Cyclin A participates in the *TSO1-MYB3R1* regulatory module to maintain shoot meristem size and fertility in *Arabidopsis*

Fuxi Wang, Wanpeng Wang and Zhongchi Liu
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Original submission

First decision letter

MS ID#: DEVELOP/2022/200612

MS TITLE: Cyclin A participates in the TSO1-MYB3R1 regulatory module to maintain shoot meristem size and fertility in Arabidopsis

AUTHORS: Fuxi Wang, Wanpeng Wang, and Zhongchi Liu

Dear Dr. Liu,

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Reviewer 1

Advance Summary and Potential Significance to Field

The TSO1 protein is part of the DREAM complex, being a key regulatory machinery ensuring cellular quiescence by repressing cell cycle regulators and coordinating phase-specific gene expression. The corresponding mutants display an increase in shoot stem cell divisions, whereas the apical root meristem shows an opposite defect. Additionally, loss of function mutants displays reduced

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fertility, which has been used in a forward genetics screen to identify complementing mutations. This has resulted before in the identification of a mutant in the MYB3R1 transcription factor, encoding another component of the DREAM complex. Here, the cyclin CYCA3;4 was identified as another complementation mutant (at least for the sterility phenotype). Reporter and overexpression analysis suggests that CYCA3;4 is hyperactivated in the tso1 mutant background, accounting for its phenotype.

Although the data do not really help in understanding the cellular origin of the tso1 mutant phenotypes, the identification of CYCA3;4 as a DREAM target is an interesting observation. Likewise, combined with the data of a recent complementary paper, the data suggest a unique role for this cyclin in cell cycle progression, despite the presence of three related genes (CYCA3;1 to CYC3;3).

Comments for the author

The relationship between CYCA3;4 and the MYB3B1 subunit of the DREAM complex (Figure 5) could be strengthened by including in the work the tso1-3 myb3r1 cyca3;4 triple mutant (the tso1-3 myb3r1 double mutant has been published by the team before). Likewise, the authors could show CYCA3;4 expression levels in mybr3r1 mutant background (using the CYCA3;4 reporter line or alternatively by RT-qPCR).

How does TSO1 activation in the 35S:TSO-GR line affect the CYCA3;4 levels (this would strengthen the idea that TSO1 suppressed cyclin expression)? Similarly, it would be nice to complement the CYCA3;4:GUS reporter data in the tso1 mutant background with data on a transcriptional reporter or by RT-qPCR.

At page 7 two possible interpretations are given for the tso1 mutant phenotype in relation to CYCA3;4: too much cell proliferation or a cell cycle arrest because the cells cannot exit the G2/M cell cycle phase. It is unclear to my why the authors believe the first interpretation is most likely. This needs clarification.

The CYCA3;4-GUS accumulation pattern is reported to become uniform in the tso1 mutant roots. Which conclusion is drawn from these data?

What is the relevance of Figure 1C? Is it to correlate expression levels with the strength of the silique phenotype shown in 1D?

The first paragraph of the introduction needs editing

Reviewer 2

Advance Summary and Potential Significance to Field

The manuscript from Wang et al. describes identification of CYCA3;4, a member of CYCA3 class cyclins, as a potential effector protein of TSO1-MYB3R1 regulatory module for cell cycle regulation in shoot apical meristems (SAM). They conducted a genetic screen for suppressor mutations of tso1 phenotype in Arabidopsis, and a mutant allele of CYCA3;4 was found to have suppressing effect on tso1 abnormality in SAM. CYCA3;4 was shown to be a candidate of TSO1-MYB3R1 direct target in the experiment using CYCA3;4-GUS and in LUC reporter assay in N benthamiana. The presented data are consistent with their conclusion that TSO1-MYB3R1 repress transcription of CYCA3;4 for properly controlling cell proliferation in SAM.

Comments for the author

The presented data are consistent with their conclusion, but several key experiments seem to be lacking in the present form of manuscript as listed below and should be considered for improving this paper. I feel most important for improving manuscript are comments #1, #3 and #4. Other comments are optional but highly recommended.

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1) Lack of quantitative data showing tso1 suppression and enhancement
In Fig. 1 and Fig. 2, suppressing effect of cyca3;4 is shown only as representative images of fluorescent stems and meristems. Only Fig. 2D shows quantitative data, which is, however, representing root phenotype unsuppressed by cyca3;4, instead of shoot phenotype that is most strongly affected in tso1. Because suppression of tso1phenotype by cyca3;4 is not very clear, I would like to see whether and to what extent this suppression is quantitatively and statistically significant. Similarly to this concern, the enhanced tso1 phenotype caused by CYCA3;4 overexpression should be also quantitatively analyzed. In the present manuscript, quantification was made only in Fig. 4D where seed number was counted and compared.

2) Potential redundancy among CYCA3 members
Related to the above comment, weak suppressing effect of cyca3;4 may be due to the presence of other members of CYCA3 genes that may be functionally overlapping with CYCA3;4. Although the authors made an argument in Discussion that CYCA3;4 is a unique member among CYCA3s, it is still highly probable that additional mutations of cyca3s would further suppress the tso1 phenotype. This possibility should be worth testing to show clear suppression and to make clear conclusion that CYCA3s are critical downstream effector of TSO1-MYB3R1. Although I do not thick it is necessary to create mutants in which all CYCA3 members ae mutated. I would like to see whether, at least, double or triple mutation may affect more strongly compared to cyca3;4 single mutation.

3) Binding of TSO1-MYB3R1 not fully demonstrated
Binding of TSO1-MYB3R1 to CYCA3;4 is not fully demonstrated in the present form of manuscript. The authors only showed the published data from DAP-seq where MYB3R1 protein has a potential for binding to upstream region of CYCA3;4 in vitro. I would like to see whether this binding is also observed in vivo by conducting ChIP-qPCR experiment with MYB3R1 and TSO1. To show in vivo binding of transcription factors (TF) in ChIP has become a current standard for properly evaluating the involvement of TF on the target genes, and thus seems to be essential in this manuscript. It is also important to see whether such binding is observed in other member of CYCA3s, related to above comment on functional overlap among CYCA3s.

4) Quantitative analysis CYCA3;4 expression required
The effect of tso1 on CYCA3;2 expression is only addressed in CYCA3;4-GUS reporter experiment in Arabidopsis plants. Again the data is only shown as representative images of GUS-stained plants without quantitative analysis. One possible way for improvement may be quantitatively analyze the GUS expression and statistically evaluate the difference between wild type and tso1. In this line of argument, it is highly recommended to analyze increased CYCA3;4 expression by qRT-PCR experiments, which should also include other members of CYCA3s. It seems to be also interesting to use cell cycle-regulated CYCA3;4 reporter fused to GFP and analyze the effect of tso1 on CYCA3;4 expression in the cell cycle. This is especially important because the authors made arguments on effect of TSO1 specific to G1/S regulation in Discussion and Fig. 6.

5) Effect of MYB3R1 should be also examined
Throughout the manuscript, the authors used only tso1 for analyzing its effect on CYCA3;4 expression. To fully support that CYCA3;4 is under the control of TSO1-MYB3R1 module, it is important to see the effect of myb3r1 as well. More interesting experiment may be to analyze the expression of CYCA3;4 when both myb3r1 and tso1 are mutated and compare with the data from each single mutation.

6) Fig. 5 shows binding of MYB3R1 in vitro to CYCA3;4 promoter. It is important to determine if the region of DAP-seq peak contain consensus binding motif of MYB3R (MSA element). If there exits in the promoter region, then recomended experiment is to analyze whether the mutation in the MSA element influences the expression of CYCA3;4 in GUS reporter experiment and also in LUC reporter experiment. Additionally, it is also worth examining whether other members of CYCA3s also show DAP-seq peak in their promoter regions, and contain the MSA element, in addition to CYCA3;4.

Reviewer 3

Advance Summary and Potential Significance to Field

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The overall reading of the manuscript was clearly logical and well-written. How the DREAM protein complex regulates the cycle is an important topic in development, in particular in plants that often own multiple paralogues of each individual component. The genetic connection of CYCA3;4 with TSO1 was established with solid and convincing data. The discoveries give new insights into this important pathway; therefore the manuscript should be a great fit for Development. However, the connection between MYB3R1 with CYCA3;4 was relatively weak. The manuscript should be strengthened by providing more experimental evidence.

Comments for the author

The manuscript entitled "Cyclin A participates in the TSO1-MYB3R1 regulatory module to maintain shoot meristem size and fertility in Arabidopsis" reports the identification of CYCA3;4 downstream of TSO1, one key component of the DREAM complex in the regulation of cell cycle. By genetically screening for suppressors of tso1 in Arabidopsis, cyca3;4 belonged to one of the suppressors that rescued the loss of fertility phenotype of tso1 (a read-out of cell proliferation in the shoot apical meristem). Furthermore, overexpression of CYCA3;4 enhanced the fertility defects of a weak mutant allele tso1-3. The genetic evidence supports that CYCA3;4 is downstream of the TSO1-associated DREAM complexes in the regulation of cell proliferation in the SAM. In addition, provided with published in vitro data that MYB3R1 (the previously characterized transcription factor functioning downstream of TSO) could bind to the CYCA3;4 promoter, the authors showed this connection by the promoter Luciferase assay in heterologous tobacco cells. In the end, the authors proposed a novel mechanism for the DREAM complex component TSO1 and MYB3R1 through CYCA3;4 to regulate cell proliferation and differentiation in the shoot.

Major points:

- Fig. 5, regarding MYB3R1 binding to the CYCA3;4 promoter regions, this should be further supported by motif deletion experiments in tobacco luciferase assay. It was not clear how long the promoter region of CYCA3;4 was used. This should be clearly specified in the figure and how it is different from/similar to what has been used for the GUS expression. The most convincing data for MYB3R1 binding to the CYCA3;4 promoter in vivo should be 1) Arabidopsis plants expressing MYB3R1 used for ChIP PCR assay on the CYCA3;4 promoter. 2) the expression patterns of CYCA3;4-GUS in myb3r1 mutants.
- According to the model, MYB3R1 connects TSO1 and CYCA3;4, this is an important interaction. To establish the model, I would ask to show more genetic data between myb3r1 with CYCA3;4 loss-of-function and overexpression.

Minor points:

- The fact that cyca3;4 mutation alone in the wild type background does not show discernable phenotype should be mentioned earlier in the Results parts before further elaborated in the Discussion.
- In figure 4A, the overexpression phenotype of CYCA3;4 was not revealed in the WT but dependent on tso1 mutation. Does this mean other partners of CYCA3;4 require TSO1-mediated function? More discussion should be added.
- In the discussion part, authors have speculated the phosphorylation of MYB3R1 by CYCA3;4, are there any CDK phosphorylation consensus sites present in MYB3R1 sequence?

Original submission

Second decision letter

MS ID#: DEVELOP/2022/200612

MS TITLE: Cyclin A participates in the TSO1-MYB3R1 regulatory module to maintain shoot meristem size and fertility in Arabidopsis

AUTHORS: Fuxi Wang, Wanpeng Wang, and Zhongchi Liu
ARTICLE TYPE: Research Article

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Dear Dr. Liu,

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

Reviewer 1

Advance Summary and Potential Significance to Field

A specific cyclin is identified as a putative regulator of a cell cycle critical transcriptional complex

Comments for the author

I'm in doubt about this resubmission. At one hand, the identification through an EMS suppressor screen of the CYCA3;4 cyclin to be putatively part of the DREAM complex is novel and interesting (as stipulated before) and the authors have addressed (in part) previous comments (mine as well as those of the independent reviewers). But at the other hand, these extra experiments have not yielded exactly the expected outcome (emphasizing that the requested experiments were useful): rather than supporting and clarifying the previous working model, they added more uncertainty to why exactly the cyclin accumulates in the TSO1 mutant and how the complementation data can be explained. In particular:

- The newly included RT-PCR data demonstrate that the previous assumption of TSO1 being a transcriptional activator was wrong. Now it rather appears that the CYCA3 protein is stabilized, whereas the gene is repressed by TSO1. Accordingly, the authors have adopted their model, speculating that TSO1 simultaneously downregulates CYCA3;4 expression and upregulates an activator of CYCA3;4 degradation.

Unfortunately, the latter part of this hypothesis is only supported by a single RT-PCR experiment.

- The requested promoter deletion analysis demonstrates that binding of MYB3B1 occurs independently from MSA cis-acting element that it normally binds. This is a quite unexpected result as up to date MYB3R has only found/reported to bind MSA elements. So again, because of being unexpected (and potentially interesting) I think this observation needs some follow-up or complementary experiments.

Here is is a pity that authors didn't follow up on the requested genetic experiments.

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Therefore, although I realize that the mapping of a suppressor mutant is no small feat and that the authors did their best to comply with the reviewer's comments, I'm not really convinced on the putative mechanism put forward.

Reviewer 2

Advance Summary and Potential Significance to Field

The manuscript from Wang et al. describes identification of CYCA3;4, a member of CYCA3 class cyclins, as a potential effector protein of TSO1-MYB3R1 regulatory module for cell cycle regulation in shoot apical meristems (SAM). They conducted a genetic screen for suppressor mutations of tso1 phenotype in Arabidopsis, and a mutant allele of CYCA3;4 was found to have suppressing effect on tso1 abnormality in SAM. TSO1-MYB3R1 may be a part of large protein complex known as DREAM complex, and this report may add a piece of knowledge on the divergence and physiological functions of these protein complexes in plant cell cycle and development.

Comments for the author

I appreciate very much for the authors' effort to carefully respond to my previous concerns. Reading the authors' responses, however, I was confused to realize that the authors changed their basic idea on the action of MYB3R-TSO1 during their revision. In the previous version of the manuscript, my understanding was that CYC3;4 is transcriptionally repressed by MYB3R-TSO1, and loss of tso1 upregulates CYC3;4, which further causes meristem phenotype in tso1. Based on the new data showing that CYCA3;4 was not transcriptionally repressed by MYB3R-TSO1, I feel that the manuscript should be largely changed in order to support the new idea. More specifically, please see following suggestions.

Upon the request from reviewers, the authors conducted new experiment for quantitative analysis of CYCA3;4 transcript levels. The data shows that CYCA3;4 transcript levels do not increase in tso1 (Fig. 3J) unlike the observed upregulation of CYCA3;4-GUS (Fig. 3A-D). Instead, the new experiment shows that CCS52A2, encoding activating protein of APC/C ubiquitin ligase, is downregulated in tso1. Upon these findings, the authors hypothesized that upregulation of CYCA3;4-GUS is due to downregulation of CCS52A2, which causes reduced protein degradation of CYCA3;4. The downregulation of CCS52A2 may be due to direct transcriptional regulation by MYB3R1-TSO1. This idea is interesting but significantly different from the context of their original submission. If the authors changed the idea and are trying to conclude in this way, many new experiments are required. Following the new idea, transcriptional regulation of CYCA3;4 by TSO1- MYB3R1 is not important, instead, the authors should experimentally show how TSO1-MYB3R1 directly regulates CCS52A2, which should be the direct downstream target responsible for tso1 suppression.

Questions immediately come to mind include 1) CCS52A2 is really functionally important for the observed suppression of tso1? To clarify this, it is necessary to analyze the effect of ccs52a2 mutation as in Fig. 2. 2) CCS52A2 is really critical for the protein levels of CYCA3;4? 3) CCS52A2 is really directly regulated by TSO1-MYB3R1? To answer these questions, it is necessary to conduct new ChiP experiment and reporter assays (as in Fig. 5). I feel these necessary revisions may cause a significant change of the manuscript as a whole, and I am afraid that significant re-writing may be required.

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Reviewer 3

Advance Summary and Potential Significance to Field

This manuscript revision addresses an important question: what the cell cycle regulator is downstream of the DREAM complex in the regulation of shoot apical meristem.

Comments for the author

In this study, an elegantly designed mutant screen identified CYC3;4 as such a key regulator. Compelling genetic evidence demonstrated that CYC3;4 functions downstream of TSO1, one component of the DREAM complex in plants, to promote cell division in the shoot meristem. Additional data in the revision further suggested that TSO1 promotes CYC3;4 expression at the transcription level, whilst suppressing CYC3;4 expression at the post-transcriptionally level. Thus, the overall regulation of TSO1 on CYC3;4 is suppressive. Taken together, the determination of a key cell cycle regulator in the DREAM complex and the demonstration of sophisticated regulation of TSO1 on CYC3;4 in shoot meristem development make the story a great fit for publishing at Development. In the last section of the manuscript, the authors attempted to fill the gap between TSO1 and CYC3;4 by testing direct regulations between MYB3R1, another component of the DREAM complex, and CYCA3;4. However, this part was felt less compelling given the data provided in the revised form. Evidence supporting MYB3T1's suppression role on CYC3;4 transcription was either from large-scale ChIP screens or from transient promoter-binding assays. As this connection appeared to be one of the core components in the model, genetic data of higher order mutants combining myb3r1 and cyca3;4 or more in vivo testing whether MYB3R1 binds to the promoter of CYC3;4 would be greatly helpful.

Minor points:
Fig. 3J, Y-axis should be labeled. 3K, the positive arrow from TSO1 to CYCA3;4 can be thinner as apparently, based on the phenotype, the negative regulation through CCS52A2 is predominant.

Author response to reviewers' comments

Dear reviewers,

Thank you so much for your time and valuable comments and suggestions. We have revised the manuscript as a “Research Report”, which allows us to delete several figures that were of concerns by you and focus on the most important findings. Specifically, we removed Figure 5 and Figure 6 as well as supplementary Figures 4 and 5 from the prior version. We moved Figure 3H-J to supplemental Figure S4 and mentioned it briefly during discussion. We added a revised model to Figure 4D. We combined Results and Discussion based on the journal rule. Following is a point-by-point response (**in bold**) to your comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
A specific cyclin is identified as a putative regulator of a cell cycle critical transcriptional complex
Reviewer 1 Comments for the Author:
I’m in doubt about this resubmission. At one hand, the identification through an EMS suppressor screen of the CYCA3;4 cyclin to be putatively part of the DREAM complex is novel and interesting (as stipulated before) and the authors have addressed (in part) previous comments (mine as well as those of the independent reviewers). But at the other hand, these extra experiments have not yielded exactly the expected outcome (emphasizing that the requested experiments were useful):

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rather than supporting and clarifying the previous working model, they added more uncertainty to why exactly the cyclin accumulates in the TSO1 mutant and how the complementation data can be explained. In particular:

Thank you. We agree with your assessment. We have since requested the Editor to allow submission of a “research report”. In this short report, we have removed data that required further experimental support concerning the relationship between TSO1 and CYCA3;4.

-The newly included RT-PCR data demonstrate that the previous assumption of TSO1 being a transcriptional activator was wrong. Now it rather appears that the CYCA3 protein is stabilized, whereas the gene is repressed by TSO1. Accordingly, the authors have adopted their model, speculating that TSO1 simultaneously downregulates CYCA3;4 expression and upregulates an activator of CYCA3;4 degradation. Unfortunately, the latter part of this hypothesis is only supported by a single RT-PCR experiment.

Thank you for your comments. While the observation of ectopic and abundant CYCA3;4-GUS accumulation in *tso1-1* is solid (Fig. 3), its mechanism and significance are uncertain. We discussed this uncertainty in page 8 (line 208-217). Further, we modified the model significantly in Fig. 4D to reflect this uncertainty. We moved the RT-qPCR data to supplementary Figure S4 and discussed it only briefly (line 210).

-The requested promoter deletion analysis demonstrates that binding of MYB3B1 occurs independently from MSA cis-acting element that it normally binds. This is a quite unexpected result as up to date MYB3R has only found/reported to bind MSA elements. So again, because of being unexpected (and potentially interesting) I think this observation needs some follow-up or complementary experiments. Here is is a pity that authors didn’t follow up on the requested genetic experiments.

Thank you for your encouragement. We removed Figure 5, Figure S4 and S5 completely from this version. Future follow-up experiments will be necessary to make conclusions regarding the MYB3R1-CYCA3;4 relationship.

Therefore, although I realize that the mapping of a suppressor mutant is no small feat and that the authors did their best to comply with the reviewer’s comments, I’m not really convinced on the putative mechanism put forward.

We thank you for recognizing our efforts. We think the short research report format will allow us to publish the most important data without delay. We will spend more time to develop the molecular mechanisms.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript from Wang et al. describes identification of CYCA3;4, a member of CYCA3 class cyclins, as a potential effector protein of TSO1-MYB3R1 regulatory module for cell cycle regulation in shoot apical meristems (SAM). They conducted a genetic screen for suppressor mutations of tso1 phenotype in Arabidopsis, and a mutant allele of CYCA3;4 was found to have suppressing effect on tso1 abnormality in SAM. TSO1-MYB3R1 may be a part of large protein complex known as DREAM complex, and this report may add a piece of knowledge on the divergence and physiological functions of these protein complexes in plant cell cycle and development.

Thank you for your positive comments.

Reviewer 2 Comments for the Author:
I appreciate very much for the authors’ effort to carefully respond to my previous concerns. Reading the authors’ responses, however, I was confused to realize that the authors changed their basic idea on the action of MYB3R-TSO1 during their revision. In the previous version of the manuscript, my understanding was that CYC3;4 is transcriptionally repressed by MYB3R-TSO1, and loss of tso1 upregulates CYC3;4, which further causes meristem phenotype in tso1. Based on the new data showing that CYCA3;4 was not transcriptionally repressed by MYB3R-TSO1, I feel that the manuscript should be largely changed in order to support the new idea. More specifically, please see following suggestions.

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Yes, the data did not show what we originally proposed despite extensive efforts in the follow-up experiments. Reviewer 1 has similar comments. We removed Figure 5, Figure S4 and S5 completely from this version and moved Fig. 3H-J to Figure S4. We also modified and simplified the model (See Fig. 4D) and discussed it (see the last section "A proposed model").

Upon the request from reviewers, the authors conducted new experiment for quantitative analysis of CYCA3;4 transcript levels. The data shows that CYCA3;4 transcript levels do not increase in tso1 (Fig. 3J) unlike the observed upregulation of CYCA3;4-GUS (Fig. 3A-D). Instead, the new experiment shows that CCS52A2, encoding activating protein of APC/C ubiquitin ligase, is downregulated in tso1. Upon these findings, the authors hypothesized that upregulation of CYCA3;4-GUS is due to downregulation of CCS52A2, which causes reduced protein degradation of CYCA3;4. The downregulation of CCS52A2 may be due to direct transcriptional regulation by MYB3R1-TSO1. This idea is interesting but significantly different from the context of their original submission. If the authors changed the idea and are trying to conclude in this way, many new experiments are required. Following the new idea, transcriptional regulation of CYCA3;4 by TSO1-MYB3R1 is not important, instead, the authors should experimentally show how TSO1-MYB3R1 directly regulates CCS52A2, which should be the direct downstream target responsible for tso1 suppression. Questions immediately come to mind include 1) CCS52A2 is really functionally important for the observed suppression of tso1? To clarify this, it is necessary to analyze the effect of ccs52a2 mutation as in Fig. 2. 2) CCS52A2 is really critical for the protein levels of CYCA3;4? 3) CCS52A2 is really directly regulated by TSO1- MYB3R1? To answer these questions, it is necessary to conduct new ChiP experiment and reporter assays (as in Fig. 5). I feel these necessary revisions may cause a significant change of the manuscript as a whole, and I am afraid that significant re-writing may be required.

We agree with you that additional experiments will be needed to support the TSO1-CCS52A2-CYCA3;4 regulation. Since this will require significant time to complete, we seek your understanding that we has shortened the manuscript into a "research report" focusing on the main findings. We removed CCS52A2 completely from the manuscript and discussed the uncertainty in the TSO1-CYCA3;4 relationships (page 8, line 208-217) and need for additional experiments (Page 10, line 271).

Reviewer 3 Advance Summary and Potential Significance to Field:
This manuscript revision addresses an important question: what the cell cycle regulator is downstream of the DREAM complex in the regulation of shoot apical meristem.

Reviewer 3 Comments for the Author:
In this study, an elegantly designed mutant screen identified CYC3;4 as such a key regulator. Compelling genetic evidence demonstrated that CYC3;4 functions downstream of TSO1, one component of the DREAM complex in plants, to promote cell division in the shoot meristem. Additional data in the revision further suggested that TSO1 promotes CYC3;4 expression at the transcription level, whilst suppressing CYC3;4 expression at the post-transcriptionally level. Thus, the overall regulation of TSO1 on CYC3;4 is suppressive. Taken together, the determination of a key cell cycle regulator in the DREAM complex and the demonstration of sophisticated regulation of TSO1 on CYC3;4 in shoot meristem development make the story a great fit for publishing at Development.

Thank you for your positive comments

In the last section of the manuscript, the authors attempted to fill the gap between TSO1 and CYC3;4 by testing direct regulations between MYB3R1, another component of the DREAM complex, and CYCA3;4. However, this part was felt less compelling given the data provided in the revised form. Evidence supporting MYB3T1's suppression role on CYC3;4 transcription was either from large-scale ChIP screens or from transient promoter-binding assays. As this connection appeared to be one of the core components in the model, genetic data of higher order mutants combining myb3r1 and cyca3;4 or more in vivo testing whether MYB3R1 binds to the promoter of CYC3;4 would be greatly helpful.

We agree that the regulatory mechanisms of CYCA3;4 by MYB3R1 and by TSO1 are not supported by strong data and require substantially more experiments including genetic data

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and in vivo data. This view is shared by reviewer 1 and 2 as well (see our responses to reviewer 1 and 2). To summarize, we have removed Figures 5-6 as well as Figure S4-S5 and shortened the manuscript to a "research report." The newly revised manuscript does not focus on the regulatory mechanisms of CYCA3;4 by MYB3R1 nor by TSO1. This is reflected in a revised model shown in Fig. 4D and the discussion of the model (see the last section "A proposed model).

Minor points:
Fig. 3J, Y-axis should be labeled.

Fig. 3J is moved to Fig. S4, and the Y axis is added.

3K the positive arrow from TSO1 to CYCA3;4 can be thinner as apparently, based on the phenotype, the negative regulation through CCS52A2 is predominant.

This figure is deleted.

Resubmission

First decision letter

MS ID#: DEVELOP/2022/201405

MS TITLE: Cyclin A participates in the TSO1-MYB3R1 regulatory module to maintain shoot meristem size and fertility in Arabidopsis

AUTHORS: Fuxi Wang, Wanpeng Wang, and Zhongchi Liu

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

As highlighted before, the finding that the mutation of this particular cyclin (CYCA3;4) can complement the cellular differentiation phenotype of the TSO1 mutant is interesting and novel.

Comments for the author

The manuscript represents a shortened version of a previously submitted manuscript, to be published as a report, rather than full article. Hence, I reviewed it independently from the previous submissions and response to the reviewer's comments (tabula rasa). Overall, I believe that the short format fits the data well, as it brings the data in a clear way without overinterpretation and without introducing a putative working mechanism for which data was lacking. As highlighted before, the finding that the mutation of this particular cyclin (CYCA3;4) can complement the cellular differentiation phenotype of the TSO1 mutant is interesting and novel, and hence I support

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publication of this work in the current format, following implementation of the following suggestions:

1. The newly written abstract does a poor job in selling the work. I do not think that the fact the TSO1 and MYB3R are homologs to components of the animal DREAM complex makes it important to study these genes in plants, but rather the fact that these genes (in both kingdoms) link to uncontrolled cell division and differentiation defects, thus giving a putative handle to study the link between meristem activity, cell cycle regulation, and cell differentiation. Second, I'm also not sure whether the work tells something specific on the role of CYCA3;4 in shoot meristem control, as the knockout and overexpression in the wild type background did not yield a shoot phenotype (thus I would suggest to reduce the last sentence to "The work reveals how the TSO1-….”). Third, a grammar check is required (in contrast to the rest of the manuscript which is well written).

2. At line 125 it is mentioned that the A144 mutation rescues as well the shoot meristem size. The way this conclusion is made is written out in the figure legend only. I would propose to do this rather in the results section.

3. Line 211: "… directly or indirectly" might be better suited.

4. Related to the expression of the CYCA3;4 gene in the root of WT versus tso1-1: it would be nice to show any root phenotypic data of tso1-1 versus tso1-1 cyca3;4 (is there a complementation phenotype of not?).

5. Line 243: I would suggest to explicitly mention the tissues examined (thus inflorescences and carpel), as (relative mild) root and leaf phenotypes following CYCA3;4 overexpression have been reported before by Willems et al. (although using another promoter to achieve overexpression). Here, the leaf and root were not examined, so it might be that the statement of lack of phenotype does not hold true for these tissues. Alternatively, authors can include such leaf and root data.

6. Concerning the mentioned phosphorylation sites within MYB3R1: it would be good to explicitly state whether these sites match the CDK consensus phosphorylation site (S/T-P-X-K/R).

<u>Reviewer 2</u>

Advance summary and potential significance to field

The revision became much more concise and clearer, and demonstrated the new genetic contribution of CYC3;4 in the TSO-MYB3R1 pathway. It is appropriate to publish as a Report in Development.

Comments for the author

Minor edits are suggested below.
cyc3;4CR3 should be annotated or explained in the main text and in the legend for Fig 1B.
The order of Fig. S3 and S4 should be switched to align with the text.

<u>Reviewer 3</u>

Advance summary and potential significance to field

The cell cycle machinery in plants is complex and linking meristem regulators to the complexities of cell cycle regulation remains a major challenge. The manuscript describes the identification of one of the cyclinA gene family (CycA 3;4) in a genetic screen aimed at identifying the suppressors of tso-1-1 allele an antimorphic mutant.

Comments for the author

The cell cycle machinery in plants is complex and linking meristem regulators to the complexities of cell cycle regulation remains a major challenge. Though cell cycle regulators gene families have

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been over amplified in plants, but the core cell cycle regulation is largely conserved between plants and animal systems.
The manuscript describes the identification of one of the cyclinA gene family (CycA 3;4) in a genetic screen aimed at identifying the suppressors of tso-1-1 allele, an antimorphic mutant. TSO1 is a member of the multigene family of proteins that share homology to animal-LIN54, which is art of MUVB core consisting of LIN9, LIN37, LIN52, LIN54 LIN64, RBBP4. MUVB core along with B-MYB, RB, DP1-3 proteins form DREAM complex that prevents quiescent cells from entering cell cycle. While the MMB complex devoid of RB promotes G2-M transition. Authors provide cloning data on the Cyc A 3;4, along with the genetic analysis showing that TSO1-1 mutant phenotypes depend on Cyc A 3;4 activity. This analysis has been integrated into the existing knowledge in proposing TSO1 could repress MYB21 expression while the Cyc A 3;4 (by binding CDK A;1) Phosphorylates MYB21. While the study identifies a role for CyC 3;4 only in certain genetic backgrounds, the biochemical and developmental roles of such a conditional regulation are not clear.

1. tso1 loss of function mutants (tso1-3) do not display SAM and floral morphogenesis defects unlike the peculiar antimorphic allele, tso1-1. Is there an explanation for this disparity such as genetic redundancy etc. If so, efforts must be made to generate higher-order mutants involving other family members.
Likewise analyzing the expression patterns of TSO1 family members may provide some clarity. In this context, the reference to the regulation of SAM and FM size in the title and the abstract is somewhat misleading.
2. Is there a loss of function phenotype for CycA 3;4? I see that authors show that its overexpression leads to no phenotype in wild-type background while seems to enhance sterility associated with the loss of function tso1-3 allele.
The sterility is a minor phenotype, therefore, authors should present data on the overexpression of CycA 3;4 in tso1-3 background. This is because the authors claim in the model that Cyc 3;4 could target MYB21 for phosphorylation. If this assumption is correct, one expects enhancement of tso1-3 phenotype.
3. Biochemical data analyzing the phosphorylation status of MYB21 in tso1 and tso1;cycA 3;4 must be provided to make the study mechanistic.

First revision

Author response to reviewers' comments

Thank you so much for your time to review our manuscript multiple times and for so many helpful suggestions! We hope that the following point-by-point responses have addressed your comments satisfactorily.
Sincerely, Zhongchi Liu

Reviewer 1 Advance Summary and Potential Significance to Field:
As highlighted before, the finding that the mutation of this particular cyclin (CYCA3;4) can complement the cellular differentiation phenotype of the TSO1 mutant is interesting and novel.

Reviewer 1 Comments for the Author: The manuscript represents a shortened version of a previously submitted manuscript, to be published as a report, rather than full article. Hence, I reviewed it independently from the previous submissions and response to the reviewer's comments (tabula rasa). Overall, I believe that the short format fits the data well, as it brings the data in a clear way without overinterpretation and without introducing a putative working mechanism for which data was lacking. As highlighted before, the finding that the mutation of this particular cyclin (CYCA3;4) can complement the cellular differentiation phenotype of the TSO1 mutant is interesting and novel, and hence I support publication of this work in the current format, following implementation of the following suggestions:

1. The newly written abstract does a poor job in selling the work. I do not think that the fact the TSO1 and MYB3R are homologs to components of the animal DREAM complex makes it important to study these genes in plants, but rather the fact that these genes (in both kingdoms) link to uncontrolled cell division and differentiation defects, thus giving a putative handle to study the link between meristem activity, cell cycle regulation, and cell differentiation. Second, I'm also not sure whether the work tells something specific on the role of CYCA3;4 in shoot meristem control, as the knockout and overexpression in the wild type background did not yield a shoot phenotype

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(thus I would suggest to reduce the last sentence to "The work reveals how the TSO1-….”). Third, a grammar check is required (in contrast to the rest of the manuscript which is well written).
Thanks for the helpful comments. We have revised the abstract based on your suggestions. We revised the abstract with specific attentions to the grammar.

2. At line 125 it is mentioned that the A144 mutation rescues as well the shoot meristem size. The way this conclusion is made is written out in the figure legend only. I would propose to do this rather in the results section.

Thanks for the suggestion. This information is moved from Figure 1 legend to the result section.

3. Line 211: "… directly or indirectly" might be better suited.

It is hard for us to image a "direct" regulation as TSO1 encodes a transcriptional regulator, yet the observed effect was on CYCA3;4-GUS protein accumulation. We have softened the "indirect" statement in the text by adding "may".

4. Related to the expression of the CYCA3;4 gene in the root of WT versus tso1-1: it would be nice to show any root phenotypic data of tso1-1 versus tso1-1 cyca3;4 (is there a complementation phenotype of not?).
The phenotype of WT, *tso1-1,* and *tso1-1; cyca3;4* root is shown in Fig. 2C, D, which showed that the *cyca3;4* CRISPR mutation did not suppress *tso1-1*'s short root phenotype.

5. Line 243: I would suggest to explicitly mention the tissues examined (thus inflorescences and carpel), as (relative mild) root and leaf phenotypes following CYCA3;4 overexpression have been reported before by Willems et al. (although using another promoter to achieve overexpression). Here, the leaf and root were not examined, so it might be that the statement of lack of phenotype does not hold true for these tissues. Alternatively, authors can include such leaf and root data.

Thank you very much for this important point. The tissues examined are now specified in the manuscript (page 9; line 269-270).

6. Concerning the mentioned phosphorylation sites within MYB3R1: it would be good to explicitly state whether these sites match the CDK consensus phosphorylation site (S/T-P-X-K/R).

MYB-S656 is the phosphorylation site followed by the sequence "**PVLDRR**", which matches the minimal CDK consensus phosphorylation site (S/T-P). This is added to the "A proposed model" section of the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:
The revision became much more concise and clearer, and demonstrated the new genetic contribution of CYC3;4 in the TSO-MYB3R1 pathway. It is appropriate to publish as a Report in Development.

Thank you very much for your positive comments.

Reviewer 2 Comments for the Author:
Minor edits are suggested below.

1. cyc3;4CR3 should be annotated or explained in the main text and in the legend for Fig 1B.

Thanks for the suggestion. We added legend about *cyca3;4CR3* in Figure 1B.

We included quantitative data of *cyca3;4CR3* in Figure 1B for easy comparison with A144. However, the manuscript text does not describe this data until after A144 was shown to reside in *CYCA3;4*. Therefore, the main text explains this *cyca3;4CR3* data in the next section (Page 6; line 178).

2. The order of Fig. S3 and S4 should be switched to align with the text.

Thanks. This is corrected.

Reviewer 3 Advance Summary and Potential Significance to Field:

The cell cycle machinery in plants is complex and linking meristem regulators to the complexities of cell cycle regulation remains a major challenge. The manuscript describes the identification of one of the cyclinA gene

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family (CycA3;4) in a genetic screen aimed at identifying the suppressors of tso-1-1 allele, an antimorphic mutant.

Reviewer 3 Comments for the Author:

The cell cycle machinery in plants is complex and linking meristem regulators to the complexities of cell cycle regulation remains a major challenge. Though cell cycle regulators gene families have been over amplified in plants, but the core cell cycle regulation is largely conserved between plants and animal systems. The manuscript describes the identification of one of the cyclinA gene family (CycA 3;4) in a genetic screen aimed at identifying the suppressors of tso-1-1 allele, an antimorphic mutant. TSO1 is a member of the multigene family of proteins that share homology to animal-LIN54, which is art of MUVB core consisting of LIN9, LIN37, LIN52, LIN54 LIN64, RBBP4. MUVB core along with B-MYB, RB, DP1-3 proteins form DREAM complex that prevents quiescent cells from entering cell cycle. While the MMB complex devoid of RB promotes G2-M transition. Authors provide cloning data on the Cyc A 3;4, along with the genetic analysis showing that TSO1-1 mutant phenotypes depend on Cyc A 3;4 activity. This analysis has been integrated into the existing knowledge in proposing TSO1 could repress MYB21 expression while the Cyc A 3;4 (by binding CDK A;1) Phosphorylates MYB21. While the study identifies a role for CyC 3;4 only in certain genetic backgrounds, the biochemical and developmental roles of such a conditional regulation are not clear.

1. tso1 loss of function mutants (tso1-3) do not display SAM and floral morphogenesis defects unlike the peculiar antimorphic allele, tso1-1. Is there an explanation for this disparity such as genetic redundancy etc. If so, efforts must be made to generate higher-order mutants involving other family
members. Likewise analyzing the expression patterns of TSO1 family members may provide some clarity. In this context, the reference to the regulation of SAM and FM size in the title and the abstract is somewhat misleading.

Thank you for this comment. Yes, *tso1-1* is an antimorphic allele, and its phenotype is much stronger than the *tso1-3*, a null allele. While *tso1-1* causes meristem fasciation, *tso1-3* does not. To understand this disparity, we previously performed extensive genetic analyses, the result of which was published in Sijacic et al., PLOS Genetics (2011).

To summarize the result in Sijacic et al., (2011), as reviewer 3 suspected, the main cause of such phenotypic discrepancy is due to genetic redundancy. One Arabidopsis homolog encoded by *SOL2* is both highly similar to *TSO1* in sequence and expression pattern. We showed that *tso1-3; sol2-1* double mutants exhibited a similar phenotype to *tso1-1* in meristem fasciation and floral organ morphogenesis. On the contrary, knockdown or knockouts of *TSO1-1* in *tso1-1* mutant background (silencing via an artificial microRNA or recently via intragenic nonsense mutation) caused *tso1-1* mutants to become *tso1- 3*-like in phenotype. We also showed that the TSO1-1 mutant protein can bind SOL2 protein. These prior analyses suggest that TSO1-1 mutant proteins (with a cysteine to tyrosine missense mutation in a conserved CXC domain) can poison the function of SOL2 and that *tso1-1* single mutant is equivalent to *tso1-3 (null); sol2-1* double mutants.

In this revised manuscript, we added above information in the Introduction. This should help understanding the distinct phenotypes between *tso1-1* and *tso1-3*.

2. Is there a loss of function phenotype for CycA 3;4? I see that authors show that its overexpression leads to no phenotype in wild-type background while seems to enhance sterility associated with the loss of function tso1-3 allele. The sterility is a minor phenotype, therefore, authors should present data on the overexpression of CycA 3;4 in tso1-3 background. This is because the authors claim in the model that Cyc 3;4 could target MYB21 for phosphorylation. If this assumption is correct, one expects enhancement of tso1-3 phenotype.

Thanks for this comment. No phenotype in root or shoot was observed in two different *cyca3;4* T-DNA insertion mutants, both of which had a strong reduction of transcript levels (Willems et al., Plant Cell 2020). We added this information to the manuscript text (page 7; line 204-207)

If we understand reviewer question correctly; reviewer asked for the presentation of data on overexpressing *CYCA3;4* in *tso1-3* background. We did in fact show the phenotype of over-expressing *CYCA3;4* in *tso1-3* background (Figure 4B). Could the reviewer actually mean that we should over- express *CYCA3;4* in *tso1-1* background? We did not overexpress *CYCA3;4* in *tso1-1* because the *tso1-1* mutant exhibits an extremely strong and severe phenotype that will likely mask any effect of over- expressing *CYCA3;4*.

3. Biochemical data analyzing the phosphorylation status of MYB21 in tso1 and tso1;cycA 3;4 must be provided to make the study mechanistic.

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We agree that biochemical data analyzing the phosphorylation of MYB3R1 in *tso1* and *tso1; cyca3;4* mutants will provide stronger mechanistic insights. Since our lab lacks biochemical experience, we contacted Dr. Masaaki Umeda in Japan and established a collaboration to demonstrate the role of CYCA3;4 in phosphorylating MYB3R1. Dr. Umeda's lab had the most relevant biochemical experience as they already worked with Arabidopsis MYB3Rs and CDKs (Chen et al., Nature Communications, 2017), in which they showed phosphorylation of MYB3R3 and MYB3R5 by CDK/CYC using an in vitro kinase assay. Unfortunately, through more than one year of effort during the pandemic, they were unable to purify the CDKA/CYC3;4 complex from E. coli. It was likely due to the unstable nature of the CYCA3;4 protein. Therefore, we regret that we could not provide the biochemical data here.

Second decision letter

MS ID#: DEVELOP/2022/201405

MS TITLE: Cyclin A participates in the TSO1-MYB3R1 regulatory module to maintain shoot meristem size and fertility in Arabidopsis

AUTHORS: Fuxi Wang, Wanpeng Wang, and Zhongchi Liu
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

See previous review reports

Comments for the author

The authors have incorporated my suggestions very well, including a much better abstract and inclusion of root data (that interestingly reveals no complementation within the root). My only remaining suggestions are for the "A proposed model" section. As it is speculatory, I would suggest writing:

Line 263: "…activity might be reduced…" (rather than "…activity is reduced…")

Line 265: "The might explain…" (rather than "This explains…").

Reviewer 2

Advance summary and potential significance to field

This manuscript revision addresses an important question: what cell cycle regulator is downstream of the DREAM complex to control the shoot apical meristem.

Comments for the author

I have no other comments. The manuscript is appropriate to publish in Development.

Reviewer 3

Advance summary and potential significance to field

The summary of advance has been provided in my previous review.

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Comments for the author

The authors have clarified the redundancy associated with the TSO1 gene family in the updated manuscript. They mentioned that they have tried biochemical analysis.
However, they ran into technical issues. The genetic analysis resulted in the identification of one of the Cyc A within the TSO1-MYB3R1 regulatory module which is sufficient enough advance to publish the results as a research report.

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