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DNA methylation in an intron of the IBM1 histone demethylase gene stabilizes chromatin modification patterns

Mélanie Rigal, Zoltan Kevei, Thierry Pélissier and Olivier Mathieu

Corresponding author: Olivier Mathieu, UMR 6293 CNRS

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(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.)

1st Editorial Decision 01 December 2011

Thank you once more for the submission of your manuscript to The EMBO Journal. I would like to apologize again for the delay in getting back to you. We have now received the full set of reports from the referees.

In addition to their comments, which you will find below, we have asked the referees to cross-comment on the other referees reports. As a result, they agree that two major issues with which you will need to deal prior to acceptance. First, as suggested below by referees #2 and #3, the mechanism by which DNA methylation regulates IBM-1 transcript accumulation is completely unclear and, second, referee #2 considers that the conclusions need to be strengthen by genome wide analyses of DNA methylation and H3K9me2.

I understand the considerable amount of effort and time needed to generate the experimental data required. Thus, although we normally would ask for a three-month revision time, I will be willing to extend this period in case additional time is needed. I would also like to remark that competing manuscripts published during the review/revision process will not negatively impact on our assessment of the conceptual novelty presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Please be aware that your revised manuscript must address the remaining referees' concerns - experimentally or not, as appropriate - and their suggestions should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also

remind you that it is 'The 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 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 iniciative, 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 REPORTS:

Referee #1:

This is an interesting publication that will be of wider interest. I have one major issue about the lack of locus-specific ChIP to address the proposed model, and I am a bit concerned about some rather blunt statements that should be modified.

The paper addresses an old enigma in plant DNA methylation research - the establishment of CNG methylation patterns in body methylation regions that have lost their CG methylation marks in a met1 mutant. The authors argue that MET1 or CG methylation established by MET1 controls alternative transcript production for IBM1, which is responsible for H3K9me/me2 demethylation and protection of body methylation regions from CNG methylation.

The authors show that the catalytically active splice variant IBM1-L is dominant in wildtype but absent in met1 and in a vim triple mutant (and in CNG specific mutants). They detect an unusual CG/CNG body methylation pattern in the large IBM1 intron, and a correlation between methylation at the intron and the presence of the IBM1-L transcript. Expression of a IBM1-L cDNA in met1 removes H3K9me2 from gene-rich euchromatic (based on immunocytological data), and removes CNG methylation from body methylation regions of some genes.

Major question:

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Other questions:

Figure 1B:
IBM1-S transcripts are still detectable in an ibm1 mutant. Is this a T-DNA readthrough effect or does the ibm1 mutant still produce truncated transcripts?

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The authors state that 'Together, these observations demonstrate that DNA methylation in the large intron of IBM1 is required for the proper accumulation of the IBM1-L transcript that encodes the jmjC domain'. This is a strong claim based on the observation of a correlation between CNG methylation and IBM1-1 transcript levels. The simple observation of two parameters occurring together does not justify claims that this demonstrates the dependence of one factor on the other. The authors have no control over other genomic changes induced by the reintroduction of CNG functions. At least, I would prefer to replace the term 'demonstrates' by 'indicates'.

Page 11:
The authors state that 'Of the three genes that were transcribed in the wild type, two (At2g24370 and At2g19520) were transcriptionally downregulated in met1, which suggests that ectopic CHG methylation in the body of these genes impairs transcription and does not compensate for the loss of CG methylation (Figure 5B).' Again a statement based on the simple correlation of two parameters. There is no evidence that the change in transcript levels is a consequence of changes in CNG methylation within the body of these genes.
The authors state that 'Together, these observations show that CHG body methylation impairs transcription at selected genes only in the absence of proper patterns of CG methylation'. Again, a speculative but potentially correct hypothesis is presented as conclusive.

Page 17:
The authors state that 'Here, we demonstrated that the transcription of a gene that encodes another demethylase, the IBM1 H3K9 demethylase, surprisingly also requires DNA methylation.' The term 'transcription' is misleading as this work shows an effect of MET1 on the accumulation of a transcript variant, which may be due to differential polyadenylation, transcript elongation, splicing or transcript stability but the authors do not investigate transcription.

The authors argue that IBM can target silent genes. How do they know if these genes are continuously silenced at all developmental stages? Do we know which developmental stage is decisive for the establishment of body methylation patters?

Referee #2:

In Arabidospsis thaliana, approximately one-third of genes have CG methylation in their coding region, which is maintained by MET1. Surprisingly, loss of gene body CG methylation in met1 mutant plants is often associated with a corresponding gain of CHG methylation as well as with a redistribution of H3K9me2 from heterochromatin to euchromatin in met1 plants. It was also shown that the IBM1 gene encodes a H3K9 demethylase that is required for the removal of CHG and H3K9me2 from the body of many genes and that there is a considerable overlap between IBM1 and MET1 target genes. The present manuscript reports evidence that the gain of CHG methylation over genes and the redistribution of H3K9me2 from heterochromatin to euchromatin seen in met1 results

from met1 being defective for IBM1 activity.

Specifically, the authors first confirm previous results of genome-wide analyses performed by others (Lister et al 2009; http://neomorph.salk.edu/epigenome/epigenome.html) indicating that the full-length IBM1 transcript is much reduced in met1 and replaced by an alternatively spliced, shorter transcript. This transcript stops in or very close to an intronic region that is heavily methylated at CG and CHG sites in wild type plants. The shorter IBM1 transcript does not encode the conserved jmjC domain, which in other histone demethylases was shown to be necessary for their activity. This suggests that met1 plants produce no or low levels of functional IBM1. Although this is peripheral, the authors also show that whereas accumulation of the longer transcript is unaffected in plants that are compromised in the RNA-dependent DNA methylation pathway, it is much reduced in mutants for the H3K9 methyltransferase gene KYP1 or the CHG maintenance methyltransferase gene CMT3, which control both H3K9 and CHG methylation. Given that unlike CG methylation, CHG methylation is limited to the region of IBM1 where the shorter transcript stops, the latter results point to methylation of this region being necessary for the production of the longer transcript.

To test whether reduced accumulation of the long IBM1 transcript (designated IBM1-L) could explain the gain of CHG methylation observed over genes and the redistribution of H3K9me2 from heterochromatin to euchromatin in met1, a construct containing a full-length, cDNA copy of IBM1 under its own promoter was introduced in met1 (and ibm1) mutant plants. In both ibm1::IBM1-L and met1::IBM1-L plants, near wild type H3K9m2 distribution patterns were observed in a large number of nuclei, demonstrating that the construct is functional and that the down-regulation of IBM1-L in met1 is responsible for much of the redistribution of H3K9me2 from heterochromatin to euchromatin. Similarly, in both ibm1::IBM1-L and met1::IBM1-L plants, near wild type methylation patterns were observed over the few genes (one for ibm1 and three for met1) that were examined. Additional experiments are presented, which assess transcription of three genes and three transposable elements in the different genetic backgrounds tested. However, no clear conclusion emerges from these limited analyses and the results are over-interpreted. Finally, it is shown that the two DNA demethylase genes DME and ROS1, which are silenced in met1 mutant plants, are reactivated in met1::IBM1-L plants.

In summary, this manuscript presents several interesting and important observations, which all point to the epigenetic control of IBM1 pre-mRNA alternative splicing being critical for the proper deposition of DNA and H3K9me2 methylation in Arabidopsis. However, in the absence of genome-wide analyses of DNA methylation by BS-Seq and of H3K9me2 by ChIP-chip or ChIP-Seq, it is not clear how general the conclusions reached by the authors are. Similarly, a genome-wide assessment of transcription is lacking.

Referee #3:

In this manuscript Rigal et al. study the relationship between DNA methylation status and expression and function of the IBM1 protein. They find that expression of functional IBM1 requires CG and CHG DNA methylation on a long intron within IBM1. They further show that in met1 mutant IBM1 is repressed which in turn causes CHG hypermethylation and repression of target genes.
Overall, this manuscript shows mostly high quality data, focuses on an interesting topic and provides an explanation for the intriguing phenomenon of DNA hypermethylation in DNA methyltransferase mutants. Unfortunately, this manuscript does not go far in explaining the molecular mechanism of its main finding - regulation of IBM1 by intronic DNA methylation.

Major criticism:
1. As mentioned above, this manuscript does not explain how DNA methylation affects IBM1 expression. It is unknown if DNA methylation affects alternative termination, alternative splicing or some other transcriptional or co-transcriptional events. Although some of those possibilities are mentioned in the discussion, lack of experimental data makes this manuscript incomplete.
2. The relationship between CHG methylation and H3K9me2 is only shown using cytology, which is insufficient, as IBM1 is a H3K9 demethylase. H3K9me2 should be tested on specific loci using ChIP in parallel to DNA methylation assays.

3. DNA methylation assays on IBM1 targets are missing an important control - the ibm1 mutant.

Minor criticism:
1. Fig. 1A would be more helpful if the position of T-DNA in ibm1-4 was indicated.
2. RT-PCR assays would be more convincing if visualized by real-time PCR instead of agarose electrophoresis. At least when no change is reported to be important for the conclusions and the possibility of PCR saturation has to be eliminated.
3. Analysis of genome-wide data mentioned in the Discussion as “unpublished" should be shown in the supplement.
4. The article lacks a summary model figure.
5. Both the introduction and discussion sections are long and written in a way more suitable for a review article.
6. The title refers to stablization of epigenetic patterns at genes, which is not well defined and therefore not too informative.

Author Correspondence 05 December 2011

Thank you for your message regarding the decision on our manuscript.

I am getting back to you as we are concerned about the request for genome-wide analyses by the referee #2. Indeed, performing genome-wide analysis of DNA methylation patterns by BS-seq is a rather challenging and delicate procedure that is currently mastered by only a few groups in the field. We are convinced that setting up this approach efficiently in our lab and then applying this technique to all the genotypes included in our manuscript would require a very long period of time that we think would be incompatible with a reasonable manuscript revision time.

We are currently using locus-specific bisulfite sequencing in order to extend our DNA methylation analyses to a significantly higher number of genomic targets. In parallel, as suggested by all three referees, we are addressing transcriptional activity and H3K9 methylation enrichment at these same target loci by ChIP. Although we are confident that these experimental data will considerably strengthen our previous conclusions, we will not reach the genome-wide level requested by referee #2.

Given that a revised version of a competing manuscript in currently under consideration, we would like to ask whether you consider the genome-wide analyses "sine qua non" for considering a revised version of our manuscript or whether a significant extension of our locus-specific analyses would represent an acceptable alternative. This, of course, provided that we address all other referee's concerns.

Editorial Correspondence 06 December 2011

I have contacted again referee #2 about the issue you raise in your e-mail.

S/he agrees with you that genome-wide studies can be technically challenging, and believes that your proposal of evaluating a limited number of loci is more feasible. However, s/he believes that a significant number of loci need to be evaluated:

"They need to look at more than a dozen of genes and as many transposable elements, chosen randomly among potential targets and non-targets".

I hope this clarifies the concerns of this referee.

I look forward to seeing the revised version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 29 March 2012

Referee #1:

This is an interesting publication that will be of wider interest. I have one major issue about the lack of locus-specific ChIP to address the proposed model, and I am a bit concerned about some rather blunt statements that should be modified.

The paper addresses an old enigma in plant DNA methylation research - the establishment of CNG methylation patterns in body methylation regions that have lost their CG methylation marks in a met1 mutant. The authors argue that MET1 or CG methylation established by MET1 controls alternative transcript production for IBM1, which is responsible for H3K9me/me2 demethylation and protection of body methylation regions from CNG methylation.

The authors show that the catalytically active splice variant IBM1-L is dominant in wildtype but absent in met1 and in a vim triple mutant (and in CNG specific mutants). They detect an unusual CG/CNG body methylation pattern in the large IBM1 intron, and a correlation between methylation at the intron and the presence of the IBM1-L transcript. Expression of a IBM1-L cDNA in met1 removes H3K9me2 from gene-rich euchromatic (based on immunocytological data), and removes CNG methylation from body methylation regions of some genes.

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In *met1* mutants, reduction in *IBM1-L* accumulation results in the establishment of CHG methylation and H3K9me2 at genes. In wild-type plants, gene-body DNA methylation is almost exclusively restricted to CG sites and the concomitant presence of CG and CHG methylation is a hallmark of repeated heterochromatic sequences and transposable elements, which are also enriched for H3K9me2. In this respect, the *IBM1* gene represents an interesting exception, as its large intron contains a region enriched in both types of DNA methylation. A previous genome-wide study from the Kakutani's group has shown that IBM1 does not target transposable elements (Inagaki et al, 2010); therefore, CHG methylation at these sequences is likely maintained owing to the absence of IBM1-mediated H3K9 demethylation and to the action of different H3K9 methyltransferases (KYP plus others). At the *IBM1* gene, we show here that CHG methylation is drastically reduced in a *kyp* H3K9 methyltransferase mutant (Figure 2); therefore, CHG methylation at this genomic region is not independent of H3K9me2 but strongly depends on KYP-mediated H3K9me2.

In the revised version of the manuscript, we have determined H3K9me2 enrichment at numerous transposable elements and genes, including *IBM1*, using chromatin immunoprecipitation of WT, *met1*, *met1::IBM1-L* and *ibm1* samples. This analysis revealed that IBM1 targets the DNA methylated *IBM1* intronic region for H3K9 demethylation. Therefore, the presence of high level of

CHG methylation at this genic region is likely a consequence of H3K9 methylation activity overriding IBM1-mediated demethylation. These new data are now included in the revised figures 5, 6 and 7 of the revised manuscript and commented page 11.

Other questions:

Figure 1B:

IBM1-S transcripts are still detectable in an ibm1 mutant. Is this a T-DNA readthrough effect or does the ibm1 mutant still produce truncated transcripts?

This is an interesting point. In our study, we used the *ibm1-4* T-DNA mutant allele (SALK_035608), which has been previously described in Saze et al, 2008. In this mutant line, the T-DNA is inserted in the 8th exon located right after the large intron of the *IBM1* gene; the position of the T-DNA is now indicated in the revised version of Figure 1. Our Northern blot and RT-PCR analyses indeed showed that the *IBM1-S* transcript accumulates to wild-type levels in *ibm1-4* (and in all other mutant backgrounds tested) while *IBM1-L* transcripts are undetectable. We are currently investigating the putative function of IBM1-S.

Page 6:

Why do the authors argue that inhibition of IBM1-L transcript production after 5-aza-dC application suggests involvement of non-CG methylation. As 5-aza-dC inhibits MET1, these date are in accordance with the already demonstrated role of MET1 and CG-methylation.

5-aza-dC is incorporated into DNA and is expected to lead to a rapid loss of all DNA methyltransferase activities since these enzymes become irreversibly bound to 5-aza-dC residues in DNA. Because the downregulation of *IBM1-L* accumulation in the *met1-3* null mutant (deficient for CG methylation) can be further enhanced when *met1* plants are treated with this drug, we had to consider that other types of DNA methyltransferases/DNA methylation are involved in the control of *IBM1-L* accumulation.

Page 7:

As DDM1 is not required for body methylation, why do the authors consider it surprising that DDM1 mutation does not alter IBM1 transcript levels?

The word “surprising” has been removed.

Page 8:

The phrase 'In the kyp mutant CG and CHH methylation remained largely intact' is misleading. There is no CHH methylation other than CHG in wildtype. It can therefore not remain intact.

We apologize for this confusing statement. CHH methylation levels are indeed very low (*likely in the range of bisulfite conversion errors)* both in WT and *kyp* plants; therefore we have removed this statement about CHH methylation in the revised manuscript.

The lengthy discussion of SUPERMAN (SUP) and BONSAI (BNS) should be moved to the discussion section.

As this paragraph was not essential to the manuscript, we have rather decided to remove it from the revised version.

Page 9:

The authors state that 'Together, these observations demonstrate that DNA methylation in the large intron of IBM1 is required for the proper accumulation of the IBM1-L transcript that encodes the jmjC domain'. This is a strong claim based on the observation of a correlation between CNG methylation and IBM1-1 transcript levels. The simple observation of two parameters occurring together does not justify claims that this demonstrates the dependence of one factor on the other. The authors have no control over other genomic changes induced by the reintroduction of CNG functions. At least, I would prefer to replace the term 'demonstrates' by 'indicates'.

It is a valid point and we have replaced ‘demonstrates’ by ‘indicates’ in this sentence.

Page 11:

The authors state that 'Of the three genes that were transcribed in the wild type, two (At2g24370 and At2g19520) were transcriptionally downregulated in met1, which suggests that ectopic CHG methylation in the body of these genes impairs transcription and does not compensate for the loss of CG methylation (Figure 5B).' Again a statement based on the simple correlation of two parameters. There is no evidence that the change in transcript levels is a consequence of changes in CNG methylation within the body of these genes.

The authors state that 'Together, these observations show that CHG body methylation impairs transcription at selected genes only in the absence of proper patterns of CG methylation'. Again, a speculative but potentially correct hypothesis is presented as conclusive.

The Reviewer is totally right. Because we provide numerous additional experimental data in the revised version of the manuscript, these sections have been extensively modified. We have been particularly careful in rewording these sentences in order to better convey the speculative nature of our conclusions.

Page 17:

The authors state that 'Here, we demonstrated that the transcription of a gene that encodes another demethylase, the IBM1 H3K9 demethylase, surprisingly also requires DNA methylation.' The term 'transcription' is misleading as this work shows an effect of MET1 on the accumulation of a transcript variant, which may be due to differential polyadenylation, transcript elongation, splicing or transcript stability but the authors do not investigate transcription.

We agree with this point and have modified this sentence by replacing “transcription” with “proper expression”. We also have extended our analysis of *IBM1* transcription using additional sets of primers along the large intron of *IBM1* in order to compare accumulation of the *IBM1* pre-mRNA in WT and *met1* plants. This confirms that the drop in *IBM1* pre-mRNA transcript accumulation in *met1* occurs inside the intronic region that is heavily methylated in the wild type. The *IBM1* pre-mRNA upstream of the methylated region accumulates to similar levels in the WT and in *met1*, suggesting that pre-mRNA transcript elongation is impaired in the *met1* mutant. Additionally, we did not detect stable differentially polyadenylated *IBM1* transcript variant in the *met1* and other mutants by Northern blot. Although this favors the hypothesis that DNA methylation influences transcript elongation in the *IBM1* intronic region, we believe that numerous additional studies will be necessary to precisely and completely elucidate the underlying molecular mechanism by which CG/CHG methylation favors *IBM1-L* pre-mRNA accumulation.

The authors argue that IBM can target silent genes. How do they know if these genes are continuously silenced at all developmental stages? Do we know which developmental stage is decisive for the establishment of body methylation patters?

This is a good point from the Reviewer and there is to date no experimental data to confidently answer these questions. Therefore, we have modified all statements about targeting of IBM1 to silent loci in the revised version of the manuscript (e.g. page 13, line 3).

Referee #2:

In Arabidospsis thaliana, approximately one-third of genes have CG methylation in their coding region, which is maintained by MET1. Surprisingly, loss of gene body CG methylation in met1 mutant plants is often associated with a corresponding gain of CHG methylation as well as with a redistribution of H3K9me2 from heterochromatin to euchromatin in met1 plants. It was also shown that the IBM1 gene encodes a H3K9 demethylase that is required for the removal of CHG and H3K9me2 from the body of many genes and that there is a considerable overlap between IBM1 and MET1 target genes. The present manuscript reports evidence that the gain of CHG methylation over genes and the redistribution of H3K9me2 from heterochromatin to euchromatin seen in met1 results from met1 being defective for IBM1 activity.

Specifically, the authors first confirm previous results of genome-wide analyses performed by others (Lister et al 2009; http://neomorph.salk.edu/epigenome/epigenome.html) indicating that the full-length IBM1 transcript is much reduced in met1 and replaced by an alternatively spliced, shorter transcript. This transcript stops in or very close to an intronic region that is heavily methylated at CG and CHG sites in wild type plants. The shorter IBM1 transcript does not encode the conserved jmjC domain, which in other histone demethylases was shown to be necessary for their activity. This suggests that met1 plants produce no or low levels of functional IBM1. Although this is peripheral, the authors also show that whereas accumulation of the longer transcript is unaffected in plants that are compromised in the RNA-dependent DNA methylation pathway, it is much reduced in mutants for the H3K9 methyltransferase gene KYP1 or the CHG maintenance methyltransferase gene CMT3, which control both H3K9 and CHG methylation. Given that unlike CG methylation, CHG methylation is limited to the region of IBM1 where the shorter transcript stops, the latter results point to methylation of this region being necessary for the production of the longer transcript.

To test whether reduced accumulation of the long IBM1 transcript (designated IBM1-L) could explain the gain of CHG methylation observed over genes and the redistribution of H3K9me2 from heterochromatin to euchromatin in met1, a construct containing a full-length, cDNA copy of IBM1 under its own promoter was introduced in met1 (and ibm1) mutant plants. In both ibm1::IBM1-L and met1::IBM1-L plants, near wild type H3K9m2 distribution patterns were observed in a large number of nuclei, demonstrating that the construct is functional and that the down-regulation of IBM1-L in met1 is responsible for much of the redistribution of H3K9me2 from heterochromatin to euchromatin. Similarly, in both ibm1::IBM1-L and met1::IBM1-L plants, near wild type methylation patterns were observed over the few genes (one for ibm1 and three for met1) that were examined. Additional experiments are presented, which assess transcription of three genes and three transposable elements in the different genetic backgrounds tested. However, no clear conclusion emerges from these limited analyses and the results are over-interpreted. Finally, it is shown that the two DNA demethylase genes DME and ROS1, which are silenced in met1 mutant plants, are reactivated in met1::IBM1-L plants.

In summary, this manuscript presents several interesting and important observations, which all point to the epigenetic control of IBM1 pre-mRNA alternative splicing being critical for the proper deposition of DNA and H3K9me2 methylation in Arabidopsis. However, in the absence of genome-wide analyses of DNA methylation by BS-Seq and of H3K9me2 by ChIP-chip or ChIP-Seq, it is not clear how general the conclusions reached by the authors are. Similarly, a genome-wide assessment of transcription is lacking.

We have discussed this point with the editor, and have significantly increased the number of genomic targets analyzed in the revised version of the manuscript. We now provide DNA methylation patterns analyses at a total of 13 protein-coding genes as well as at 10 transposable elements. Additionally, we have used chromatin immunoprecipitation assays to determine H3K9me2 enrichment at all these genomic loci and we have assessed their transcriptional status by RT-PCR.

These new additional experimental data, which are included in the revised figures 5, 6 and 7, strongly support our conclusion that control of *IBM1-L* accumulation by DNA methylation is critical

for protecting genes from ectopic non-CG and H3K9 methylation.

Referee #3:

In this manuscript Rigal et al. study the relationship between DNA methylation status and expression and function of the IBM1 protein. They find that expression of functional IBM1 requires CG and CHG DNA methylation on a long intron within IBM1. They further show that in met1 mutant IBM1 is repressed which in turn causes CHG hypermethylation and repression of target genes.

Overall, this manuscript shows mostly high quality data, focuses on an interesting topic and provides an explanation for the intriguing phenomenon of DNA hypermethylation in DNA methyltransferase mutants. Unfortunately, this manuscript does not go far in explaining the molecular mechanism of its main finding - regulation of IBM1 by intronic DNA methylation.

Major criticism:

1. As mentioned above, this manuscript does not explain how DNA methylation affects IBM1 expression. It is unknown if DNA methylation affects alternative termination, alternative splicing or some other transcriptional or co-transcriptional events. Although some of those possibilities are mentioned in the discussion, lack of experimental data makes this manuscript incomplete.

We have extended our analysis of *IBM1* transcription using additional sets of primers along the large intron of *IBM1* in order to compare accumulation of the *IBM1* pre-mRNA in WT and *met1* plants. This confirms that the decrease in *IBM1* pre-mRNA transcript accumulation in *met1* occurs inside the intronic region that is heavily methylated in the wild type. The *IBM1* pre-mRNA upstream of the methylated region accumulates to similar levels in the WT and in *met1*, suggesting that pre-mRNA transcript elongation is impaired in the *met1* mutant. Additionally, Northern blot assays did not reveal differentially polyadenylated *IBM1* transcript variant in the *met1* and other mutants analyzed. Although this favors the hypothesis that DNA methylation influences transcript elongation in the *IBM1* intronic region, we believe that numerous additional studies will be necessary to precisely and completely elucidate the underlying molecular mechanism by which CG/CHG methylation favors *IBM1-L* pre-mRNA accumulation.

However, we do not feel that this makes our manuscript incomplete. Our results provide the first report of a role for intronic DNA methylation in controlling accumulation of a transcript variant in Arabidopsis, and further highlight the critical role played by this original type of regulation in controlling epigenetic stability. Additionally, another equally important part of our findings is the molecular explanation for the establishment of CHG/H3K9 methylation patterns in gene bodies that have lost their CG methylation marks in a *met1* mutant. So far, this has remained as a long-standing enigma in the plant DNA methylation research field.

2. The relationship between CHG methylation and H3K9me2 is only shown using cytology, which is insufficient, as IBM1 is a H3K9 demethylase. H3K9me2 should be tested on specific loci using ChIP in parallel to DNA methylation assays.

In addition to extending our previous DNA methylation and transcription analyses to a total of 13 genes and 10 transposable elements, we have assayed H3K9me2 enrichment at all these genomic targets using chromatin immunoprecipitation as requested. These data support or previous conclusions and are now included in the revised versions of figures 5, 6 and 7.

3. DNA methylation assays on IBM1 targets are missing an important control - the ibm1 mutant.

Upon Reviewer's request, DNA methylation patterns (as well as H3K9me2 enrichment) in the *ibm1* mutant have been determined at all genomic targets analyzed in this study. These data are now

included in the revised versions of figures 5, 6 and 7.

Minor criticism:

1. Fig. 1A would be more helpful if the position of T-DNA in ibm1-4 was indicated.

Figure 1A has been modified accordingly.

2. RT-PCR assays would be more convincing if visualized by real-time PCR instead of agarose electrophoresis. At least when no change is reported to be important for the conclusions and the possibility of PCR saturation has to be eliminated.

We have performed again all RT-PCR analyses not supported by northern blot assays using much less PCR cycles to minimize PCR saturation possibility. This reveals the same transcriptional patterns. However, because slight variations, which could only be detected by real-time PCR, cannot be excluded, we have reworded all our conclusions regarding the absence of changes in order to clearly indicate that we refer to the absence of "detectable" variation.

3. Analysis of genome-wide data mentioned in the Discussion as "unpublished" should be shown in the supplement.

This analysis is now included as a new supplemental figure (supplemental Figure S8).

4. The article lacks a summary model figure.

A model figure has been added in the revised manuscript (Figure 8).

5. Both the introduction and discussion sections are long and written in a way more suitable for a review article.

Both sections have been reworded and shortened as requested.

6. The title refers to stablization of epigenetic patterns at genes, which is not well defined and therefore not too informative.

Because of the constraints with the total length of the title, we would prefer to keep the title unchanged.

2nd Editorial Decision 18 April 2012

Thank you for the submission of your revised manuscript to The EMBO Journal. It has been sent back to two of the original reviewers, who now consider that their major concerns have been properly addressed and your manuscript is almost ready for publication.

Nevertheless, as you will see below, referee #2 still points out to some minor issues that need your attention before your manuscript can be accepted. Following his/her suggestion, I would like to propose the following title for the paper: "MET1-dependent DNA methylation in a conserved intron of the IBM1 histone demethylase gene stabilizes chromatin modification patterns".

Browsing through the manuscript myself I have noticed minor problems with the description of your statistical analyses. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed. In this case, the number of independent experiments performed in figures 2, 5A, 6A and 7B is not stated. For figures 2, 5A and 6A it is enough to mention whether only one experiment was performed of the result shown is

representative of n experiments. However, for figure 7B, if the number of independent experiments is less than 3, the use of error bars is not appropriate.

I would also like to mention that we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or just the key gels presented? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much again for your contribution to The EMBO Journal and congratulations in advance on a successful publication. Once you send us the final revised version, you will receive an official acceptance letter with further instructions on how to proceed with the publication process.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #2:

The revised manuscript is much improved and should be of broad interest.

Referee #3:

The manuscript has been significantly improved and I am satisfied with most of the changes. The notable exception is lack of successful explanation of the mechanism by which DNA methylation affects IBM1. Although I accept the authors' reasoning that explanation of this mechanism could be beyond the scope of this manuscript, this loose end significantly decreases my enthusiasm for this paper.

There are a few minor issues that still need fixing:
1. In Fig. 4 panels A-C use IBM1 driven by its native promoter and panels D-E use IBM1 driven by MET1 promoter. This should be explained.
2. The new model figure is very complex, which makes it not too helpful.
3. On page 9 the paragraph about no alternative polyadenylation detected by northern blot is not clear.
4. The title should be changed to eliminate the phrase “epigenetic patterns”, which I find ambiguous and confusing. I would suggest ”patterns of chromatin modifications” instead.

2nd Revision - Authors' Response 19 April 2012

Dear Editor.

Please find enclosed the revised version of our manuscript #EMBOJ-2011-79825R and our answers to the referee’s remarks.

Following your and referee’s request, we have modified the title of the manuscript for:

“DNA methylation in an intron of the IBM1 histone demethylase gene stabilizes chromatin

modification patterns".

We have also carefully considered your point about statistical analyses and provide the information about the number of independent experiments and the definition of the error bars used in the Material and Methods section and in the legend of the figures (figures 5B, 6B, 7B). In the case of bisulphite sequencing data shown in figures 2, 5A and 6A, the graphs represent the methylation percentage calculated from 8-12 individual clones (as mentioned in the material and methods).

Point-by-Point-Response:

Referee #3:

The manuscript has been significantly improved and I am satisfied with most of the changes. The notable exception is lack of successful explanation of the mechanism by which DNA methylation affects IBM1. Although I accept the authors' reasoning that explanation of this mechanism could be beyond the scope of this manuscript, this loose end significantly decreases my enthusiasm for this paper.

There are a few minor issues that still need fixing:

1. In Fig. 4 panels A-C use IBM1 driven by its native promoter and panels D-E use IBM1 driven by MET1 promoter. This should be explained.

There is a misunderstanding here. As mentioned in the manuscript, we used a single construct that is IBM1-L cDNA driven by its native promoter *(pIBM1:IBM1-L)*.

As explained in the main text page 9, *ibm1::IBM1-L* (figure 4 panels A-C) and *met1::IBM1-L* (figure 4 panels D-E) respectively refer to *ibm1* and *met1* mutant plants that are transformed with the same *pIBM1:IBM1-L* construct.

2. The new model figure is very complex, which makes it not too helpful.

We would prefer to keep this figure unchanged.

3. On page 9 the paragraph about no alternative polyadenylation detected by northern blot is not clear.

As mentioned in the Material and Methods section of the manuscript, the northern blot was performed using poly(A)+ RNA samples; this is now also indicated in this paragraph.

4. The title should be changed to eliminate the phrase "epigenetic patterns", which I find ambiguous and confusing. I would suggest "patterns of chromatin modifications" instead.

The title has been modified accordingly.