anti WOX11, and subsequently analyzed the precipitations by immunoblotting using anti-WOX11 and anti-OsADA2 antibodies: OsADA2 could be immunoprecipitated from the WOX11 overexpression line but not from the *wox11* mutant.

Point 2. The authors analyzed the transcript levels of OsGCN5 and WOX11 common target genes by qRT-PCR. If WOX11 recruits OsGCN5 through OsADA2 (scaffold for HAT interaction with transcription factors as mentioned in the discussion), the analysis of the transcript levels of those target genes in *OsADA2* RNAi lines will strengthen the conclusion of WOX11-OsADA2-OsGCN5 complex for regulating those targets.

RESPONSE: We performed analysis of target gene transcripts in *OsADA2* RNAi lines and the corresponding wild type. The data are presented in Figure 6B.

Point 3. The authors performed ChIP assays using polyclonal antibodies of WOX11 and OsADA2, and showed that both WOX11 and OsADA2 bound same regions. Moreover, the OsADA2 binding was reduced in *wox11* mutant roots. This is a strong evidence that OsADA2 may partially rely on WOX11 for binding to the targets. However, statistical analysis is needed for those ChIP assays.

RESPONSE: Statistical analysis was added in Fig. 7C to E

Point 4. The conclusion of "*OsGCN5* RNAi and *wox11* mutant display reduced auxin concentration in root" is only supported by the DR5-GUS reporter. More information is needed for the DR5-GUS reporter line and the GUS staining protocol is also missing in the methods. Moreover, DR5-GUS reporter is more related to auxin response rather than auxin concentration.

RESPONSE: GUS staining method was added.

Point 5. The authors characterized two *OsGCN5* overexpression lines (OG-21 and OG-25) in detail (Figure S4) and observed no obvious difference of root development or plant growth. Are those lines from the OsGCN5-fh lines? In 9 OsGCN5-fh transgenic lines (1, 2, 3, 4, 21, 22, 23, 24, 25), only 4 lines (3, 4, 21, 22) have detectable signals using anti-HA antibody (Figure S2), but line 21 signal is very weak. If OG-21 and OG-25 lines have very weak (OG-21) or no (OG-25) detectable protein expression of the transgene, the phenotype analysis of these two lines may be misleading. Are there any growth phenotypes of Gfh3 and Gfh4 lines, which were used to confirm OsGCN5 and OsADA2 interaction in vivo (Figure 1D) and had relatively strong protein expression level (Figure S2)?

RESPONSE: OG-21 and OG-25 are different from OsGCN5-fh lines. They over-express OsGCN5 without tags. Neither Gfh3 and Gfh4 nor OG-21 and OG-25 plants produced an obvious phenotype.

TPC2016-00908-RAR1 2nd Editorial decision – *revision requested* March 22, 2017

The Reviewers and Editors appreciate your efforts to address their prior concerns. However, a few of the key points remain unaddressed in this revision, and so we ask you to pay attention to the following points in preparing your revision:

1) Figure 2A: The earlier critique regarding the quality of the in situ hybridization data has not been addressed in this revised manuscript. The resolution of the in situ hybridization images shown in Fig 2A are not of sufficient quality to evaluate in which cells in the root apex *OsGCN5* and *OsADA2* are expressed. Cross sections or better DIC images are needed. In fact this is also true for WOX11. Looking at the earlier published in situ data it is entirely unclear in which cell layers in the root *WOX11* is expressed. Therefore, it remains unclear in which cell types WOX11 overlaps with OsGCN5 and OsADA2.

2) Figure 3A, B: data should be presented from 3 independent biological replicates with statistical analysis.

3) Figure 6D: See the comment to Figure 2A above. Also, the legend is unclear, which panel belong to which genotype?

4) Figure 7A, B: data from all biological reps should be included in the manuscript. Authors can include the explanation provided in the rebuttal in the results section of the manuscript and include the other biological reps in supplemental data for reviewers and readers to see. All data sets should include statistical analysis. The figure legend states that Fisher's test was used to assess statistical significance; this is not the correct test in this case.

5) Please also address the few minor editorial changes highlighted by the Reviewers.

Note that the sampling and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

I should stress that we are reluctant to see manuscripts undergoing multiple rounds of revision and would be unlikely

to offer you more than one chance to satisfy the reviewers. Please contact us if there are ambiguous comments or if you wish to discuss the revision.

TPC2016-00908-RAR2 2nd Revision received April 11, 2018

RESPONSE TO EDITOR: Thank you for giving us a second chance to revise our manuscript.
After carefully reading your comments and that of reviewer 2, we have repeated the in situ hybridization experiments of *OsGCN5*, *OsADA2*, and *WOX11* genes in wild-type crown root tips, and *OsPIN9* and *OsCSLF* in *OsGCN5* RNAi and *wox11* mutant crown root tips compared to the respective wild type. This time we got better DIC images with higher resolution.

The data clearly show that *OsGCN5*, *OsADA2*, and *WOX11* are expressed in all cell types in the crown root tip (Fig 2A).

***OsPIN9* and *OsCSLF* transcripts are found to be uniformly reduced in the crown root tip of *OsGCN5* RNAi and *wox11* mutant (due to oversize of the images, this part of data is now presented in Fig S8).**

Concerning RT-PCR data in Fig 3A, B, we have included data from the other two biological repeats (with mRNA isolated from 10 d-old seedlings of 3 different cultures). The mean values in the RNAi plants are about 20% of that in wild type. The significance of differences are indicated (p value <0.001). There seems to us no doubt about the effectiveness of the RNAi.

Regarding to Fig 7, B, we have included the other 2 biological repeats (with seedling crown roots of 3 different cultures) in Fig S9.

RESPONSE TO EDITOR Point 1: We have performed new in situ hybridization experiments and obtained better DIC images. *OsGCN5*, *OsADA2*, and *WOX11* transcripts are detected in nearly all type of cells in the root tip (except *OsADA2* is unlikely to be expressed in root cap cells).

RESPONSE TO EDITOR Point 2: We have added 2 biological repeats of RT-qPCR with mRNA extracted from different individual plants of the lines. The data in Fig 3A, 3B are now calculated from 3 biological repeats. In the RNAi lines, there are only about 20% of WT levels for GCN5 and ADA2 transcripts. Statistical analysis has been added.

RESPONSE TO EDITOR Point 3: We have repeated the *in situ* hybridization experiments and obtained better quality DIC images. *OsPIN9* and *OsCSLF* transcripts are found to be reduced in all types of root tip cells in the *OsGCN5* RNAi and *wox11* crown root tips. Due to the bulky size of the images, we present the data as Figure S8.

RESPONSE TO EDITOR Point 4: We have added the other 2 biological replicates of the data in Figure S9. Yes, we did use Student's t-test for analysis. This is now corrected.

RESPONSE TO EDITOR Point 5: All corrected.

Reviewer #1:

I judge that the authors revised the manuscript appropriately in compliance with the reviewer comments

Reviewer #2:

The authors have addressed the majority of my concerns during the revision of this manuscript, but I have noticed a few other minor points that need to be addressed. These are outlined below:

Point 1. Figure 2A legend; the probes are mislabelled - the sense probe was used for (a) and (c) and antisense in (b) and (d).

RESPONSE: Actually, the labeling was right. Antisense probes detect mRNA of transcripts in the tissue, but sense probes do not. This Figure is completely revised by presenting new in situ hybridization images.

Point 2. Figure 3A & B; the legend states that the data come from 3 technical replicates; does this mean that you repeated the measurements of the same seedlings 3 times?? If so then I recommend that the experiment be repeated twice more with more plants. The bar showing statistical difference is purported to show significance

between wt and transgenic lines and yet for some of the comparisons (eg RL between ZH11 and RA2 or CN between ZH11 and RG6) it is hard to believe that there is a difference given the overlap of the error bars.

RESPONSE: We did two more biological replicates (with mRNA isolated from seedlings of 3 different cultures). The RT-PCR data shown in Fig 3A and 3B are from 3 biological repeats.

For root length and number data, according to student *t*'s test, P value for RL comparison of ZH11 and RA2 is 0.00797, P value for CN comparison of ZH11 and RG6 is 0.0004588. So, they did have significant differences.

Point 3. Figure 7; I recommend that the additional data provided for the reviewers should be included as Supplemental data.

RESPONSE: The biological replicates of the experiments are now shown in Figure S9.

Reviewer #3:

This is a much-improved version of the manuscript. The authors have addressed all my concerns, having included new data to support the proposed role of WOX11-ADA2-GCN5 complex in regulating rice crown root development. The data is sound and provides novel insights into crown root development and the mechanisms of gene regulation that control it. On page 6, Figure 1F, Input, Anti-WOX11, line 3 needs to be labeled.

RESPONSE: Labeled.

TPC2016-00908-RAR2 3rd Editorial decision – *accept with minor revision* April 18, 2017

The board of reviewing editors have reviewed your revised manuscript entitled "Rice homeodomain protein WOX11 recruits a histone acetyltransferase complex to establish programs of cell proliferation of crown root meristem", and would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision of the following point:

Considering data in Fig. 7A, B and Fig. S9 are based on single biological replicates, it is not possible to add statistical significance to these figures. Please remove significance values from these graphs and make this point clear in the legends. However, for all other data presented, statistical significance should be calculated based on at least three independent biological replicates -- see previous decision letters for instructions on defining the nature of these replicates.

TPC2016-00908-RAR3 3rd Revision received April 19, 2017

RESPONSE TO EDITOR: Thank you very much for the decision letter. We are very sorry for the mistakes still present in the previous version. We have removed the statistical analysis from Fig 7A, 7B, and Fig S9 and have modified the figure legends. We have added statistical analysis of biological replicates in Fig 6, and have revised legends of Fig 3A, 3B, and Fig 6 to detail the nature of the biological replicates. Thank you for having given us this opportunity,

TPC2016-00908-RAR3 4th Editorial decision – *acceptance pending* April 21, 2017

We are pleased to inform you that your paper entitled "Rice homeodomain protein WOX11 recruits a histone acetyltransferase complex to establish programs of cell proliferation of crown root meristem" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor May 7, 2017