Flower meristem maintenance by *TILLERS ABSENT1* is essential for ovule development in rice

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DOI: 10.1242/dev.199932

Editor: Yka Helariutta

Review timeline
Original submission: 8 July 2021
Editorial decision: 5 August 2021
First revision received: 13 October 2021
Accepted: 15 November 2021

Original submission

First decision letter

MS ID#: DEVELOP/2021/199932

MS TITLE: Stem cell maintenance by *TILLERS ABSENT1* is essential for ovule development in rice

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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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Tanaka et al. investigate the function of the rice WUSCHEL (WUS) ortholog TAB1 in ovule development. It has been long known that the rice WUS gene is essential for axillary meristem formation rather than the well-known role in shoot apical meristem maintenance reported in Arabidopsis. However, a female sterility phenotype was also observed in rice tab1 mutants, but the underlying molecular mechanisms are unknown. Here, the authors carefully analyze the defects of ovule development in tab1-1 mutants and check the expression pattern of several marker genes (e.g. DL, OSH1, FON2) by in situ hybridizations in different stages of flower development and ovule development, as well as characterize the expression of TAB1 in early and late floral meristems and in the ovule development stage. The tab1-1 defects can be partially rescued by fon2, carrying a mutation in a CLAVATA3 homolog. Overall, this study suggests that TAB1 plays an important role in maintaining stem cell fate through late stages of floral development and thus promotes ovule formation in rice, including integument formation.

Comments for the author

This is a well-written paper and the tab1-1 ovule phenotype is carefully analyzed.
Comments:
- It should be indicated how many samples were checked by in situ hybridizations, in particular when no signal is detected e.g. Fig 4F and 5D.
- Specify which type of mutation is tab1-1 at the beginning (line 112); complete or partial loss-of-function?
- Line 119: “We found that the tab1-1 mutant was completely sterile”. This observation was made previously in rice moc3 mutants (another tab1 allele), and the following citation should be included:
http://dx.doi.org/10.1016/j.jgg.2014.12.005.
- Line 222: I don’t think it is obvious that the expression of TAB1 is expanded in Fig. 6E compared to 6A.

Reviewer 2

Advance summary and potential significance to field

Little prior information on the genetic regulation of ovule development in rice is available. This study provides clear evidence of a critical role for TAB1 in rice ovule formation. It further shows that TAB1 affects stem cell maintenance and the expression of stem cell marker genes. Evidence of gene redundancy and a gene circuit regulating ovule development is also presented. This is critical information toward a description of the molecular regulation of this critical reproductive structure in rice.

Comments for the author

The authors examine the role of the rice TAB1 gene in ovule development. TAB1 is related to Arabidopsis WUS. In Arabidopsis, WUS is important for maintenance of all SAM, whether they are the primary SAM or derived from axillary buds. WUS is also important in development of Arabidopsis ovule integuments. Prior work by some of these authors (and others) showed that there was subfunctionalization of the “WUS” function in rice where multiple paralogous rice genes carry out roles of a single WUS gene in Arabidopsis.
They showed that TAB1 was important in axillary meristem formation, but not for maintenance of the primary shoot apical meristem (SAM). In contrast the related WOX4 was necessary for maintenance of the primary SAM. This made a strong case for subfunctionalization of WUS-like genes in rice in different meristems. This conclusion of the prior work is kind of obscure in the introduction and discussion of the current manuscript and it is up to the reader to glean this from going back to the included references.
While the prior work addressed two meristems partly governed by WUS-like genes, it did not characterize effects on ovules. Hence, the current work. The authors conclusively show that

effects of tab1 mutations on rice ovule development are even more profound that those of (specially constructed ovule-specific) wus mutants in Arabidopsis. Ovules are most commonly eliminated in the, and occasionally highly aberrant structures are produced in their place. Thus, tab1 is shown to be a critical regulator of ovule formation in rice.
Pistil formation is also slightly altered. However, in rice the ovule makes up a very large part of the total gynoecial tissue and it is possible that altered pistil formation is a secondary effect of the severe disruption of ovule formation.
The ovule forms from the final flower meristem in rice, and the effect of tab1 is visible at an early stage prior to ovule initiation. Using marker genes, the authors convincingly show that tab1 maintains cells with meristem identity and stem cell identity in this region. That is, they show that TAB1 promotes stem cell fate in the final flower meristem through demonstration of the absence of OSH1 and FON2 expression in the tab1 mutant. The preservation of stem cell fate is a necessary precondition to ovule formation.
Based on a larger region of expression of TAB1 in the fon2-3 background, the authors say this "suggests" that FON2 is a negative regulator of TAB1 expression. However, the fon2-3 mutant exhibits an increase in size of the final flower meristem. The expanded region of TAB1 expression just seems to follow the expansion of this region. Since TAB1 is expressed in the final flower meristem this seems rather a secondary effect of expansion of this region, rather than a direct effect of de-repression of TAB1. So this is not really evidence that FON2 is a repressor of TAB1.
Ovule development loss in tab1 was largely restored in the fon2 tab1 double mutant. This also is not really consistent with FON2 being a negative regulator of TAB1. Rather, this could imply that FON2 is an inhibitor of some factor (they call it "factor X" in other contexts) that could act to substitute for TAB1 activity. In fact, the model in Fig. 8 could be augmented to completely explain these results. At left in Fig. 8, TAB1 and X act in parallel to promote stem cells in early stage flowers. In late stage flowers they say only TAB1 is acting. If FON2 is the repressor of X in late stage flowers then X would be activated in the fon2 mutant, increasing stem cells in a TAB1 background, and substituting for TAB1 in a tab1 background. This would completely explain pretty much all the results. It is also consistent with the previously reported role of FON2 in other meristems.
The discussion brings out all of the major points but in something of an indirect fashion. Shortening could make this section more clear. If the discussion were to start from the previously demonstrated roles of the subject genes, and the also previously shown subfunctionalization of the WUS-like clade, then it could be more concise.
While some of the analysis can be more clearly presented, the manuscript provides clear evidence that strongly supports the main conclusions about the roles of the studied genes in ovule development. It provides important new information on TAB1, FON2, and their roles in reproductive development. It provides a substantial initial set of studies on genetic regulation of ovule development in rice, and indicates useful avenues for further study.
Some specific comments:
Lines 83 – 87 Arabidopsis WUS is said to express in ovules only in the embryo sac. This is close to correct but not quite accurate. As shown in the cited references, WUS is actually expressed in the entire nucellus, not just in the embryo sac. This should be corrected by replacing "embryo sac" with "nucellus" in these sentences. This does not affect the conclusions of this section.
Lines 219 – 226 TAB1 express in fon2 results are not convincing for direct regulation. The area of TAB1 is expanded in fon2 mutants, but the meristem is expanded. The expanded TAB1 expression area would appear more to be an effect of the expanded meristem rather than its cause.

Reviewer 3

Advance summary and potential significance to field

This manuscript describes a novel function of the homeodomain transcription factor TAB1 in rice ovule development. TAB1 is a rice ortholog of Arabidopsis WUSCHEL and promotes stem cells during axillary meristem development. Here they report that no ovule-like structure was observed in tab1-1. Since early ovule marker OsMADS13 is expressed in tab1-1, the ovule identity appears to be specified. However, OSH1 expression as well as FON2 in ovules was not maintained in tab1. Interestingly, the ovule defect in tab1 was partially rescued by FON mutations, which causes overproliferation of stem cells. They proposed that TAB1 promotes ovule formation by maintaining stem cells at a later stage of flower development. This is a simple but interesting work to highlight

the evolutionary diversification and conservation of the homeodomain transcription factor in floral meristem maintenance at the later stage of flower development.

Comments for the author

1) “In tab1-1, no ovule-like structure was observed. In severe cases, the ovary had a hollow structure, implying that it lacked an ovule (Line 142)” “TAB1 promotes stem cell fate to maintain the final flower meristem at the late stage of flower development. (Line 268)”
Although OsMADS13 is reported to be expressed in tab1, how is it quantitatively affected? Q-PCR analysis should be done using a proper control. Please also discuss when ovule identity is specified by OsMADS13 and till when stem cell fate are maintained by TAB1.
2) They previously showed that the tab1 defects in axillary meristem development were partially rescued by fon2 mutation (Tanaka and Hirano,335 2020). This rescue was found to be due to precocious expression of WOX4. Here they also should test whether WOX4 is involved in ovule development and whether FON2 negatively regulates WOX4 in ovules. Accordingly, the summary Figure 8 should include FON2, and possibly WOX4.

First revision

Author response to reviewers' comments

Response to Reviewer 1

Thank you very much for your valuable suggestions to improve our manuscript.

- *It should be indicated how many samples were checked by in situ hybridizations, in particular when no signal is detected e.g. Fig 4F and 5D.*
Reply: We confirmed the reproducibility of the in situ hybridization results by testing more than 6 samples. In addition, if no signal was detected, we confirmed the result in more than 10 samples. We have added this information to the appropriate figure legends (page 28 lines 589-590, page 29 lines 600-602, page 29 lines 609-611, page 30 lines
620-621).

- *Specify which type of mutation is tab1-1 at the beginning (line 112); complete or partial loss-of-function?*
Reply: *tab1-1* has a complete loss-of-function mutation that cause premature termination (Tanaka et al., Plant Cell, 2015). We have added a description of the mutation to the “RESULTS” (page 7 line 115) and the “MATERIALS AND METHODS” (page 18 lines 370-372).

- *Line 119: “We found that the tab1-1 mutant was completely sterile”. This observation was made previously in rice moc3 mutants (another tab1 allele), and the following citation should be included: http://dx.doi.org/10.1016/j.jgg.2014.12.005.*
Reply: Thank you for pointing out the *moc3* mutant paper. We have cited this reference in the revised manuscript (page 7 lines 122-123).

- *Line 222: I don’t think it is obvious that the expression of TAB1 is expanded in Fig. 6E compared to 6A.*
Reply: To answer this question, we repeated the in situ hybridization and obtained clearer results. We have replaced the old image in Fig 6E with a new one, which shows more clearly that *TAB1* is expressed in a wider region in the enlarged flower meristem of *fon2-3* as compared with wild type.

Response to Reviewer 2

Thank you very much for your valuable suggestions to improve our manuscript.

- *This conclusion of the prior work is kind of obscure in the introduction and discussion of the current manuscript, and it is up to the reader to glean this from going back to the included*

references.
(omission)
- *The discussion brings out all of the major points but in something of an indirect fashion. Shortening could make this section more clear. If the discussion were to start from the previously demonstrated roles of the subject genes, and the also previously shown subfunctionalization of the WUS-like clade, then it could be more concise.*
Reply: We deeply appreciate that reviewer 2 has closely followed our papers. In response to this useful suggestion, we have added a description of the background of *WOX4* to the "INTRODUCTION" (page 5 lines 72-77). In addition, we have completely revised the first section of the "DISCUSSION" by providing a summary of our prior studies (pages 13-15 lines 263-305).

- *Lines 83 – 87 Arabidopsis WUS is said to express in ovules only in the embryo sac. This is close to correct, but not quite accurate. As shown in the cited references, WUS is actually expressed in the entire nucellus, not just in the embryo sac. This should be corrected by replacing "embryo sac" with "nucellus" in these sentences. This does not affect the conclusions of this section.*
Reply: We have revised the manuscript (page 5 lines 87, 88) in accordance with this comment.

- *Lines 219 – 226 TAB1 express in fon2 results are not convincing for direct regulation. The area of TAB1 is expanded in fon2 mutants, but the meristem is expanded. The expanded TAB1 expression area would appear more to be an effect of the expanded meristem rather than its cause.*
Reply: Thank you for this valuable suggestion. We agree with the reviewer's comments that the expanded *TAB1* expression area is a secondary effect of an enlarged meristem. But also we think that we cannot exclude the possibility that *TAB1* is negatively regulated by *FON2*. However, because the *TAB1-FON2* relationship is not a main message of this manuscript, to reduce complication, we have deleted the sentence relating to the negative regulation of *TAB1* by *FON2* (page 12 lines 225-226 in the original manuscript) from the "RESULTS". In addition, we have deleted the paragraph that described the *TAB1-FON2* relationship (page 14 lines 280-286 in the original manuscript) from the "DISCUSSION" in the revised manuscript.

Response to Reviewer 3

Thank you very much for your valuable suggestions to improve our manuscript.

1) Although OsMADS13 is reported to be expressed in tab1, how is it quantitatively affected? Q-PCR analysis should be done using a proper control.
Reply: It would be preferable if we could obtain Q-PCR data for *OsMADS13* and compare them between *tab1* and wild type. However, it is very difficult to obtain precise quantification data for the following reasons. The development of each flower is not synchronized in rice and the developing inflorescence contains flowers at various stages. Collecting flowers at exactly the same stage when development of the ovule initiates would have to be done under a microscope and would need much labor and time. Induction of the *AP1* gene in the *ap1 cal* double mutant is a very useful tool for the synchronization of flower development in Arabidopsis (Wellmer et al., Plos Genet., 2006; Sun et al., Genes Dev., 2009). Unfortunately, however, we do not have such an excellent experimental system in rice.

We repeated the in-situ-hybridization experiment and obtained similar patterns of *OsMADS13* expression in wild type and *tab1* in most cases, except for its expression in ovule primordia in wild type. Although *OsMADS13* signals were evident in both *tab1* and wild type, we have tried to avoid mentioning the strengths of signals in the text.

Please also discuss when ovule identity is specified by OsMADS13 and till when stem cell fate are maintained by TAB1.

Reply: This is an interesting issue to clarify in rice flower development. Unfortunately, however, it is difficult to examine when the stem cells disappear from the flower meristem because expression of the stem cell marker *FON2* is very weak. To address this comment, *FON2* expression needs to be examined by a more sensitive method than in situ hybridization, such as GFP reporter. In addition, a marker for ovule identity other than *OsMADS13* would be needed to examine this issue. At present, however, we do not have such a marker. We are planning to address this issue in the future by solving these problems.

2) Here they also should test whether WOX4 is involved in ovule development and whether FON2 negatively regulates WOX4 in ovules. Accordingly, the summary Figure 8 should include FON2, and possibly WOX4.
Reply: Thank you very much for your following our past research papers. We appreciate the suggestion concerning *WOX4*. We examined *WOX4* expression by in situ hybridization using wild-type samples. *WOX4* was expressed in the flower meristem at the late stage, in addition to the early-to-middle stages (Fig. 6G, H). This result implies that *WOX4* alone is insufficient to maintain the flower meristem at the late stage in *tab1*. Thus, *WOX4* does not seem to have an essential function in ovule development. We have revised the "RESULTS" (page 12 lines 229-232) and the "DISCUSSION" (pages 13-15 lines 263-305) by incorporating the result of *WOX4* expression.

Concerning the negative regulation of *TAB1* by *FON2*, reviewer 2 has pointed out the possibility that the expanded expression of *TAB1* in *fon2* is a secondary effect of the enlarged meristem. While we still consider that *TAB1* is negatively regulated by *FON2*, we have decided that this matter of controversy should not be included in the model (Fig. 8).

Second decision letter

MS ID#: DEVELOP/2021/199932

MS TITLE: Flower meristem maintenance by *TILLERS ABSENT1* is essential for ovule development in rice

AUTHORS: Wakana Tanaka, Suzuha Ohmori, Naoto Kawakami, and Hiro-Yuki Hirano
ARTICLE TYPE: Research Article

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

The authors adequately addressed my previous concerns.

Comments for the author

I have two comments on this new version:
- New in situ hybridization data on WOX4 were added in Fig. 6G,H in response to reviewer 3. However, the new results do not appear particularly convincing. There is a lot of background signal in these in situ hybridizations.
- I would suggest combining the first two sections in the Results under the title "Pistil and ovule formation are compromised in tab1-1 flowers", since currently the "Ovule formation is severely affected in the tab1-1 flower" section is very short.

Reviewer 2

Advance summary and potential significance to field

Little prior information on the genetic regulation of ovule development in rice is available. This study provides clear evidence of a critical role for TAB1 in rice ovule formation. It further shows that TAB1 affects stem cell maintenance and the expression of stem cell marker genes. Evidence of gene redundancy and a gene circuit regulating ovule development is also presented. This is critical information toward a description of the molecular regulation of this critical reproductive structure in rice.

Comments for the author

I enjoyed reading the revised manuscript. The manuscript is much improved. The authors have done an excellent job of modifying the manuscript to address comments of the reviewers. The changes completely address my comments. The authors appear to also have effectively addressed the comments of the other two reviewers.

Reviewer 3

Advance summary and potential significance to field

This manuscript describes a novel function of the homeodomain transcription factor TAB1 in rice ovule development. I was convinced by their reply to the first part of my comments.

Comments for the author

Their response to my 2nd comment is not clear enough. Based on their new data of in situ hybridization assay of WOX4 in wild-type samples (Fig. 6G, H), they wrote, “This result implies that WOX4 alone is insufficient to maintain the flower meristem at the late stage in tab1. Thus, WOX4 does not seem to have an essential function in ovule development.” This statement should be based on the observation that WOX4 expression is not affected in tab1. If WOX4 expression is reduced in tab1 and enhanced in tab1 fon2, their idea could be wrong. Partial rescue of tab1 by fon2 in ovules is still an exciting part of this paper. Please discuss more accurately by providing data.