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Manuscript EMBOR-2013-37915

Meristem-specific expression of epigenetic regulators safeguards transposon silencing in Arabidopsis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 27 September 2013

Thank you for the submission of your manuscript to our journal. I am very sorry for the unusual delay in getting back to you; we are still waiting for the third referee report to come in. However, as two referees have submitted their report and think that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. I will be in touch as soon as we will receive the final report on your manuscript.

As you will see, both referees acknowledge that the findings are interesting and the data convincing. However, both referees also point out that DNA methylation should be analyzed directly to examine tissue specific differences between true leaves and cotyledons. Referee 1 further suggests to investigate TGS in lateral roots and well-developed rosette leaves. Both referees also indicate where additional discussion of the data is required.

Given these constructive comments, we would like to invite you to revise your manuscript with the

understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values (if applicable) in the respective figure legends? This information is currently missing and needs to be provided in the figure legends.

We recently decided to offer authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) for all or your key experiments together with the revised manuscript.

When submitting your revised manuscript, please include:

A Microsoft Word file of the manuscript text, editable high resolution TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format), a letter detailing your responses to all the referee comments, and a two sentence summary of your findings and their significance.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any further questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

The manuscript by Baubec et al places RNA-directed DNA Methylation (RdDM) as an important checkpoint mechanism during vegetative development in order to silence repetitive sequences in the genome such as transposons.
The authors show that zebularine treatments triggers the reactivation of a silent reporter transgene specifically in the cotyledons and not in true leaves in wild type seedlings. This tissue-specific reactivation after zebularine treatment is abolished in RdDM mutants that show reactivation also in true leaves. A similar observation is shown for different endogenous repetitive elements. The authors show that, in the absence of an active RdDM pathway, zebularine treatments cause

reactivation of the reporter even after several weeks of the removal of the treatment both in newly produced tissues, such as cauline leaves, and even in the next generation. Finally they show that different component of the Transcriptional Gene Silencing (TGS) such as MET1, DDM1 or RdDM components are highly expressed in the SAM compared to other tissues.
The manuscript describes for the first time the role of the RdDM pathway in silencing repetitive sequences in newly produced organs derived from the SAM during vegetative growth and its relevance, since changes in DNA methylation at this stage can be transmitted to the next generation. The paper is well written and is of general interest for a broad audience of readers interested in the regulation of DNA methylation and chromatin silencing during development. The paper is suitable for EMBO reports although the following comment/suggestions should be addressed.
Comments:
- The authors claim that even in the presence of zebularine, true leaves do not show any reactivation of the TS-GUS reporter due to the activity of the RdDM pathway. Indeed, inactivation of this pathway triggers the activation of the reporter. Thus, it would be interesting to profile DNA methylation by bisulfite sequencing of the reporter in cotyledons and true leaves of mock and zebularine-treated WT and drm2 (or drd1 or ago4) mutant seedlings to study whether there are tissue-specific differences in the methylation profile of the reporter, that could be attributed to differential expression of TGS components. If the activity of RdDM is more relevant during true leaves formation, one would expect an increased content of CHH methylation in young true leaves of WT plants compared to the cotyledons.
- The authors claim that TS-GUS is activated in the cotyledons and not in true leaves because there is differential expression of TGS components between these two tissues. I wonder if that occurs also in lateral root formation. It would be interesting to examine lateral roots of mock and zebularine-treated TS-GUS and maybe TS-GUS in different RdDM mutant backgrounds.
- Young leaves do not show activation of the reporter because the TGS machinery is more active in the SAM and thus, all new tissue originated from there go through this checkpoint that keeps the reporter and other repetitive elements silent. It would be nice if authors could show the effect of zebularine treatments in well-developed rosette leaves. One would expect reactivation of the reporter since the expression of TGS component is low in this tissue based in the microarray data.
- Supp Fig 4-A. It is difficult to appreciate what the authors describe in the text based on these images. I would suggest retaking the photographs using a different background.
- Fig 3A-B experiment shows that S1 generation after self-pollination of zebularine-treated plants shows a certain degree of TS-GUS activation that is dependent on the RdDM pathway activity. The effect of drd1 and ago4 mutants, especially ago4, is much stronger that drm1/2 mutant. Since DRM2 is the last step of the RdDM pathway, how do the authors explain the weaker effect of the drm1/2 mutant? Is it because of the different genetic mix? Col0 VS, Col0/WS VS Col0/Ler? If this is the case it should be discussed in the text.

Referee #3:

This manuscript by Baubec et al builds on previous work published by the same group (Baubec et al 2009) that showed that the DNA methylation inhibitor zebularine releases transcriptional gene silencing (TGS) in cotyledons but not in true leaves of Arabidopsis seedlings. This pattern of reactivation is reminiscent of that reported earlier by others in some mutants affected in DNA methylation (Elmayan et al 2005). Here the authors extend the latter observations to several mutants defective in RNA-directed DNA methylation (RdDM) and show that transcriptional reactivation of a reporter transgene as well as of several endogenous repeats does also occur in true leaves when zebularine treatment is applied to these mutants. In addition, the authors demonstrate that this reactivation can be sustained during vegetative growth and be inherited in the absence of zebularine in some of the mutants, but that it is erased when those mutants are crossed with wild type plants. Finally, using data mining, the authors show that RdDM as well as other chromatin regulators of TGS have elevated expression in meristems.

While a role for RdDM in restoring DNA methylation and transcriptional repression of demethylated/reactivated repeats across generations has already been established (Teixeira et al, 2009; a reference omitted by the authors), this is the first time that it is shown that RdDM plays a similar role during vegetative growth. For this reason, this manuscript should be of wide interest.

The paper is generally well written and the data appear sound and convincing. However, there are three points that the authors should consider.

1. The authors equate zebularine treatment with release of TGS maintenance, which leads them to write in the introduction that RdDM and TGS maintenance have redundant roles, which is not quite true. The seond part of that same sentence (Page 4 lines 70-72) is also confusing.

2. An analysis of DNA methylation levels/patterns by Bisulphite sequencing in mutant plants treated or not with zebularine is lacking for the various endogenous repeats studied. This could in particular provide an explanation as to why reactivation in true leaves is not observed despite similar hypomethylation in both tissues and why this reactivation is inherited only in some RdDM mutants.

3. The observation that RdDM is required for restoring DNA methylation and transcriptional repression of demethylated/reactivated repeats during vegetative growth is at odds with previous results indicating that restoration by RdDM is most effective during the reproductive phase and progressive across several generations (Teixeira et al, 2009; Teixeira & Colot, 2010). Could the authors comment?

Correspondence - editor 02 October 2013

We have now received the comments from referee 2, who is more concerned about the novelty of the findings than the other two referees. All her/his concerns need to be addressed in the manuscript text, and additional chromatin marks should be compared between cotyledons and true leaves. It should also be experimentally investigated whether a synergistic effect between DNA methylation and other chromatin modifications might be responsible for the more efficient loss of TGS in cotyledons.

REFEREE REPORT:

Referee #2:

The manuscript by Baubec et al. is focused in the cooperative action of transcriptional gene silencing (TGS) pathways to maintain and reinforce the silencing of repetitive elements such as transposable elements (TE) during vegetative development and more particularly in meristems. This is of especial relevance in plants given that all of the somatic tissues are continuously developed from the undifferentiated cells located at the meristems, furthermore, gametes arise from vegetative tissues later on development. Therefore, reinforcing the TGS of potentially threatening invasive or mobile TE is important to maintain genome integrity during plant development and across generations. By following the activity of a silenced reporter transgene and endogenous repeated sequences and TE, and by using a combination of genetics and chemical interference with DNA methylation, the authors conclude that, while interfering with DNA methylation (zebularine treatment) is enough to alleviate TGS in embryo-derived tissues (cotyledons and hypocotyl), it is not enough to do so in post-embryonic vegetative tissues. Only the combination of zebularine treatment with mutations in the de novo DNA methylation pathway (i.e. RNA-directed DNA methylation; RdDM) allows the release from TGS in vegetative tissues. The authors further support the hypothesis of a reinforced TGS via RdDM in meristematic tissue by analysing publically available microarray data where they observe increased expression levels of a subset of RdDM components.

The results presented about the weakness of TGS in embryonic tissues and its

reinforcement in post-embryonic and meristematic tissues are interesting, however I have a some problems regarding the significance and novelty of the work presented here and that should be addressed given that, the observation of cotyledon-specific reactivation of both reporter systems (GUS-based L5 and GFP) by zebularine was previously reported by the same authors (Baubec et al., 2008). Tissue-specific release from TGS of the same L5 reporter in different mutant backgrounds has also been previously reported (Elmayan et al., 2005).

Comments:

1) The role of mobile siRNAs in reinforcing TE silencing during gametogenesis and embryogeneis is mentioned twice in the text (lines 56-58; 232-233) as an example of RdDM role in reinforcing TGS in critical time-points of development. Yet different components of TGS have been shown to be differentially expressed in gametophytes and their companion cells together with different methylation levels, and following the report of mobile siRNAs in pollen from the vegetative cell to the sperm cells (Slotkin et al., 2009) it is tempting to suggest that loss of methylation in companion cells can lead to the release of TE silencing and production of siRNAs to reinforce TGS in the gametes. However, a recent report (Grant-Downtown et al., 2013) casts serious doubts on the interpretation of the Slotkin et al. paper. It is also not entirely clear to me how relevant this might be to the work presented here.

2) In lines 111-113 the authors claim no differences in nuclei decondensation by zebularine between cotyledons and true leaves. However in fig S2B, cotyledons display double levels of dispersed nuclei than leaves. Do the authors think this might be relevant?

3) In lines 123 to 125: "During mock treatment, we observed full TS-GUS reactivation in ddm1 and met1 and weak, predominantly cotyledon-specific activation in cmt3, hda6, fas1 and RdDM mutants (Fig 2A, upper panel)". However in the referenced panel cmt3 is the only mutant shown. Of special importance is met1 given that later on in the text (lines 140 to 148) the authors claim that in cotyledons, "DNA methylation maintenance is sufficient for TE repression" but not in the rest of the post-embryonic growth tissues. However, if I understood well, met1 leads to a full TS-GUS reactivation (line 124) Therefore it seems that loss of RdDM leads to partial reactivation only in cotyledons and is enhanced by interference with DNA methylation maintenance (and DNA methylation in general) by zebularine. Hence I find the conclusions in lines 140 to 148 a bit confusing. Please clarify.

4) One of the most interesting observations is the fact that zebularine treatment seems to cause the same level of loss of DNA methylation in cotyledons and true leaves (Supp Fig 2A) yet silenced transgenes or endogenous loci are only released from TGS in cotyledons. However, considering the effect of not only DNA methylation but also chromatin modifications on TGS, it would be of interest to know if both tissues have the same histone marks at the inspected loci and transgenes in both tissues, and to know the impact of mutations in chromatin modification other than drd1, including kyp in the TS-GUS expression.

5) Linked to the previous comment and to point 2, another fact that the authors should consider is the possibility that cotyledons are a sensitized tissue for loss of DNA methylation or undergo special epigenetic reprogramming during development (germination) that might be exacerbated by zebularine. Indeed, several studies should be of interest to, and perhaps mentioned by, the authors. It has been known for a long time that in different plant species there is a progressive loss of DNA methylation during germination (Drozdenyuk et al., 1976; Follmann et al., 1990; Portis et al., 2004). Moreover, this loss of DNA methylation has been reported to happen in cotyledons during germination and the first days of post germination growth (Ziuvova et al., 2001; Douet et al., 2008) in a Pol IV (NRPD1) and ROS1 dependent manner where ROS1 drives active demethylation that is later restored by the NRPD1 pathway (Douet et al., 2008). This loss of DNA methylation is

accompanied by chromocenter decondensation, which might explain the almost double amount of dispersed foci observed in cotyledons compared to leaves upon zebularine treatment (Supp Fig 2B). Therefore a possible scenario would be that while germinating seeds in the presence of zebularine, the synergistic effect with ROS1 activity (or other chromatin modifications that might take place in cotyledons during germination) leads to a more efficient loss of TGS. The latter can be easily tested by germinating the seeds in medium without zebularine and transferring the seedlings to zebularine-containing medium one week after (after DNA methylation changes in cotyledons are meant to be stabilized, Douet et al., 2008). A second approach would be to introgress the TS-GUS from L5 into the ros1 mutant background and treat with zebularine.

6) In figure S4A I fail so see TS-GUS active sectors in the plants shown (line 154 to 155).

1st Revision - authors' response 08 January 2014

Baubec et al., Point by point response (EMBOR-2013-37915V1)

Comments from the Editor:

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 main plus 5 supplementary figures, which should directly relate to their corresponding main figure. Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values (if applicable) in the respective figure legends? This information is currently missing and needs to be provided in the figure legends.

Response: We have addressed all points raised by the editor and performed substantial shortening of the manuscript and reduced the number of main and supplemental figures to four each. However, we have been asked for including additional experiments, descriptions and references. This resulted in the current manuscript length of approximately 31,000 characters. In case further shortening is needed we will transfer part of the method descriptions into the supplementary data. Adding new experiments required substantial changes in the manuscript text including the abstract. Concerning your offer for adding the source data, we would like use this opportunity for the primary data of the bisulfite sequencing analysis.

Manuscript short summary: *De novo* DNA methylation reinforces silencing maintenance at transposable elements in Arabidopsis shoot apical meristems. This secures correct transmission of epigenetic information during development and between generations.

Referee #1:

The manuscript by Baubec et al places RNA-directed DNA Methylation (RdDM) as an important checkpoint mechanism during vegetative development in order to silence repetitive sequences in the genome such as transposons.
The authors show that zebularine treatments triggers the reactivation of a silent reporter transgene specifically in the cotyledons and not in true leaves in wild type seedlings. This tissue-specific reactivation after zebularine treatment is abolished in RdDM mutants that show reactivation also in true leaves. A similar observation is shown for different endogenous repetitive elements. The authors show that, in the absence of an active RdDM

pathway, zebularine treatments cause reactivation of the reporter even after several weeks of the removal of the treatment both in newly produced tissues, such as cauline leaves, and even in the next generation. Finally they show that different component of the Transcriptional Gene Silencing (TGS) such as MET1, DDM1 or RdDM components are highly expressed in the SAM compared to other tissues.
The manuscript describes for the first time the role of the RdDM pathway in silencing repetitive sequences in newly produced organs derived from the SAM during vegetative growth and its relevance, since changes in DNA methylation at this stage can be transmitted to the next generation. The paper is well written and is of general interest for a broad audience of readers interested in the regulation of DNA methylation and chromatin silencing during development. The paper is suitable for EMBO reports although the following comment/suggestions should be addressed.

- We appreciate the positive comments.

Comments:
***1)** - The authors claim that even in the presence of zebularine, true leaves do not show any reactivation of the TS-GUS reporter due to the activity of the RdDM pathway. Indeed, inactivation of this pathway triggers the activation of the reporter. Thus, it would be interesting to profile DNA methylation by bisulfite sequencing of the reporter in cotyledons and true leaves of mock and zebularine-treated WT and drm2 (or drd1 or ago4) mutant seedlings to study whether there are tissue-specific differences in the methylation profile of the reporter, that could be attributed to differential expression of TGS components. If the activity of RdDM is more relevant during true leaves formation, one would expect an increased content of CHH methylation in young true leaves of WT plants compared to the cotyledons.*

Response:
As requested, we provide now DNA methylation analysis by bisulfite sequencing in mock- and zebularine-treated true leaves and cotyledons of wild type and *drd1* plants. Primers for conclusive bisulfite analysis could not be designed for the highly repetitive TS-GUS transgene, therefore we focused on defined copies of the endogenous repeats LINE1-4 and MULE2 that respond similarly (New Figure 2C). The results support the requirement of RdDM for re-methylating TEs in true leaves after zebularine treatment, as combined treatment and lack of DRD1 lead to increased hypomethylation.
We do not observe increased levels of CHH methylation in true leaves of mock-treated wt plants when compared to cotyledons. This is expected since RdDM and CHH methylation are dispensable for TE silencing during true leaf formation in mock treatments: the majority of TEs does not depend on RdDM for silencing maintenance (see e.g. Huettel et al., EMBO J, 2006). Rather the opposite is observed as we detect higher levels of CHH in cotyledons (New Fig 2C). This is most likely due to low replication rates in cotyledons that favors accumulation of asymmetric methylation.

***2)** - The authors claim that TS-GUS is activated in the cotyledons and not in true leaves because there is differential expression of TGS components between these two tissues. I wonder if that occurs also in lateral root formation. It would be interesting to examine lateral roots of mock and zebularine-treated TS-GUS and maybe TS-GUS in different RdDM mutant backgrounds.*

Response: Plants grown on zebularine do not develop lateral roots, likely due to toxic side effects of the inhibitor. Therefore, we were unfortunately not able to evaluate TS-GUS expression in emerging lateral roots.

***3)** - Young leaves do not show activation of the reporter because the TGS machinery is more active in the SAM and thus, all new tissue originated from there go through this checkpoint that keeps the reporter and other repetitive elements silent. It would be nice if authors could show the effect of zebularine treatments in well-developed rosette leaves. One would expect reactivation of the reporter since the expression of TGS component is*

low in this tissue based in the microarray data.

Response:
GUS staining of three weeks-old wild type and *drd1* plants at the bolting stage, with or without zebularine treatment, did not reveal an increased reactivation of the TS-GUS reporter construct (see new sup figure S3A). This suggests that the silencing is sufficient even in the well-developed rosette leaves. Further support comes from the microarray data analysis: the gene expression of TGS components is nearly identical for all leaf samples covered by the AtGenExpress dataset, with no differences between young and developed leaves (New Figure S4D). We have now made this point clearer in the manuscript on pages 5 and 10, and we are sorry for the misunderstanding.

4) *- Supp Fig 4-A. It is difficult to appreciate what the authors describe in the text based on these images. I would suggest retaking the photographs using a different background.*

Response: There must have been a misunderstanding concerning the extent of the activation. We have now corrected the description in the text and indicate specific regions by arrowheads (New Figure S3B).

5) *- Fig 3A-B experiment shows that S1 generation after self-pollination of zebularine-treated plants shows a certain degree of TS-GUS activation that is dependent on the RdDM pathway activity. The effect of drd1 and ago4 mutants, especially ago4, is much stronger that drm1/2 mutant. Since DRM2 is the last step of the RdDM pathway, how do the authors explain the weaker effect of the drm1/2 mutant? Is it because of the different genetic mix? Col0 VS, Col0/WS VS Col0/Ler? If this is the case it should be discussed in the text.*

Response: We agree that the effect is most likely due different silencing efficiencies of the used Arabidopsis accessions and made this point clear on page 8 of the manuscript.

Referee #2:

The manuscript by Baubec et al. is focused in the cooperative action of transcriptional gene silencing (TGS) pathways to maintain and reinforce the silencing of repetitive elements such as transposable elements (TE) during vegetative development and more particularly in meristems. This is of especial relevance in plants given that all of the somatic tissues are continuously developed from the undifferentiated cells located at the meristems, furthermore, gametes arise from vegetative tissues later on development. Therefore, reinforcing the TGS of potentially threatening invasive or mobile TE is important to maintain genome integrity during plant development and across generations. By following the activity of a silenced reporter transgene and endogenous repeated sequences and TE, and by using a combination of genetics and chemical interference with DNA methylation, the authors conclude that, while interfering with DNA methylation (zebularine treatment) is enough to alleviate TGS in embryo-derived tissues (cotyledons and hypocotyl), it is not enough to do so in post-embryonic vegetative tissues. Only the combination of zebularine treatment with mutations in the de novo DNA methylation pathway (i.e. RNA-directed DNA methylation; RdDM) allows the release from TGS in vegetative tissues. The authors further support the hypothesis of a reinforced TGS via RdDM in meristematic tissue by analysing publically available microarray data where they observe increased expression levels of a subset of RdDM components.

The results presented about the weakness of TGS in embryonic tissues and its reinforcement in post-embryonic and meristematic tissues are interesting, however I have a some problems regarding the significance and novelty of the work presented here and that should be addressed given that, the observation of cotyledon-specific reactivation of both reporter systems (GUS-based L5 and GFP) by zebularine was previously reported by the same authors (Baubec et al., 2008). Tissue-specific release from TGS of the same L5

reporter in different mutant backgrounds has also been previously reported (Elmayan et al., 2005).

Response: We appreciate that also this referee finds our work interesting. Indeed, the tissue-specific activation can be found in figures of both publications mentioned (which are cited in our manuscript). However, this observation was neither mentioned nor explained there. Now, we describe this previously neglected observation, identify its mechanistic basis and suggest a function in the vegetative tissue-specific transcriptional gene silencing. We consider this as novel and significant beyond previous work, as done by reviewers 1 and 3.

Comments:

***1)** The role of mobile siRNAs in reinforcing TE silencing during gametogenesis and embryogeneis is mentioned twice in the text (lines 56-58; 232-233) as an example of RdDM role in reinforcing TGS in critical time-points of development. Yet different components of TGS have been shown to be differentially expressed in gametophytes and their companion cells together with different methylation levels, and following the report of mobile siRNAs in pollen from the vegetative cell to the sperm cells (Slotkin et al., 2009) it is tempting to suggest that loss of methylation in companion cells can lead to the release of TE silencing and production of siRNAs to reinforce TGS in the gametes. However, a recent report (Grant-Downtown et al., 2013) casts serious doubts on the interpretation of the Slotkin et al. paper. It is also not entirely clear to me how relevant this might be to the work presented here.*

Response: We thank the reviewer for this note. Correct transmission of epigenetic states is currently a relevant topic and we agree that, although the activation of TEs in companion cells is well documented, the mechanism and its involvement in TE silencing in the gametes remains less clear. Therefore we have modified the corresponding sentences accordingly and included the reference to Grant-Downton et al., 2013 as a critical view on this issue.

***2)** In lines 111-113 the authors claim no differences in nuclei decondensation by zebularine between cotyledons and true leaves. However in fig S2B, cotyledons display double levels of dispersed nuclei than leaves. Do the authors think this might be relevant?*

Response: We agree that extreme decondensation, as depicted in the example, is less pronounced in zebularine-treated nuclei of true leaves. The reason for that is most likely the slightly less effective zebularine-mediated de-methylation of 180 bp repeats in true leaves (Figure S1C), which consequently leads to reduced numbers of nuclei showing strong decondensation. This could be further promoted by slightly elevated expression of MET1 and DDM1 in true leaves (Fig 4A), the enzymes responsible for DNA methylation maintenance and condensation of 180 bp repeats. Nevertheless, the ratios of affected nuclei are similar between the compared tissues, although - as noted by the reviewer - with different rates of strong decondensation. We now included a comment on this effect in the main text (page 5), and thank the reviewer for pointing this out.

***3)** In lines 123 to 125: "During mock treatment, we observed full TS-GUS reactivation in ddm1 and met1 and weak, predominantly cotyledon-specific activation in cmt3, hda6, fas1 and RdDM mutants (Fig 2A, upper panel)". However in the referenced panel cmt3 is the only mutant shown. Of special importance is met1 given that later on in the text (lines 140 to 148) the authors claim that in cotyledons, "DNA methylation maintenance is sufficient for TE repression" but not in the rest of the post-embryonic growth tissues. However, if I understood well, met1 leads to a full TS-GUS reactivation (line 124) Therefore it seems that loss of RdDM leads to partial reactivation only in cotyledons and is enhanced by interference with DNA methylation maintenance (and DNA methylation in general) by zebularine. Hence I find the conclusions in lines 140 to 148 a bit confusing.*

Please clarify.

Response: We have now added more images of activation patterns in several other mutants (New Figure S2B, see also next response). In addition, we agree that the statement concerning maintenance methylation was too categorical, and we changed it accordingly.

4) *One of the most interesting observations is the fact that zebularine treatment seems to cause the same level of loss of DNA methylation in cotyledons and true leaves (Supp Fig 2A) yet silenced transgenes or endogenous loci are only released from TGS in cotyledons. However, considering the effect of not only DNA methylation but also chromatin modifications on TGS, it would be of interest to know if both tissues have the same histone marks at the inspected loci and transgenes in both tissues, and to know the impact of mutations in chromatin modification other than drd1, including kyp in the TS-GUS expression.*

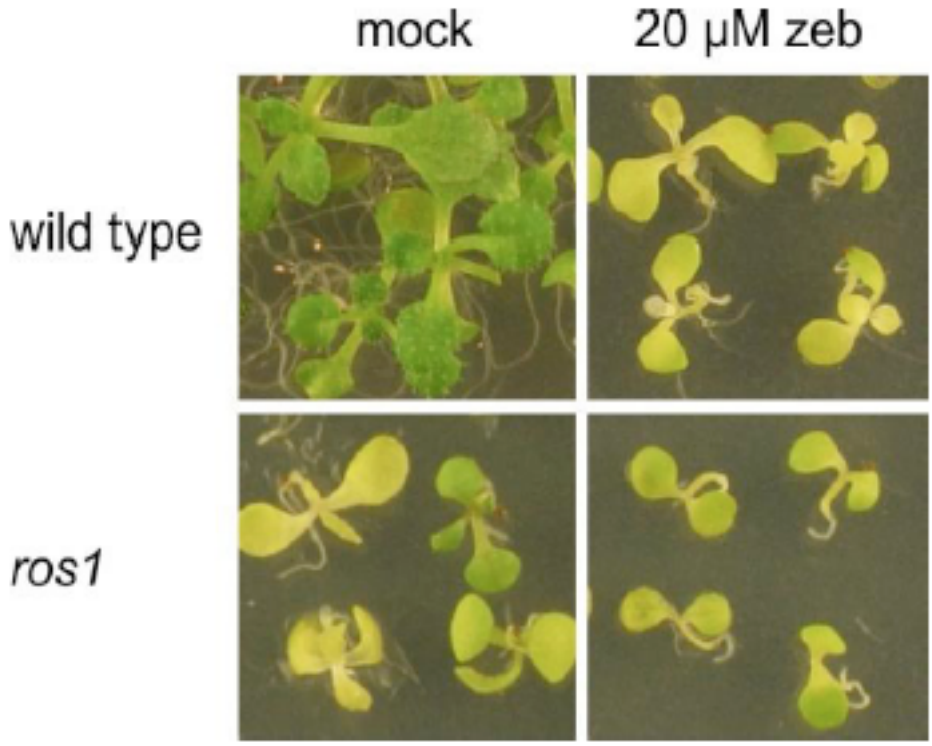
Response: We fully agree with the reviewer that it would be interesting to address the roles of histone modifications in regulating the observed tissue-specific response. However, cotyledons and true leaves of 14 days-old seedlings grown on zebularine-containing media provide only very limited amounts of material that does not allow addressing this by chromatin immunoprecipitation in a meaningful way. Rather, in order to address this important question, we have now included several mutants lacking specific histone-modifying enzymes, namely TS-GUS *kyp*, TS-GUS *lhp1* and TS-GUS *hda6*(*rts1-1*) hybrids grown under mock and zebularine treatment (New Fig S2B). The results show that the loss of H3 lysine 9 di-methylation, H3 lysine 27 tri-methylation or histone hyperacetylation, respectively, do not lead to GUS expression in true leaves, even in presence of zebularine. Corresponding changes were made in the text on page 6.

5) *Linked to the previous comment and to point 2, another fact that the authors should consider is the possibility that cotyledons are a sensitized tissue for loss of DNA methylation or undergo special epigenetic reprogramming during development (germination) that might be exacerbated by zebularine. Indeed, several studies should be of interest to, and perhaps mentioned by, the authors. It has been known for a long time that in different plant species there is a progressive loss of DNA methylation during germination (Drozdenyuk et al., 1976; Follmann et al., 1990; Portis et al., 2004). Moreover, this loss of DNA methylation has been reported to happen in cotyledons during germination and the first days of post germination growth (Ziuvova et al., 2001; Douet et al., 2008) in a Pol IV (NRPD1) and ROS1 dependent manner where ROS1 drives active demethylation that is later restored by the NRPD1 pathway (Douet et al., 2008). This loss of DNA methylation is accompanied by chromocenter decondensation, which might explain the almost double amount of dispersed foci observed in cotyledons compared to leaves upon zebularine treatment (Supp Fig 2B). Therefore a possible scenario would be that while germinating seeds in the presence of zebularine, the synergistic effect with ROS1 activity (or other chromatin modifications that might take place in cotyledons during germination) leads to a more efficient loss of TGS. The latter can be easily tested by germinating the seeds in medium without zebularine and transferring the seedlings to zebularine-containing medium one week after (after DNA methylation changes in cotyledons are meant to be stabilized, Douet et al., 2008). A second approach would be to introgress the TS-GUS from L5 into the ros1 mutant background and treat with zebularine.*

Response: We thank for suggesting these experiments. We agree that cotyledons, as terminally differentiated tissue, may undergo special epigenetic reprogramming during maturation. However, gene expression data suggest little differences between cotyledons and true leaves, independent of the gene sets analyzed (TGS components, TEs etc.). This suggests that the epigenetic reprogramming mentioned (Douet et al 2008) may be restricted to 5S rDNA and does not affect other genomic regions. We would also like to highlight that the cotyledon-specific heterochromatin de-condensation and de-methylation reported by Douet et al. was only described for 5S rDNA repeats which are clearly distinct

from repeats analyzed in this study.
Based on our DNA methylation analysis (Southern blots, bisulfite data) we do not detect reduced DNA methylation in cotyledons at the targets analyzed in this study (Fig S1C and new Figure 2C). In fact, DNA methylation at LINE and MULE elements is rather elevated in cotyledons compared to true leaves. Although this does not exclude that DNA methylation may be reduced elsewhere, it allows to conclude that the reactivation in cotyledons is predominantly triggered by the zebularine treatment.

As suggested, we have included *ros1* as a potential factor involved in cotyledon de-methylation. Surprisingly, loss of ROS1 leads to hypersensitivity to zebularine treatments as the mutant plants did not develop true leaves (see figure). This may be linked to a potential role of ROS1 in repair of DNA damage caused by zebularine treatment. Although these results are interesting, we have decided not to include them in the manuscript, as they do not add essential information to the current study.



Initial growth on drug-free medium and transfer to zebularine-containing media at day 6 after germination resulted in TS-GUS activation only in tissues that developed/proliferated in the presence of the inhibitor. This is expected, as zebularine-mediated de-methylation requires incorporation of the agent into DNA during replication (New Figure 3A). These experiments further strengthened our conclusions that replication-dependent zebularine incorporation is required for reactivation in cotyledons, roots and hypocotyl, while the proliferating SAM and true leaf tissues are not affected. After 6 days growth under mock conditions, zebularine reactivated the GUS gene only in newly formed roots while it failed reactivation in non-replicating cotyledons, adult roots and hypocotyl (New Figure 3A). Further, RdDM is required for protecting reactivation in true leaves. Zebularine treatment of drd1 after 6 days mock results in reactivation of TS-GUS in true leaves only, but not in non-replicating tissues (e.g. cotyledons). We have included these important results as Figure 3A and thank the reviewer for the suggestion.

6) *In figure S4A I fail so see TS-GUS active sectors in the plants shown (line 154 to 155).*

Response: As pointed out in response to point 4 of reviewer 1, we have now corrected the description in the text and indicate specific regions by arrowheads (New Figure S3B).

Referee #3:

This manuscript by Baubec et al builds on previous work published by the same group (Baubec et al 2009) that showed that the DNA methylation inhibitor zebularine releases transcriptional gene silencing (TGS) in cotyledons but not in true leaves of Arabidopsis seedlings. This pattern of reactivation is reminiscent of that reported earlier by others in some mutants affected in DNA methylation (Elmayan et al 2005). Here the authors extend the latter observations to several mutants defective in RNA-directed DNA methylation (RdDM) and show that transcriptional reactivation of a reporter transgene as well as of several endogenous repeats does also occur in true leaves when zebularine treatment is applied to these mutants. In addition, the authors demonstrate that this reactivation can be sustained during vegetative growth and be inherited in the absence of zebularine in some of the mutants, but that it is erased when those mutants are crossed with wild type plants. Finally, using data mining, the authors show that RdDM as well as other chromatin regulators of TGS have elevated expression in meristems.

While a role for RdDM in restoring DNA methylation and transcriptional repression of demethylated/reactivated repeats across generations has already been established (Teixeira et al, 2009; a reference omitted by the authors), this is the first time that it is shown that RdDM plays a similar role during vegetative growth. For this reason, this manuscript should be of wide interest. The paper is generally well written and the data appear sound and convincing.
We thank the reviewer for these positive comments.

However, there are three points that the authors should consider.

1. *The authors equate zebularine treatment with release of TGS maintenance, which leads them to write in the introduction that RdDM and TGS maintenance have redundant roles, which is not quite true. The second part of that same sentence (Page 4 lines 70-72) is also confusing.*

Response: We thank the reviewer for pointing this out. We have now corrected this part (page 4).

2. *An analysis of DNA methylation levels/patterns by Bisulphite sequencing in mutant plants treated or not with zebularine is lacking for the various endogenous repeats studied. This could in particular provide an explanation as to why reactivation in true leaves is not observed despite similar hypomethylation in both tissues and why this reactivation is inherited only in some RdDM mutants.*

Response: We thank for this important comment. As described already in response to point 1 of reviewer 1, we have now analyzed DNA methylation by bisulfite sequencing of *LINE1-4* and *MULE2* in cotyledons and true leaves of zebularine-treated wild type and *drd1* plants. The results support the importance of RdDM for re-methylation of zebularine de-methylated TEs in true leaves.

3. *The observation that RdDM is required for restoring DNA methylation and transcriptional repression of demethylated/reactivated repeats during vegetative growth is at odds with previous results indicating that restoration by RdDM is most effective during the reproductive phase and progressive across several generations (Teixeira et al, 2009; Teixeira & Colot, 2010). Could the authors comment?*

Response: While genetic mutations in key TGS genes (e.g. *DDM1*) result in strong reduction of DNA methylation, the low dose of zebularine used in this study reduces DNA methylation less effectively (Baubec et al., 2009). Therefore, the RdDM activity in the apical meristems may be sufficient for opposing de-methylating potential of zebularine but not loss of *DDM1*. We have included the reference to Teixeira et al., 2009 and discuss these points on page 7.

2nd Editorial Decision 17 January 2014

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it, and as you will see, both referees support publication of the manuscript by EMBO reports now.

I noticed, however, that some statistical information is still missing from the figures legends. The legend for figure 1H, 2B, SF2C,D, SF3C and SF4A,B do not define the error bars, or state that the error bars are calculated from three technical replicates. Does it mean that a single experiment with three technical replicates was performed? Error bars should only be calculated for three or more independent experiments, and not for technical replicates. Can you please clarify, and add the number of experiments "n" to the legends?

I also would like to suggest a few minor changes to the abstract:

In plants, transposable elements (TEs) are kept inactive by transcriptional gene silencing (TGS). TGS is established and perpetuated by RNA-directed DNA methylation (RdDM) and maintenance methylation pathways, respectively. Here, we describe a novel RdDM function specific for shoot apical meristems that reinforces silencing of TEs during early vegetative growth. In meristems, RdDM counteracts drug-induced interference with TGS maintenance and consequently prevents TE activation. Simultaneous disturbance of both TGS pathways leads to transcriptionally active repetitive sequences that are inherited by somatic tissues and partially by the progeny. This apical meristem-specific mechanism is mediated by increased levels of TGS factors and provides a checkpoint for correct epigenetic inheritance during the transition from vegetative to reproductive phase and to the next generation.

Please let me know whether you agree with these changes and if you have any questions.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The authors have addressed my concerns.

Referee #3:

I am satisfied with the revisions that the authors have made to this manuscript.

2nd Revision - authors' response 22 January 2014

Thank you and the reviewers for fast processing of our revised manuscript. We appreciate that the reviewers were satisfied with our revision.

Indeed, the error bars in some of our graphs indicated standard deviation of the technical triplicates. We agree that this does not provide information on biological variation and based on your request we removed error bars from the figures 1H, 2B, SF2C,D, SF3C and SF4A. The error bars in SF4B are based on three biological replicates and therefore were kept in figure. We now included information on the number of replicates into the legends.

We agree to the proposed changes in the abstract.

For your convenience, I am sending you the manuscript and the supplementary data file highlighting all changes performed in this revision per email.

3rd Editorial Decision 23 January 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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