# The OsRR24/LEPTO1 Type-B Response Regulator is Essential for the Organization of Leptotene Chromosomes in Rice Meiosis

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**Review timeline:**

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|---|---|---|
| **TPC2018-00479-RA** | Submission received: | June 23, 2018 |
| | 1st Decision: | Aug. 8, 2018 *revision requested* |
| **TPC2018-00479-RAR1** | 1st Revision received: | Sept. 30, 2018 |
| | 2nd Decision: | Oct. 13, 2018 *accept with minor revisions* |
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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.)

## TPC2018-00479-RA 1st Editorial decision – *revision requested* Aug. 8, 2018

We have received reviews of your manuscript entitled "A type-B response regulator, LEPTO1, mediates chromosomes developing into leptotene status in rice meiosis." Thank you for submitting your best work to *The Plant Cell*. 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 potential re-review.

We ask you to pay attention to the following points in preparing your revision. As you will see from the detailed comments of the reviewers, they make several suggestions how to improve the manuscript, to erase some problems in the interpretation of the data and to emphasize the most important points. While the requirements of LEPTO1 for fertility and meiosis are well documented, this is likely not a direct effect, and the current title is indeed misleading and should be changed (#1). The nature of the cytokinin link can be addressed with some of the suggested experiments. We agree also that the quality of several images should be improved, and the phylogenetic analysis extended. All reviewers state that it is necessary to define the time point of meiosis arrest more precisely. It would strengthen the manuscript to extend some analysis to one of the other mutant alleles and some other meiosis mutants would be analysed for similar effects on the tapetum (#3).

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

[Reviewer comments shown below along with author responses]

## TPC2018-00479-RAR1 1st Revision received Sept. 30, 2018

Reviewer comments and **author responses**:

Reviewer #1:

Zhao et al. report the analyses of the rice LEPTO1 gene encoding a type B response regulator for its role in regulating meiotic leptotene chromosome formation. They further showed that meiocytes of the *lepto1* mutant exhibit defects in DSBs formation, recruitment of meiosis-specific proteins (OsREC8, PAIR3, OsMER3 and ZEP1), and the deposition of the carbohydrate callose in the meiocyte cell wall. In addition, the *lepto1* mutant is defective in anther somatic cell development and megaspore formation (female meiosis). Therefore, LEPTO1 is required for multiple aspects of male and female fertility. Moreover, the authors showed that LEPTO1 is not required for the expression of some of the genes encoding proteins (such as *OsAM1* and *OsREC8*) that were not loaded onto the chromosomes, but is required for normal expression of some cyclin genes.

These are important and informative results that reveal significant novel functions of a response regulator (and possibly cytokinin) in promoting male and female fertility, with specific results that suggest some possible mechanisms of such regulation.

**Q1** The title is too narrowly focused on the role of LEPTO1 in regulating leptotene chromosome formation; a more inclusive title that informs the reader of the functions of LEPTO1 is better. In addition, LEPTO1 does not seem to directly regulate leptotene chromosome formation, but rather likely regulate gene expression and other aspects of the meiocytes, including possibly "chromosome state", which is not clearly defined here. The current title suggests a more direct function on leptotene chromosome.

**Response: We definitely agree that the title is not rigorous, so we have replaced this statement. We have retitled the manuscript with "A type-B response regulator, LEPTO1, is essential for setting up leptotene status in rice meiosis" to make it more appropriate. Thank you very much for your valuable suggestions.**

**Q2** The results in this paper suggest that cytokinin plays an important role in male meiosis and male fertility. Has this been reported? The authors did not mention any previous work on this, so it is likely that this is not known. On lines 100-101, the authors indicated that the role of type B RRs in meiosis is not clear, but the role (the lack of knowledge) of cytokinin should also be mentioned here.

**Response: Thank you very much for your advice. So far, very few studies have been focused on the relationships between meiosis and cytokinin or RRs. In Arabidopsis, the triple mutant of cytokinin AHK receptors may complete meiosis in both male and female reproductive organs, but exhibits reproductive defects in the specification of functional megaspore. However, it can still complete the lifecycle, indicating that cytokinin may not be important enough, or there may still exist residual cytokinin signals or redundantly cytokinin signaling pathways in this triple mutant [Cheng CY *et al.* Plant J. 2013,73(6):929-40; Kinoshita-Tsujimura K *et al*. Plant Signal Behav. 2011,6(1):66-71.]. Deng et al. has reported that CKI1 is able to activate cytokinin signaling independent of cytokinin receptors in certain developmental stages [Deng Y *et al.* Plant Cell. 2010,22(4):1232-48.]. Hutchison CE et al mention that there is still residual cytokinin signaling in *ahp1,2,3,4,5* mutant, although the mutant has seriously reduced cytokinin function [Hutchison CE *et al*. Plant Cell. 2006,18(11):3073-87.]. This possibility may make it hard to explore the real relationship between meiosis and cytokinin signaling. In our study, we uncovered a type-B RR involved in the establishment of leptotene chromosomes, suggesting a role of type-B RRs in meiosis. However, we still lack evidences to demonstrate that cytokinin is directly involved in meiotic regulation. According to your suggestions, we tend to use the description "the role of RRs in meiosis is not clear", when we mention the purpose of our study.**

**Q3.** Related to the above, it would be important to test whether cytokinin is required for normal male meiosis and male fertility. Is it possible to treat rice flowers with cytokinin and/or cytokinin inhibitors, and examine meiosis after the treatment?

**Response: Yes, we are also puzzled by the question you mentioned. It is indeed important and challenging to explore the function of cytokinin in meiotic regulation. In Arabidopsis, studies on the function of cytokinin in reproductive development are done mainly using plant materials harboring defects in cytokinin signaling. In *ahk2,3,4* triple mutants, *cki* mutant, and *ahp1,2-2,3,4,5* quintuple mutants, the male and female reproductive organs exhibit defects in megasporogenesis, but not meiosis. These studies indicate that the residual cytokinin signaling or some unknown bypassing cytokinin signaling pathways are enough to regulate meiosis.**

**According to your suggestion, we tried to reduce the level of cytokinin signaling in rice flowers using the cytokinin inhibitor lovastatin [Frébort I *et al.* J Exp Bot. 2011,62(8):2431-52.]. After treating rice panicles before meiosis**

**initiation with 50 μM or 200 μM lovastatin, we found that these treated flowers failed to mimic the meiotic defects in *lepto1* and were still able to generate pollen normally. Moreover, treating *lepto1* flowers with synthetic cytokinin 6-BA or CPPU (N-2-(chloro-4-pyridyl)-N-phenyl urea) also failed to recover their meiotic defects. As these results did not meet our expectations, we then tried to think about the possible reasons. In plants, there are a variety of cytokinin forms, and multiple possible biosynthetic pathways are involved. Lovastatin is an inhibitor of cytokinin biosynthetic and can only inhibit the mevalonate pathway. Plant materials treated with lovastatin still have a certain ability to synthesize cytokinin. And this part of cytokinin is enough to achieve meiotic process.**

**To get more information about cytokinin and meiosis, we have also tried to obtain *Osahp1,2* double mutants using CRISPR-Cas9 tool. But we did not identify homozygous recessive mutations in these two genes in both T0 and T1 generations, suggesting *Osahp1,2* double mutants may be lethal in rice. Thank you very much for your valuable suggestions.**

**Q4.** If information regarding the role of cytokinin is not available, then the possibility that LEPTO1 has a function other than mediating cytokinin regulation should be discussed.

**Response: Thank you very much for your suggestions. Based on our results, we agree that the viewpoint "LEPTO1 has a function in meiotic process" would be more rigorous. Accordingly, the discussion part about the possible function of cytokinin has been removed.**

**Q5.** Many of the images in Figures 1-3 intending to show meiotic defects contain mostly somatic cells, with only a few PMCs. This makes it hard to see the details of the meiotic defects in the mutant. It would be better to replace these images with ones for a single PMCs (like Fig. 3A and 3B), or just a few PMCs, but without the somatic cells.

**Response: Thank you very much. The valuable suggestions really helped us to improve the quality of our pictures. We have replaced Figures 1D, 1E, 3B, 3C and Supplemental Figure 2D, 2E, 2H, and 2I with enlarged images to better present meiotic defects in PMCs.**

**In Figure 2A and 2B, the CENH3 foci in PMCs were compared with those in somatic cells to examine whether meiosis-specific CENH3 loading pattern occurred in the *lepto1* mutant. In Figure 2C and 2D, anther sections were used to present EDU signals. Because the experimental materials need to be fixed and embedded first, it is difficult for us to eliminate the background when taking pictures. Thanks a lot.**

**Q6.** The authors showed that the expression of *OsAM1* and *OsREC8* was not affected in the *lepto1* anthers. Can this be done using only PMCs?

**Response: Yes, we agree that it will be more rigorous to detect the expression of *OsAM1* and *OsREC8* in *lepto1* PMCs. By RNA *in situ* hybridization, we found that *OsAM1* and *OsREC8* were mainly expressed in PMCs. And the expression levels of these two genes in PMCs showed no significant differences between the wild type and *lepto1* (Supplemental figure 11). The result is consistent with the data obtained by real-time PCR assays. Therefore, loss of function of LEPTO1 did not affect the transcription of *OsAM1* and *OsREC8*.**

**Q7.** The expression of other meiotic genes, such as *SPO11*, *RAD51*, *DMC1*, *ZEP1*, and *PAIR3*, should also be examined in the *lepto1* mutant meiocytes.

**Response: Yes, we have detected the expression of these meiotic genes in *lepto1* PMCs by RNA *in situ* hybridization. At preleptotene, the expression levels of *OsSPO11-1*, *DMC1A*, and *PAIR3* in *lepto1* PMCs showed no significant difference with those in the wild type (Supplemental figure 11). *ZEP1* mRNA signal started to accumulate at zygotene, and the expression of *ZEP1* was not detectable in wild-type or *lepto1* PMCs during preleptotene stage. We have also tried to investigate the expression of *RAD51C* in the *lepto1* mutant but failed, which may be due to invalid probes or a lower expression level of *RAD51C*. Thank you very much for your kind suggestions.**

**Q8.** The *lepto1* mutant showed defects in callose deposition, and the authors mentioned that two genes (line 457) related to callose deposition are down-regulated. What roles do these genes play, are they expressed specifically in the meiocytes? How much reduction was observed in the *lepto1* cells? These results should be shown.

**Response: Thank you very much for your kind reminder. We have added descriptions about the role and expression characteristic of these two genes in the revised manuscript (Pages 8, lines 190-193). Rice UGP1 and GSL5 are important enzymes involved in callose deposition during male gametophyte development. Chen R et al showed that**

**expression of UGPase protein was mainly located in PMCs at premeiotic and meiotic stages [Chen R *et al*. Plant Cell. 2007,19(3):847-61]. Shi X et al showed that *GSL5* was widely expressed in various tissues and was highly expressed in anthers during meiosis stage and early microspore stage. At microspore stage, for example, the most obvious signal was observed in both tapetum and microspores by RNA *in situ* detection [Shi X *et al*. Plant Cell Physiol. 2015,56(3):497-509.]. Deposition of callose in PMCs was dramatically disrupted in *gsl5* mutant and *UGP1*-silenced plant.**

**In addition, we have given the down-regulated ratios of *UGP1* and *GSL5* in *lepto1* anthers in the revised manuscript (Page 8, line 193). We hope that these modifications make it more informative.**

**Q9.** The authors suggested that the anther somatic cell defects in the *lepto1* mutant was an indirect effect. However, it is known that other meiotic mutants also show this kind of indirect effects in the somatic cells? Also, it is hard to rule out low level expression of *LEPTO1* in the somatic cells, even when strong expression is found preferentially in the PMCs.

**Response: Thank you very much for your kind advice. Our previous description "the somatic cell defects in the *lepto1* mutant was an indirect effect" was indeed not appropriate. We have revised this improper description in this manuscript.**

**As there is a lack of information about the developmental defects of somatic cells in rice meiotic mutants, we selected *Osspo11-1* mutant for observation. Mutation in *OsSPO11-1* resulted that meiocytes were unable to form meiotic DSBs. As shown in Supplemental 6E, the somatic cell layers showed degradation in *Osspo11-1* anthers, although these somatic cells were not degraded completely.**

**The PMCs in *Osam1* exhibited arrest at leptotene, therefore, we then observed the development of somatic cell layers in *Osam1*. In *Osam1*, tapetum showed weakly expanded (Supplemental 6C). As with *lepto1*, *lepto1 Osam1* double mutants showed severe swollen-tapetum phenotype (Supplemental 6D and 6E). These observations suggest that the opinion "LEPTO1 have a role in the development of somatic cell layers" may be more reasonable. In addition, *LEPTO1* was weakly expressed in somatic cells (Page11, lines 270-271), and it indeed cannot be ruled out that LEPTO1 directly affects the development of somatic cells by expressing in the somatic layers. Thanks for your advice again, we really appreciate it.**

**Q10.** How conserved is the *LEPTO1* gene? The authors presented a phylogenetic tree with its homologs, but it cannot show what the author stated in the text that LEPTO1 is most closely related to the other genes in the tree, as there is not comparison with less related genes. The phylogenetic analysis should include 2-3 closest homologs from each of those plants, and show that they form 2 or more clades, with LEPTO1 and its likely orthologs (closest homologs) forming one clade, and other more distantly related genes forming a separate clade(s).

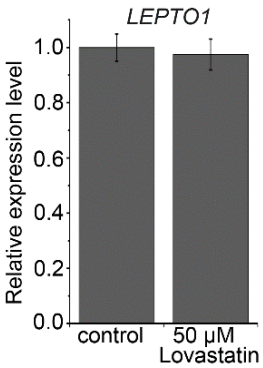
**Response: We agree that the phylogenetic tree was not informative. As shown in Supplemental figure 7, a more reasonable phylogenetic tree was constructed. The phylogenetic tree was based on the conserved N-terminal amino acid sequences (DDK and MYB domains) of LEPTO1 and other type-B subfamily-I RRs from four plant species (*Oryza sativa*, *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Zea mays*). A subfamily B-III RR, AtARR20, was set as the outgroup. In the phylogenetic tree, type-B subfamily-I RRs were divided into four clades and LEPTO1 was most closely related to ZmRR9 in maize. Thanks a lot.**

**Q11.** The author showed that *LEPTO1* is not induced by cytokinin in the seedlings, but they also showed that it is preferentially expressed in the PMCs. Thus it would be important to test whether its expression in PMC is enhanced by cytokinin treatment of the anther.

**Response: Thanks very much for your constructive advice. We agree that it is significant to test whether the expression of *LEPTO1* in PMCs is induced by cytokinin treatment. Therefore, we treated wild-type panicles with 50ppm CPPU and detected the expression level of *LEPTO1* after 12 h treatment, using *ORR1* as a positive control. Real-time PCR analysis showed that the expression of *LEPTO1* in panicle was not induced after CPPU treatment (Supplemental figure 8). As *LEPTO1* was specifically expressed in PMCs, we speculate that the expression of *LEPTO1* in PMCs may be not induced by cytokinin treatment yet. Moreover, the expression levels of type-B RRs in Arabidopsis were not affected by the application of cytokinin [Hutchison CE *et al*. Plant Cell. 2002;14.].**

**Q12.** It might be even more interesting to test whether *LEPTO1* expression in PMC depends on normal level of cytokinin signaling, using some inhibitor treatment of the anther.

**Response: Thank you very much. We have treated wild-type panicles with 50 μM lovastatin and detected the expression level of *LEPTO1* after 12 h treatment. As shown in the figure below, the expression of *LEPTO1* was not affected after locastatin treatment.**




**Q13.** A minor comment is that it appears that the legends of Figure S9 and S10 have been switched.

**Response: Thanks very much for your kind reminder. We have corrected it.**

Reviewer #2:

The manuscript "A type-B response regulator, LEPTO1, mediates chromosomes developing into leptotene status in rice meiosis" by Zhao and co-workers describes the isolation of LEPTOTENE1 (LEPTO1), a type-B RR that is required for the ultimate establishment of leptotene chromosomes in rice (*Oryza sativa*) meiosis. The authors show that *lepto1* meiotic chromosomes arrest early in meiosis and never form thread-like structures. LEPTO1 inactivation also blocks double-strand break formation, recruitment of a number of meiosis-specific proteins, and callose deposition.

LEPTO1 is able to interact with OsAHP1and OsAHP2 via a DDK domain, and the phosphorylated mutation mimic of this DDK domain can relieve repression it's transactivation activity.

The manuscript is well-written and on an important topic. This is the first time that LEPTO1 has been identified; therefore, the information provided is new and novel. The manuscript presents a considerable amount of data and a relatively thorough characterization of the gene. I have two major and one minor concerns regarding the manuscript that should be considered/addressed.

**Q1.** The quality of many of the microscopy images are not of the same high quality that typically comes out of the Cheng lab. This makes it somewhat difficult to actually determine the specific nature of the meiotic defect(s) in the mutant. For example, it is not really clear what Fig. 1 D & E add to our understanding of the mutant. Likewise, the quality of the images in Fig. 2 and Fig. 4 could be better.

**Response: Thank you very much for your kind advice. In the revised manuscript, all figures were replaced with higher resolution images to present more details. It is possible the conversion from PPT files to PDF files may loss lots of the resolution. We found the quality of the original PPT files is pretty much high. You may download the PPT files if the resolution is not as high as expected.**

**The number of somatic cell layers can be used to indicate the early stages of anther development. In Figure 1D and 1E, we want to present the number of somatic cell layers and the chromosome morphology in PMCs at the same time using DAPI-stained anther sections. In wild-type PMCs, preleptotene chromosomes will proceed to leptotene**

**stage soon after. While *lepto1* PMCs are arrested at preleptotene. To make Figure 1D and 1E more rational, we have deleted irrelevant and redundant images.**

**In Figure 2A to 2D, because the experimental materials need to be fixed and embedded first, it is difficult for us to eliminate the background when taking pictures. In Figure 2E and 2F, we have added white arrows to indicate callose signals. In Figure 4B, we have replaced the image labeled as swollen tapetum. In Figure 4C, we performed whole-mount stain-clearing laser scanning confocal microscopy (WCLSM) to examine megasporogenesis in the wild type and the *lepto1* mutant. Because the cell morphology was not displayed very clear by WCLSM assays, we have added white arrows to indicate AR, MMC, FG, and EC to make it understandable.**

**Thanks a lot. These valuable suggestions really helped us improve the quality of our pictures.**

**Q2.** The authors conclude that the *lepto1* mutation results in a block between premeiotic S-phase and the onset of leptotene. It is not clear to me that they actually demonstrated this and that the block could also be before or during pre-meiotic S-phase. I believe that additional experiments, including BrDU labelling or additional double mutant analyses could better define the actual meiotic block. While this would add to the manuscript, I don't believe that additional experiments are essential. But a re-evaluation/statement of the timing of the defect should be considered.

**Response: Thank you very much for your valuable suggestions. We definitely agree that it is essential to investigate the meiotic block point in the *lepto1* mutant. As EDU labelling is more convenient than BrDU labeling, we performed EDU labelling to detect whether meiotic DNA replication occurred in *lepto1* PMCs. EDU signals were observed in both wild-type and *lepto1* PMCs, suggesting meiotic DNA replication occurred in the *lepto1* mutant (Figure 2C and 2D).**

**In addition, the *lepto1* mutant was arrested at preleptotene, during which the chromosomes exhibited a hairy appearance. Moreover, *lepto1* PMCs presented meiosis-specific CENH3 foci, which were similar to those in wild-type preleptotene PMCs. Taken together, we suspect that the *lepto1* PMCs are arrested at preleptotene, with a status between premeiotic S-phase and typical leptotene. Thank you very much!**

**Q3.** Finally, a minor issue, the legends for Figs. S9 and S10 appear to have been switched.

**Response: Thank you very much for your kind reminder. We have corrected it.**

Reviewer #3:

A recessive sterile rice mutant, *lepto1*, was isolated from $^{60}$Co mutagenized seed, carrying a 7 bp deletion in a type B RR factor. Evidence that this mutation is causative to the sterility is deduced from a cross with *lepto1-2*, a Nipponbare line with a 10 bp deletion within the same gene and the engineering of *lepto1-3* Cas9 mutant showing sterility. The characterization was done using *lepto1-1* and it can't be ruled out that some aspects of the phenotype of this mutant are caused by extraneous mutations. In particular, the somatic anther tissue phenotype might not be linked with *LEPTO1* function.

**Response: Thank you very much for your careful review and suggestions. To verify the sterile phenotype in *lepto1-1* indeed results from the mutation of *LOC_Os02g08500*, we conducted a complementation test by transformation *Pactin::LEPTO1-GFP*, which rescued the fertility of *lepto1-1* (Supplemental Figure 1E). In addition, we investigated the development of somatic cells in the *lepto1-cas9* by observing transverse sections of anthers staining with TBO. The *lepto1-cas9* mutant also showed swelled tapetum (Supplemental Figure 6B). Taken together, our results suggest that the development of somatic cells were indeed caused by the mutation of *LEPTO1*.**

Major revisions:

**Q1.** The DNA spread of *lepto1* in fig1 shows a cell with chromosome threads formed which are not as "hairy and with blurred outlines". Based on the image shown, I do not see a strong difference with the WT and the spread from *lepto1-1* is even crispier than that of the WT. I suggest that the authors present a few more examples of the leptotene stage and if possible from degenerating spores.

**Response: We are sorry for this misleading presentation. We have replaced the image labeled as *lepto1* preleptotene with a more representative image (Figure 1C). To present more examples of preleptotene chromosome**

**morphology in both wild-type and *lepto1* PMCs, we have added images showing several nuclei in Supplemental Figure 2B and 2C. And we have added images showing degraded chromosomes in *lepto1* PMCs in Supplemental Figure 2F and 2G. Thank you very much.**

**Q2.** The cross sections through *lepto1-1* anthers show that the tapetum does not degenerate but instead expands inwards, filling up the empty space where normally the spores develop. It is not clear from the image whether the cells have expanded or whether they have divided.

**Response: We are sorry for the right panel in Figure 4B. We have changed it for a better image to present the outline of swollen tapetum in *lepto1*. And we have adjusted the image with a higher resolution. Moreover, we performed TUNEL assays to detect the PCD process of somatic cells, and no obvious PCD signals were detected in the expanded tapetum in *lepto1*, suggesting defects in the degradation of tapetum (Supplemental Figure 6F to 6I). Consistently, expression levels of *OsCP1*, *OsAP25*, and *OsAP37* were down-regulated in *lepto1* panicles (Supplemental Figure 6L to 6N). Thanks a lot.**

**Q3.** The behavior of the tapetum may be an indirect effect caused by the developmental arrest of the microspores. Do other rice mutants arrested in spore development show similar expansion of the tapetum? In any case, the cross sections of *lepto1-1* and the inward growth of the tapetum are reminiscent to the cross sections of tapetum mutants such as *dyt1* in *Arabidopsis*. A comparison with a tapetum mutant in rice may more firmly establish the proposed role for *LEPTO1* in tapetum functioning.

**Response: Thank you very much for your valuable advice. In rice, MEL2, OsAM1, and DTM1 are reported to play important roles in the progression of male meiosis. According to the descriptions in these papers, the tapetal layer showed hypertrophic phenotype [Nonomura K *et al*. PLoS Genet. 2011, 7(1): e1001265.] [Yi J *et al*. Plant J. 2012, 70(2): 256-70.]. As shown in Supplemental figure 6C and 6D, this kind of hypertrophic tapetum in *Osam1* et al is different from the swollen tapetum in *lepto1*. The inward growth of the tapetum in *lepto1* is more severe.**

**It is an important clue that the cross sections of *lepto1-1* and the inward growth of the tapetum are reminiscent to the cross sections of tapetum mutants such as *dyt1* in Arabidopsis. *DYT1* encodes a putative bHLH transcription factor and is expressed in both meiocytes and tapetum. However, meiotic nuclear divisions can proceed normally in *dyt1* [Zhang W *et al*. Development. 2006 Aug;133(16):3085-95.]. Importantly, the rice bHLH proteins TDR, TIP2, and EAT1 promotes tapetal cell death by regulating aspartic proteases. TDR is able to interact with both EAT1 and TIP2. *tdr* show similar tapetum defects with *lepto1*, while *tip2* and *eat1* show weakly expanded tapetum [Fu Z *et al*. Plant Cell. 2014,26(4):1512-1524.] [Niu N *et al*. Nat Commun. 2013,4:1445.] [Li N *et al*. Plant Cell. 2006,18(11):2999-3014.]. Rice UDT1 is the homolog of Arabidopsis DYT1 [ Jung KH *et al*. Plant Cell. 2005,17(10):2705-22.]. The tapetal cells in *udt1* are weakly expanded and continuously vacuolated. Recently, Ono S *et al* has reported that UDT could also interact with EAT1 and TIP2 [Ono S *et al*. PLoS Genet. 2018,14(2):e1007238.]. These four bHLH proteins also have different effects on the development of PMCs. PMCs in *eat1* and *tdr* can proceed to micospores, while PMCs degraded in *udt1* and *tip2*. Another two rice mutants, *Ostdf1* and *gamyb*, also exhibit hypertrophic tapetum and normal meiosis [Aya K *et al*. Plant Cell. 2009,21(5):1453-72.] [Cai C *et al*. Science Bulletin. 2015,60(12):1073-82.]. In summary of the male defects in these mutants related to arrested meiosis or expanded tapetum, we think that our previous description "The behavior of the tapetum may be an indirect effect" is not appropriate. And then we detected expression levels of genes involved in tapetum development in *lepto1* panicles. The results of real-time PCR demonstrated that the expression of *TDR* and *EAT1* were down-regulated in *lepto1*, which may partially explain swollen tapetum (Supplemental Figure 6J and 6K). Moreover, *lepto1* showed abnormal tapetal PCD and down-regulated expression of *OsCP1*, *OsAP25*, and *OsAP37*. Collectively, these results suggest that LEPTO1 may also have a regulatory role in tapetum development. And we have revised the manuscript in this version (Page 10, lines 233-245). Thank you, we really appreciate it.**

**Q4.** New insight into tapetum function suggests that the tapetum not only supports the formation of the outer cell wall and maturation of the spores but also regulates meiosis. The *in situ* hybridization data favors the view that *lepto1* function is primarily linked with the meiocytes. The same in situ hybridization does however show some staining in the tapetum and surrounding tissue as well (Fig 5). That should be notified.

**Response: Thank you very much for your kind reminder. We are sorry for inappropriate statement about the results of *in situ* hybridization and have made the change in the manuscript (Page 11, lines 270-271).**

**Q5.** Another aspect is that tapetum mutants are affected in the anther development and staging of the flowers in relation to the meiotic cell cycle phase is no longer as in WT plants. In Arabidopsis for example, the flower size carrying dividing meiocytes is much larger, which requires an adaptation of the experimental setup when preparing meiotic spreads. Was there an impact on the staging process?

**Response: Thank you very much. When preparing meiotic spreads, the staging process in *lepto1* seems to be normal before four-layer stage. After that, the defects start to be shown in *lepto1*. As shown in Supplemental Figure 5A to 5C, when wild-type spikelet had already generated isolated microspores, the PMCs in similar-sized *lepto1* anthers were still arrested in preleptotene. However, it was very hard to see the leptotene chromosomes in *lepto1*. The arrested chromosomes in *lepto1* PMCs were degraded in flowers with a larger size (Supplemental Figure 2E to 2G).**

**Q6.** In Figure 3, immunostainings are shown illustrating the lack of immunostaining of meiotic proteins, AM1, OsREC8, γH2AX, MER1 and ZEP1. This finding is exciting as it demonstrates that critical meiotic functions are not executed in *lepto1-1*. The *lepto1-1* immunostainings show nuclei with prominent nucleoli, a feature that is also in the WT cell stained with OsAM1. The other WT cells are clearly progressed to later stages of meiosis, apparent from DNA structure and the position of the CENH3 dots. The absence of immunostaining of meiosis proteins therefore seems to result from arrested meiocyte cell cycle progression. In my opinion this should be demonstrated more firmly. It can be done for example by showing spreads or immunos using meiocytes isolated from different flowering stages.

**Response: Thank you very much for your kind advice. We have added the immunostaining of meiotic proteins in wild-type PMCs which were at preleptotene. In wild type, OsAM1, OsREC8, PAIR3, γH2AX and ZEP1 signals were not detected in preleptotene PMCs, and these meiotic proteins were recruited to chromosomes with the processing of meiosis (Figure 3A). Nevertheless, there was no OsAM1, OsREC8, PAIR3, γH2AX and ZEP1 signal in *lepto1* PMCs from beginning to the end, suggesting that the chromosome status at preleptotene may result in the failure of recruitment of meiotic proteins.**

**Q7.** So far in plants, none of the cyclins were shown to lead to a meiotic cycle arrest: *Tam1* (*cyc1A1;2*) and *cyca2;2* and *cyca2;234* mutants show segregation defects yet progress into forming spores. The *lepto1* phenotype (the interpretation that it arrests the meiotic cycle) can therefore not be explained by a lack of expression or activity of cyclins. This should be more thoroughly discussed and comparisons draw with cyclin function in other organisms.

**Response: We have revised the discussion part about cyclins in this manuscript according to your suggestions (Pages 17-18, lines 431-457). Thank you very much.**

**Q8.** An alternative view is that *lepto1* regulates tapetum function and that tapetum meiocyte communication is critical for progression. This possibility should also be discussed, comparing the phenotype of tapetum mutant with that of *lepto1*.

**Response: Yes, we agree that tapetum meiocyte communication is critical for anther development. We have compared the phenotype of *lepto1* with *eat1* and *tdr*, and the proper role of LEPTO1 in tapetum meiocyte communication was discussed in this revised manuscript (Page 18, lines 458-471). Thanks a lot!**

Minor revisions.

**Q1.** On the molecular characterization of LEPTO1 and the interaction with OsAHP1 I do not have important remarks.

**Response: Thank you very much.**

**Q2.** In the conclusion is said that LEPTO1 may be a component of the cytokinin signaling pathway. Since it was shown that *LEPTO1* is not controlled by cytokinin, and there is so far no other evidence for a role of cytokinin in early meiosis progression, I suggest to remove or modify that claim.

**Response: Thank you very much for your suggestions. As we did not obtain evidence about the direct role of cytokinin in meiosis progression, we agree that our previous description is improper. Accordingly, the description about the possible function of cytokinin in meiosis has been removed. Thank you very much.**

**TPC2018-00479-RAR1 2nd Editorial decision – *accept with minor revision*** **Oct. 13, 2018**

We have received reviews of your manuscript entitled "A type-B response regulator, LEPTO1, is essential for setting up leptotene status in rice meiosis." As you will see from the comments below, the reviewers appreciate the revised version and the consideration of their former comments. On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in *The Plant Cell*. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

As pointed out by Reviewer 2, Fig 3 A is confusing in the current version: it has two rows of images for the wt (one with DAPI and CENH3, one merged with the immunostainings of the meiotic proteins). For the lepto 1 mutant, there is only one row, with DAPI and CENH3 signals similar to the wt. Is the row with the merged images missing? Or is this row the one with the merged images? If so, it is indeed puzzling why there are no proteins detectable although their RNAs are present in the *in situ* images in Fig. S11. Please clarify. The reviewers also point to the need of grammar and spelling corrections throughout. Please use a professional editor to help you with this before you re-submit.

Reviewer #1:

The revised manuscript is much improved and no further comments for changes.

Reviewer #2:

The revised manuscript "A type-B response regulator, LEPTO1, is essential for setting up leptotene status in rice meiosis" by Zhao and co-workers presents a detailed study that reports the isolation of LEPTO1, which encodes a type-B RR that is required for the establishment of leptotene in meiotic chromosomes in rice.

The manuscript is well-written, is on an important topic and it presents new and interesting information. The manuscript is much improved from the previous version. The authors have done a nice job addressing the concerns raised in the previous review cycle and present a considerable amount of new information that strengthens the manuscript and helps round out their story. I have one minor concern/correction and one inconsistency that is bothering that I need to bring up.

1) There are a number of small grammatical/English language issues throughout the manuscript that need to be cleaned up/corrected.

2) I am having a hard time reconciling the results in Fig. 3A and S11, which show that even though OSAM1, REC8, PAIR3, DMC1, SPO11 and ZEP1 transcripts are relatively normal, there is absolutely no sign of any of the proteins in the immunolocalization results. It may be that this is due to the clearing methods used, but based on my experience I would have expected some sign of at least some of the proteins on the chromosomes or in the nucleoplasm. The CENH3 signal is a strong indicator that the immunolocalization is working, but the total absence of any green signal is hard to believe. This is something that the authors should discuss. Do they believe that the proteins are not synthesized, degraded quickly or fail to bind anywhere on the chromosomes and are totally washed out of the cells during sample preparation?

Reviewer #3:

Typos:
line 26 development in *lepto1* pollen mother cells (PMCs). Furthermore, loss OF function ...
28 meiosis-specific proteins, and "lack of" deposition of the callose wall.

**TPC2018-00479-RAR2 2nd Revision received Nov. 19, 2018**

**Dear editor and reviewers,**

**Thank you very much for your valuable comments and constructive suggestions on our manuscript entitled "A type-B response regulator, LEPTO1, is essential for setting up leptotene status in rice meiosis" (ID: TPC2018-RA-00479R1). We have made adequate revisions according to your suggestions. We have also carefully proof-read the manuscript with the help of a professional language editing company, to minimize the potential typographical and grammatical errors. The revised parts were marked in red in the revised manuscript.**

**We are sorry for the misleading representation in Fig 3A of the previous version and have replaced it with a more reasonable figure in the current version. For the concerns of Reviewer 2, we have separated the immunostaining signals of OsAM1, OsREC8, PAIR3, γH2AX, and ZEP1 from the merged images to show more details. As shown in the bottom low of Fig 3B, only faint background signals of these proteins were observed in *lepto1* PMCs. The green signals in *lepto1* PMCs might be covered by the fluorescence signals of CENH3 and DAPI in our previous version. As the transcription of these meiotic genes could be detected in *lepto1* PMC by RNA *in situ* hybridization, we suspect that loss of LEPTO1 function causes defects in setting up leptotene status and may further lead to the failure in recruitment of these meiotic proteins.**

**For the concerns of Reviewer 3, we have revised the typographical and grammatical errors with the help of a professional language editing company.**

**Again, your valuable comments and wonderful suggestions are really appreciated. We hope these revisions have significantly improved our manuscript.**

**TPC2018-00479-RAR2 3rd Editorial decision – *acceptance pending* Nov. 19, 2018**

We are pleased to inform you that your paper entitled "A type-B response regulator, LEPTO1, is essential for setting up leptotene status in rice meiosis" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

**Final acceptance from Science Editor Dec. 3, 2018**