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Autocatalytic differentiation of epigenetic modifications within the Arabidopsis genome

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1st Editorial Decision 28 April 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has been evaluated by three referees and I enclose their reports below. As you will see from their comments the majority of referees find the study to be potentially interesting but require some further experimental analysis before it can be further considered for the EMBO Journal. In general the referees find the biochemical activity of IBM1 should be more rigorously demonstrated and that the link between IBM1 and transcription activity should be strengthened. Given the interest in the study should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper is interesting and fun to read. Unlike many other epigenetics groups who study factors required for heterochromatic gene silencing, the Kakutani lab has focused on factors that keep genes free of heterochromatic marks and hence transcriptionally active. In this context they discovered IBM1 (Saze et al., 2008), a predicted histone demethylase that preferentially targets protein-coding genes.
The paper by Ingaki et al. is the latest in a series examining the interplay between different epigenetic marks, in this case histone H3 lysine 9 dimethylation (H3K9me2) and DNA methylation, at genes and transposons in ibm1 mutants. Previous work from the same group (Miura et al., 2009) showed that thousands of genes acquire ectopic non-CG methylation in ibm1 plants, and the present study extends this observation to ectopic accumulation of H3K9me2, which is further shown to require the histone methyltransferase KYP/SUVH4 and the CHG methyltransferase CMT3. Results from several experimental approaches indicate that IBM1-mediated H3K9 demethylation depends on transcription of target genes, thus prohibiting them from accumulating silent epigenetic marks (H3K9me2 and non-CG methylation). The authors' data suggest a plausible model in which active genes and inactive transposons are differentially acted upon by IBM1, KYP and CMT3 (Fig. 7)

The scientific questions addressed are important and of broad interest; the data are excellent and convincing; the paper is well written and concise. I have only a few minor comments/suggestions for improvement:

1. Fig. 1A: is this experimental method (over-expression of FLAG-IBM1 in transfected leaf cells followed by isolation of nuclei and immunostaining for FLAG-IBM1 and various histone modifications) an accepted method to assess histone demethylase activity? It seems a bit primitive by only providing a crude overall estimate of histone methylation and FLAG-IBM protein in isolated interphase nuclei. Perhaps a comment on this method and how it has been used previously plus some quantification would be useful. Also, the figures in 1A are too small and would benefit by enlargement.

2. Epigenetic modifications are shown for in representative genomic regions on chr. 1 (Fig. 1B,C) and chr. 3 (Fig. 2A,B). Why were these particular regions chosen? The whole genome data from the tiling arrays are depicted only in scatter plots. It would be informative for the community to (eventually) have all of the data on a publicly accessible browser, similar to the Arabidopsis methylome data from, for example, the Ecker lab.

3. The bisulfite data in Fig. 6A-C are beautiful. However, data from RT-PCR showing disruption of transcription by the T-DNA insertions in the 3 genes should be shown in the main text. In the Fig. 6 legend, after the sentence containing RDR2 and NRPD1a, Supp. Fig. 9 should be mentioned.

Referee #2 (Remarks to the Author):

The manuscript by Inagaki et al. presents evidence that the Arabidopsis IBM1 protein is a H3K9 demethylase in vivo, acting to remove H3K9me from gene bodies. The authors show that genic H3K9me that arises in the absence of IBM1 is dependent on the H3K9 methyltransferase KYP and the DNA methyltransferase CMT3, and that CMT3 is partially required for H3K9me of transposable elements in WT plants, observations that support a previously proposed model that KYP and CMT3 form a positive feedback loop by binding to the modification catalyzed by the other enzyme. The authors go on to show that mutation of IBM1 restores H3K9me of transposons demethylated by mutating KYP or CMT, demonstrating that IBM1 can affect transposons as well as genes. Finally, the authors find that a number of such transposons are transcriptionally derepressed in KYP and

CMT mutant plants, and that several IBM1 target genes gain non-CG methylation after T-DNA insertion within the genes, and interpret these data to suggest that IBM1 targets transcriptionally active sequences.

This is an important, well-executed study that I feel is appropriate for EMBO. I do have a number of suggestions for improvement:

1. I feel that although the link between IBM1 activity and transcription is plausible, the evidence for this is fairly weak. I see no evidence that the transposons affected by IBM1 are activated to a greater extent that those that are not. The authors look at a small number of genes with T-DNA insertions, but T-DNA insertion is a radical alteration that can have consequences unrelated to interruption of transcription. Indeed the authors also find hypermethylation upstream of the T-DNA. The connection between IBM1 and transcription is given considerable weight, including in the title and abstract, and I feel that without further experimental evidence the language has to be considerably softened.

2. I found Supplementary Figure 6 very confusing. In particular, the X-axes need to be clearly defined - what does [mCG] of .1 mean?

3. Genes that have CG methylation in WT tend to be IBM1 targets. CG methylation may cause mutation of CG sites over time, so it's possible that genes with lower concentrations of CG sites appear to be preferred targets of IBM1 for this reason.

Referee #3 (Remarks to the Author):

This study presents in-depth analysis of the consequences of the loss of function of IBM1 and complements the elegant study by Miura et al 2009 by the same group, which reported that loss of IBM1 causes DNA hypermethylation on CHG primarily in genes. CHG methylation depends on its recognition by the SRA motif of KYP, which performs H3K9 methylation. Hence it was predicted that H3K9methylation would increase. The puzzling observation in the study by Miura et al was that genes and not transposons were primarily affected. In this study the authors provide some hypothesis to explain this phenomenon. It is based on the supposed H3K9 demethylase activity of IBM1. However, this is not directly demonstrated in the study and although the quality of the genome-wide analysis of chromatin marks is high, many of the conclusions drawn from the data obtained do not provide new concepts or are not compelling to argue for the models proposed.

Major points

1.The results shown in Fig1A are not convincing as they are produced using transient expression in tobacco cells. It is not clear to what level IBM1 is expressed in each tobacco cell and the signal is not quantified in a clear manner. The H3K9 demethylase activity should rather be properly assayed with the analysis of the amount of H3K9me, H3K9me2 and H3K9me3 isolated from transgenic lines that overexpress IBM1. Ideally the authors should consider attempting direct biochemical demonstration of IBM1 activity.

2. Data presented in Fig.2 is largely redundant with previously published data at isolated loci (eg Tariq et al., 2003) or genome-wide (Bernatavichute et al., 2008).

3. Figure 3 and 5 shows that H3K9 increased methylation on gene in ibm1 background depends on kyp and cmt3, which is not surprising. When H3K9methylation activity is decreased , transposon are activated and the lack of IBM causes increased H3K9 methylation on transposon, which is not seen in a background with wild type H3K9 methyltransferase activity. From this observation the authors propose that transcriptional activity might be important for IBM1 function. However it is equally possible that IBM1 function become detectable only when loci carry H3K9me2 below a certain level, which is not the case of transposon in wt KYP/CMT3 background. Alternatively IBM1 activity might be inhibited by high levels of H3K9 methylation.

4. Fig4 shows more surprisingly that sensitivity to H3K9 hypermethylation in ibm1 background is correlated with a decrease of CG density (and as expected there is a positive correlation with

increased CHG density). This suggests a competition between the DNA methylation pathways. The authors could also check whether this does not result from an anticorrelation between CG and CHG densities. As suggested by the authors this is reminiscent of the "rescuing" methylation on CHG and CHH observed in met1 mutants.

5. The authors show that CHG methylation increases on sites targeted by TDNA insertion. Although the authors do not show that the TDNA insert disrupts transcription and do not show that the increase of CHG is IBM 1-dependent they conclude that IBM1 requires transcription to demethylate H3K9 at these loci.

Many conclusions are drawn without statistical testing of hypotheses.

Several "*Corresponding authors." Are indicated but only one email address is mentioned.

1st Revision - authors' response 26 July 2010

Point-by-point response to the Referees' comments.

Referee #1

This referee was very positive and suggested only "minor" comments. Nonetheless, the comments were very constructive and helped us to improve the manuscript.

The scientific questions addressed are important and of broad interest; the data are excellent and convincing; the paper is well written and concise. I have only a few minor comments/suggestions for improvement:

1. Fig. 1A: is this experimental method (over-expression of FLAG-IBM1 in transfected leaf cells followed by isolation of nuclei and immunostaining for FLAG-IBM1 and various histone modifications) an accepted method to assess histone demethylase activity? It seems a bit primitive by only providing a crude overall estimate of histone methylation and FLAG-IBM protein in isolated interphase nuclei. Perhaps a comment on this method and how it has been used previously plus some quantification would be useful. Also, the figures in 1A are too small and would benefit by enlargement.

Our response:
As suggested by the Referee #1, we quantified the effects on H3K9me2 and H3K9me1 in large number of cells in multiple slides (Supplementary Figure S1A). The results show that the effects are highly significant and reproducible. We also added results using a mutated IBM1 protein as an additional negative control (Figure 1B, Supplementary Figure S1B), which we believe has strengthened the results. Over-expression of jmjC proteins and subsequent immunostaining to see the demethylase activity in vivo is a method used often, and we added a reference (Seward et al 2007 Nat Struct Mol Biol 14, 240-242). We made the panels in Figure 1AB bigger, as suggested by the Referee.

2. Epigenetic modifications are shown for in representative genomic regions on chr. 1 (Fig. 1B,C) and chr. 3 (Fig. 2A,B). Why were these particular regions chosen? The whole genome data from the tiling arrays are depicted only in scatter plots. It would be informative for the community to (eventually) have all of the data on a publicly accessible browser, similar to the Arabidopsis methylome data from, for example, the Ecker lab.

Our response: We have deposited the ChIP-chip data, as well as expression data, to NCBI (SuperSeries GSE23030), so that anyone can examine any

locus. The representative genomic regions shown in Figure 1 and 2 have been chosen without any specific reason. Other regions also show similar patterns.

3. The bisulfite data in Fig. 6A-C are beautiful. However, data from RT-PCR showing disruption of transcription by the T-DNA insertions in the 3 genes should be shown in the main text. In the Fig. 6 legend, after the sentence containing RDR2 and NRPD1a, Supp. Fig. 9 should be mentioned.

Our response: As suggested, we added description that transcription was disrupted in these mutants in the Figure 6 legend and quoted Supplementary Figure S10 (S9 in the previous version).

Referee #2

This referee was also very positive and the comments were constructive.

This is an important, well-executed study that I feel is appropriate for EMBO. I do have a number of suggestions for improvement:

1. I feel that although the link between IBM1 activity and transcription is plausible, the evidence for this is fairly weak. I see no evidence that the transposons affected by IBM1 are activated to a greater extent that those that are not. The authors look at a small number of genes with T-DNA insertions, but T-DNA insertion is a radical alteration that can have consequences unrelated to interruption of transcription. Indeed the authors also find hypermethylation upstream of the T-DNA. The connection between IBM1 and transcription is given considerable weight, including in the title and abstract, and I feel that without further experimental evidence the language has to be considerably softened.

Our response:
As suggested by the Referee #2, we examined expression of the transposons genome-wide in *kyp* and *cmt3* single mutants (Supplementary Figure S9). Consistent with our proposal, transposons derepressed in the *kyp* or *cmt3* mutant showed increase in H3K9me2 level as a response to the *ibm1* mutation.

2. I found Supplementary Figure 6 very confusing. In particular, the X-axes need to be clearly defined - what does [mCG] of .1 mean?

Our response: We agree that the explanation for Supplementary Figure S6 was confusing. By [mCG], we meant number of methylated CG sites in wild type Col divided by length of the transcription unit. We added more explanation in the legend of Figure S6. In addition, we added explanation about the parameters [CG], [CHG], [mCG], and [mCHG] in the Materials and Methods.

3. Genes that have CG methylation in WT tend to be IBM1 targets. CG methylation may cause mutation of CG sites over time, so it's possible that genes with lower concentrations of CG sites appear to be preferred targets of IBM1 for this reason.

Our response: That is a very interesting suggestion. We examined if the suppression of CG frequency in methylated sites is associated with the effect of the *ibm1* mutation. The results clearly suggest that such effects do exist (Supplementary Figure S8EF), and could explain substantial part of the effect of the *ibm1* mutation (Supplementary Figure S8GH). We added discussion about that.

Referee #3

Although we do not agree to some of the comments by the Referee #3, our manuscript has certainly been improved by responding to the comments by this referee.

This study presents in-depth analysis of the consequences of the loss of function of IBM1 and complements the elegant study by Miura et al 2009 by the same group, which reported that loss of IBM1 causes DNA hypermethylation on CHG primarily in genes. CHG methylation depends on its recognition by the SRA motif of KYP, which performs H3K9 methylation. Hence it was predicted that H3K9methylation would increase. The puzzling observation in the study by Miura et al was that genes and not transposons were primarily affected. In this study the authors provide some hypothesis to explain this phenomenon. It is based on the supposed H3K9 demethylase activity of IBM1. However, this is not directly demonstrated in the study and although the quality of the genome-wide analysis of chromatin marks is high, many of the conclusions drawn from the data obtained do not provide new concepts or are not compelling to argue for the models proposed.

Major points

1.The results shown in Fig1A are not convincing as they are produced using transient expression in tobacco cells. It is not clear to what level IBM1 is expressed in each tobacco cell and the signal is not quantified in a clear manner. The H3K9 demethylase activity should rather be properly assayed with the analysis of the amount of H3K9me, H3K9me2 and H3K9me3 isolated from transgenic lines that overexpress IBM1. Ideally the authors should consider attempting direct biochemical demonstration of IBM1 activity.

Our response:
We have made the results more rigorous and convincing by additional experiments. First, we quantified the loss of H3K9me2 and H3K9me1 in large number of cells in multiple slides. As shown in Supplementary Figure S1A, the quantification demonstrates that the effects are clear and reproducible. We also examined another negative control, mutated IBM1 construct, and confirmed that the mutated IBM1 did not affect H3Kme2 and H3K9me3 (Figure 1B, Supplementary Figure S1B). These have made the results more solid, together with examination of ten types of other modifications of histone H3, none of which changed in the cells expressing the IBM1 protein (supplementary Figure S1C-F). The transient assay is a standard method used very often to examine demethylase activity in vivo, and generally gives reproducible results. We added an example in the references (Seward et al 2007).

2. Data presented in Fig.2 is largely redundant with previously published data at isolated loci (eg Tariq et al., 2003) or genome-wide (Bernatavichute et al., 2008).

Our response:
Tariq et al showed that H3K9m reduces in *met1*, the CG methylase mutant. Because our results in Figure 2, effect of mutations in CMT3 (CHG methylase) and KYP (H3K9 methylase), show effects of different mutations mediating different modifications, we do not think our results are redundant with the results in Tariq et al. We do not think our results are redundant with that of Bernatavichute et al either. They showed that genomic distribution of CHG methylation and H3K9 methylation positively correlates in the wild type Arabidopsis; the causative relationship between the two epigenetic marks has not been examined. We examined the causative relationship using the

mutants, and actually our results in Figure 2, significant amount of H3K9m remaining in *kyp* and *cmt3* mutants, are rather unexpected from the simple prediction from the observation in Bernatavichute et al. In addition, it is in sharp contrast to the results in Figure 3 –the genic H3K9 methylation induced by the ibm1 were completely abolished in *kyp* or *cmt3* mutant. The main reason we put these results here is to compare them to the results in Figure 3.

3. Figure 3 and 5 shows that H3K9 increased methylation on gene in ibm1 background depends on kyp and cmt3, which is not surprising.

Our response:
We believe the results in Figure 3 are surprising, especially when compared to the results in Figure 2. The results in Figure 2 and 3 show that effects of *kyp* and *cmt3* mutations differ between genes and transposons.

When H3K9methylation activity is decreased , transposon are activated and the lack of IBM causes increased H3K9 methylation on transposon, which is not seen in a background with wild type H3K9 methyltransferase activity. From this observation the authors propose that transcriptional activity might be important for IBM1 function. However it is equally possible that IBM1 function become detectable only when loci carry H3K9me2 below a certain level, which is not the case of transposon in wt KYP/CMT3 background. Alternatively IBM1 activity might be inhibited by high levels of H3K9 methylation.

Our response:
To explain the results of Figure 5, the Referee #3 proposed the possibility that "IBM1 function become detectable only when loci carry H3K9me2 below a certain level". In order to directly see if the effects of *kyp* and *cmt3* mutations are mediated by reduction in H3K9m level itself, or it is mediated by transcriptional activation, we examined expression of these transposons in WT, *kyp* and *cmt3* plants genome-wide. The results clearly support the original interpretation; transposons with reduced H3K9me without transcriptional de-repression did not respond much to the *ibm1* mutation, while those with transcriptional de-repression responded robustly to the *ibm1* mutation (Supplementary Figure S9). We believe these new results have strengthened our proposal.

4. Fig4 shows more surprisingly that sensitivity to H3K9 hypermethylation in ibm1 background is correlated with a decrease of CG density (and as expected there is a positive correlation with increased CHG density). This suggests a competition between the DNA methylation pathways. The authors could also check whether this does not result from an anticorrelation between CG and CHG densities. As suggested by the authors this is reminiscent of the "rescuing" methylation on CHG and CHH observed in met1 mutants.

Our response:
The correlation between CG and CHG densities has been examined and shown in Supplementary Figure S8C in the original manuscript. As the correlation was positive (r=0.2324), the effect of CG density cannot be explained by indirect effect of CHG density. As this is an important point, we moved the results to Figure 4E. We also added more discussion about the possible explanation for the correlation between the *ibm1* effects and CG density with new analyses shown in Supplementary Figure S8E-I.

5. The authors show that CHG methylation increases on sites targeted by TDNA insertion. Although the authors do not show that the TDNA insert disrupts transcription and do not show that the increase of CHG is IBM1-dependent they conclude that IBM1 requires transcription to

demethylate H3K9 at these loci.

Our response:
The Referee #3 asked us to show the transcription results in T-DNA insertion lines. The transcription results have been shown in Supplementary Figure.S9E (Figure S10E in the revised version). We added quotation of these results in the Figure 6 legend, and described that transcription was disrupted in these mutants. The Referee#3 also asked us to show "that the increase of CHG is IBM1-dependent". It was not clear for us what this statement means, but if the reviewer meant to ask if absence of IBM1 function (or mutation in the IBM1 gene) can induce increase in CHG methylation, those results are included in Figure 6.

Many conclusions are drawn without statistical testing of hypotheses.

Our response:
Statistical testing would be necessary when interpretation of the results is not obvious. We feel most of the results in this manuscript are sufficiently clear even without statistics. For examples, t-test of results in Supplementary Figure S1A gives P<10-67 and P<10-20 for the reduction of H3K9me2 and H3K9me1, respectively. We feel the test is unnecessary for these types of results.

*Several "*Corresponding authors." Are indicated but only one email address is mentioned.*

Our response: We corrected the mistake. We thank the referee for pointing that out.

2nd Editorial Decision 10 August 2010

Your revised manuscript has been reviewed by one of the original referees who finds pending minor text changes the manuscript is ready for publication in The EMBO Journal.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

I did not have major concerns about the original manuscript, and the revision adequately addresses the few issues that I raised. A number of grammatical errors and inconsistencies in nomenclature (H3K9me vs. H3K9m) need to be corrected in the abstract, otherwise the manuscript is appropriate for publication.

2nd Revision - authors' response 12 August 2010

Thank you for handling our manuscript EMBOJ-2010-74405. We are glad to find that the Referee was very positive. According to comments by the Referee, we corrected grammatical errors and inconsistencies in nomenclature. Specifically, we found two “H3K9m” in the Abstract and changed them to “H3K9me”. We also corrected grammatical errors in the Abstract by incorporating opinion of a native English speaker.

We believe the manuscript is now suitable for publication in the EMBO Journal. Thank you again for your consideration.

