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Mobilization of a plant transposon by expression of the transposon-encoded anti-silencing factor

Yu Fu, Akira Kawabe, Mathilde Etcheverry, Tasuku Ito, Atsushi Toyoda, Asao Fujiyama, Vincent Colot, Yoshiaki Tarutani, Tetsuji Kakutani

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Editor: Anke Sparmann

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Thank you for submitting your research manuscript entitled "Anti-silencing and mobilization of a Mutator-like element without terminal inverted repeats" (EMBOJ-2013-85064) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. However, they do raise a number of concerns that need to be addressed prior to publication, especially since reviewer #3 points to a rather preliminary character of your study.

Nevertheless, given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that attends to the raised criticisms in full. Please keep in mind that our title caters to a broad research community and that the significance of your study should be assessable to readers not immediately connected to the field.

I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me should any particular argument require further clarification.

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, 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!

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REFEREE REPORTS:

Referee #1

Summary
The authors report about a VANDAL21 transposon that is able to mobilize in ddm1 mutants. Despite the fact that the terminal inverted long repeats have degenerated, they can observe transposition of the original copy at predicted terminal sites. Integration events are often asymmetrical and occur preferentially in the 5' promoter of the gene. When a transgenic Hi is introduced into wild type plants, it is able to induce demethylation at endogenous Hi loci and activate transposition, suggesting a mechanism of trans-activation. The authors do deletion analysis to identify each ORF's contributions to Hi mobilization and find that HiC is indispensible for a complete demethylation of Hi.

Positive experiments:

Authors present an interesting result where presence of a Hi transgene is sufficient to promote endogenous element mobilization. They correlate an increase in Hi copy number to a decrease in methylation at the original Hi loci. This methylation is reestablished in progeny that lose the transgene. The segregation experiment shows clearly that the introduction of an expressing copy of the Hi is sufficient to promote mobilization. Figure 7C is also a good proof of concept: in plants carrying a copy of the HiC gene only, you see transposition of Hi. This can be segregated away, and plants that have lost the transgene have also lost Hi transposition activity and non-CG methylation at Hi is stored. In general, these results are highly interesting and appropriate for publication in EMBO.

I suggest that the authors address the following points either in discussion or in added data.

HiB and/or HiC allow for demethylation of endogenous Hi, and show that in the absence of A or B from the transgene that endogenous HiA and HiB expression is upregulated. Is demethylation sufficient for active transcription of Hi or is HiC acting to recruit transcription machinery? Are there siRNAs still being made towards the Hi locus?

3B: Would it be more meaningful to have more samples for the methylation data?

Can you try silence the transgene in the T2/T2+ progeny? Would you expect remethylation to occur within the same generation or in the next generation?

Figure 4: Do you think that Hi replication (and therefore overall copy number) is controlled by any of these gene products? Can you check copy # in your WT plants that were transformed with transgenes missing Hi gene ORFs?

In the transgene/ORF deletion experiments, is the asymmetry of any Hi integration compromised?

Referee #2

The manuscript by Fu et al describes the remobilization of an endogenous DNA transposon (Hi) upon expression of a transgenic copy in Arabidopsis wild type plants. Interestingly, the expression of a single Hi-encoded gene (HiC) that differs from the transposase is sufficient to trigger Hi somatic mobility. The expression of the HiC transgene is correlated with a loss of DNA methylation of the endogenous Hi locus, thus releasing silencing and leading to excision of the endogenous Hi transposon. This very interesting observation is supported by clear evidence and provides a possible functional role for the HiC protein in promoting Hi mobility. Furthermore it illustrates how a single neocopy of a transposon could generate a TE burst by initiating a positive feedback loop.

Main comments

1. Did the authors analyze the excision of the Hi transgenic copy itself?

2. The Hi mobility assays are based on nested PCR detecting an empty donor site. In these assays a loading control is missing (Fig 2B, 3A, 6B). The authors mention that these assays reveal somatic mobility, however for clarity it could be nice to show the PCR product at the full donor site (as shown in 4D).

3. Page 9 Line 3. The Figure 3A shows Hi excision for 14 transgenic plants (16 in the text), are these T1? Are the 8 Hi transgene-free progenies shown?

4. Figure 5: Does the McrBC assay report on methylation at the original locus (not obvious from Figure 1)? In this epiRIL experiment Hi mobility is detected in half of the wt-originating Hi lines. Was this expected for this population?

5. Page 12 line 10. The authors conclude that HiC plays a key role in Hi mobility. However in the transgenic plants lacking the HiC transgene Hi mobility is also detected (as mentioned in the discussion). The Figure S5 shows important evidence relative to the functional role of HiC in guiding precise excision and should be included in the main Figures.

6. Figure S4 and S6: could the authors clarify if the transcript detection is specific for the endogenous or transgenic locus?

Minor points

7. Page 3. In the introduction the authors mention examples of TE that could "counteract" DNA methylation. Of note, in snapdragon the temperature dependent fixation of the Tam3 transposase to the corresponding genomic TE locus seems to prevent its DNA methylation (Hashida et al, 2006). In the case of Hi the proposed mechanism is independent of the transposase, yet the direct binding of HiC to the Hi locus could explain the effect on DNA methylation. Could the authors comment on this?

8. Page 4: Please note that not all class II TEs have long TIRs (Wicker et al., 2007, Nat Rev Genetics).

9. Please correct a few typos (e.g. p3 L17, p9 L14, p12 L18, p25 L10).

Referee #3

In this manuscript "Anti-silencing and mobilization of a Mutator-like element without terminal inverted repeats" by Fu et al., the activity of a Vandal-class transposable element is investigated in the model plant Arabidopsis. The data in this manuscript demonstrates that this silenced element can be activated in a ddm1 mutant that undergoes loss of control of heterochromatin and transcriptional silencing. More importantly, the endogenous element can be trans-activated and trans-de-methylated by a transgenic TE copy. The authors perform a deletion analysis of this transposable element and demonstrate that one of the three proteins is specifically responsible for the trans-de-methylation of silenced copies, and this protein is not the transposase. The authors argue that this protein has likely been acquired during plant evolution and now is associated with successful element activity.

The science is this manuscript is sound and well controlled. I have specific minor comments that I have made below. However, my major comments are:

1. This manuscript does not go far enough in the analysis of the HiC protein to warrant publication in a journal as good as EMBO. This manuscript leaves very easy to test experiments on the table. These experiments need to be completed.
a. Does HiC act as a general silencing suppressor? Test if other TEs or endogenous sequences undergo de-methylation in the plant with transgenic HiC.
b. Are plants expressing a HiC transgene hyper-sensitive to viral infection? I am reminded of the following paper that demonstrated that a virus was carrying a protein that interferes with DNA and histone methylation: Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM (2009)

Geminivirus AL2 and L2 Proteins Suppress Transcriptional Gene Silencing and Cause Genome-Wide Reductions in Cytosine Methylation. Journal of Virology 83: 4717-4717
c. Do HiA and HiC interact, for example in a yeast 2 hybrid assay?
d. When integrating these experiments, I think much of the first few sections of the Results (Identification, Structure, Integration & Excision in ddm1), can all be very much condensed into one Figure / section with one supplemental figure. This paper got off to a very slow start, and I think all of this can be greatly condensed.
2. I don't understand why the authors focus so much on the fact that this element has no TIRs. In fact, this point is made throughout the text, and is even in the title. Supplemental Figure 1A shows that this element does indeed have TIRs, they are just highly degenerated and short. I have no problem with the fact that these elements have short degraded TIRs, but the language of them having NO TIRs should be removed from throughout the manuscript and title. I didn't see what this added to the main story, which was all about HiC.
3. I dislike the data presentation in Figure 8 and 9. In Figure 8A, if the authors are arguing that Vandal elements are only successful when they acquire HiC or the Ulp1 protease, then there must be a more statistical way to demonstrate that, rather than just showing a tree. Please use numbers and statistics to make this argument, rather than a picture. In fact, my dislike of Figure 8 and 9 both have to do with the fact that the authors are using pictures to demonstrate large amounts of data. I think its OK for Figure 8B, but for Figure 8A and Figure 9, I don't see what ever it is that the authors want me to see. I don't think the data or the analysis is bad, I just think the display of the information is poor. For example, in Figure 8A, could you calculate the number of elements/copy number per family for Vandal families that have the ULP1 protease, DUF287 or neither, and then create 3 box plots to demonstrate that the element families that integrate these protein domains are more evolutionarily successful?

Other Comments:
1. Does the name "Hi" refer to one element at one locus, the TE family, or the group of just very closely related elements? Please be clear on the definition of the nomenclature.
2. In the last line of the Results section, the authors state that HiC plays a role in the trans-activation and mobilization of Hi. I agree with trans-activation. However, I see mobilization as an indirect consequence of the trans-activation, and I don't see evidence for HiC playing a direct role in mobilization. I would remove this claim.
3. Its been a long time since I've seen so many agarose gels in a paper. Perhaps instead of showing a band as a binary measure of excision, the authors could perform qPCR and show a quantitative measure of excision frequency. The same is true for the expression analysis in Figure 6-7.
4. I felt that the explanation of Figures 8 & 9 belong in the Results section, and not in the Discussion. I suggest moving what you have written into a new Results section topic of "HiC evolution", and discuss your analysis there, instead of the discussion section.
5. In Figure 4A, why does ddm1 have a low copy number? I though Hi transposes in ddm1 mutants (Figure 2). Please elaborate on the reason behind this discrepancy. Does HiC excise in ddm1, but not re-integrate?
6. The Figure legend of Figure 6 describes a molecular weight marker, but none is seen in the Figure.

1st Revision - authors' response 20 June 2013

Referee #1
This referee was very positive and regards the results “highly interesting and appropriate for publication in EMBO.” We thank the referee for the following specific comments, which helped us to improve the manuscript.

Summary
The authors report about a VANDAL21 transposon that is able to mobilize in ddm1 mutants. Despite the fact that the terminal inverted long repeats have degenerated, they can observe transposition of the original copy at predicted terminal sites. Integration events are often asymmetrical and occur preferentially in the 5' promoter of the gene. When a transgenic Hi is

introduced into wild type plants, it is able to induce demethylation at endogenous Hi loci and activate transposition, suggesting a mechanism of trans-activation. The authors do deletion analysis to identify each ORF's contributions to Hi mobilization and find that HiC is indispensible for a complete demethylation of Hi.

Positive experiments:

Authors present an interesting result where presence of a Hi transgene is sufficient to promote endogenous element mobilization. They correlate an increase in Hi copy number to a decrease in methylation at the original Hi loci. This methylation is reestablished in progeny that lose the transgene. The segregation experiment shows clearly that the introduction of an expressing copy of the Hi is sufficient to promote mobilization. Figure 7C is also a good proof of concept: in plants carrying a copy of the HiC gene only, you see transposition of Hi. This can be segregated away, and plants that have lost the transgene have also lost Hi transposition activity and non-CG methylation at Hi is stored. In general, these results are highly interesting and appropriate for publication in EMBO.

I suggest that the authors address the following points either in discussion or in added data.

HiB and/or HiC allow for demethylation of endogenous Hi, and show that in the absence of A or B from the transgene that endogenous HiA and HiB expression is upregulated. Is demethylation sufficient for active transcription of Hi or is HiC acting to recruit transcription machinery? Are there siRNAs still being made towards the Hi locus?

(Our response) Hi was activated by the DNA hypomethylation mutation *ddm1* (*decrease in DNA methylation*), but the *DDM1* gene encodes a chromatin remodeling factor, rather than a DNA methyltransferase. In order to directly test the role of DNA methylation in controlling Hi, we examined Hi mobility in mutants of DNA methyltransferases (new Supplementary Figure S15). Mutation in the maintenance (CpG) DNA methyltransferase MET1 induced mobilization of Hi, indicating that DNA methylation at CpG sites is necessary for the silencing. On the other hand, a triple mutant defective in the non-CpG methyltransferases DRM1, DRM2, and CMT3 did not show mobilization of Hi. Mutations in components of RdDM (RNA-directed DNA methylation) machinery did not induce mobilization either. Therefore, loss of non-CpG methylation in terminal regions does not seem to be sufficient for mobilizing Hi. Still, the possibility remains that non-CpG methylation in internal region plays a role, because, according to a recent report (Zemack et al Cell 153, 193-), another non-CpG DNA methyltransferase, named CMT2, plays a major role in methylation at CpHpH sites of internal regions of TEs. This may be important, because our new results (new Figure 9; new Supplementary Figure S14) show that Hi affects not only terminal regions but also internal regions of TEs. We added the results for the Hi mobility and a brief discussion in new Supplementary Figure S15.

3B: Would it be more meaningful to have more samples for the methylation data?

(Our response) In order to see the remethylation, we examined one more plant sample for T2/TG- and also examined TG+ and TG- plants in the T3 generation (new Supplementary Figure S7). All the results are consistent. Loss of transgene caused remethylation. Effect of transgene was also examined independently for Figure 4, which show reduced DNA methylation at comparable level. We examined more than eleven reads for each plant. We believe the results are highly reproducible and consistent.

Can you try silence the transgene in the T2/T2+ progeny? Would you expect remethylation to occur within the same generation or in the next generation?

(Our response) The timing of remethylation is a very interesting question, but it is difficult to examine. Currently we are setting up experiment to see if remethylation depends on RdDM machinery, but we feel that the topic is beyond the scope of the present study.

Figure 4: Do you think that Hi replication (and therefore overall copy number) is controlled by any of these gene products? Can you check copy # in your WT plants that were transformed with transgenes missing Hi gene ORFs?

(Our response) We tried Southern analysis of full-length Hi transgenic lines, but the increase in Hi copy number was undetectable. Genome re-sequencing would be more sensitive assay, if it were deep enough. We would like to examine that in future using full length and deletion constructs, when the deep sequencing becomes less expensive. We examined excision products in 16 independent Δ*hiC*-TG lines. That was added as new Supplementary Figure S3C.

In the transgene/ORF deletion experiments, is the asymmetry of any Hi integration compromised?

(Our response) That would also be an interesting question, especially because trasnposases are generally believed to function in dimer or other types of symmetric multimer. As stated above, that could also be examined by deep sequencing in future.

Referee #2
This referee was also very positive. We thank the referee for his/her comments, which helped us to improve the manuscript.

The manuscript by Fu et al describes the remobilization of an endogenous DNA transposon (Hi) upon expression of a transgenic copy in Arabidopsis wild type plants. Interestingly, the expression of a single Hi-encoded gene (HiC) that differs from the transposase is sufficient to trigger Hi somatic mobility. The expression of the HiC transgene is correlated with a loss of DNA methylation of the endogenous Hi locus, thus releasing silencing and leading to excision of the endogenous Hi transposon. This very interesting observation is supported by clear evidence and provides a possible functional role for the HiC protein in promoting Hi mobility. Furthermore it illustrates how a single neocopy of a transposon could generate a TE burst by initiating a positive feedback loop.

Main comments

1. Did the authors analyze the excision of the Hi transgenic copy itself?

(Our response) We examined excision of Hi transgene itself using primers flanking the Hi sequence in the transgene. Excision was detected in many, although not all, of the transgenic lines. The results were added to a new Supplementary Figure S5.

2. The Hi mobility assays are based on nested PCR detecting an empty donor site. In these assays a loading control is missing (Fig 2B, 3A, 6B). The authors mention that these assays reveal somatic mobility, however for clarity it could be nice to show the PCR product at the full donor site (as shown in 4D).

(Our response) In Southern analyses of the self-pollinated *ddm1* mutant, every band for the original copies remained, suggesting that the empty locus was not fixed. In addition, in deep sequencing, we could detect reads corresponding to Hi termini of the original locus. We added the results to footnote of Supplementary Table S1. These results indicate that the empty locus was not fixed in most of independent *ddm1* lines even after repeated self-pollinations. For most of the reads corresponding to new integration, read number was much less than the average coverage of the reads (Supplementary Table S1), again suggesting that the transposition was somatic. We added PCR results to detect full donor site in the experiment for new Supplementary Figs S6 and S9.

3. Page 9 Line 3. The Figure 3A shows Hi excision for 14 transgenic plants (16 in the text), are these T1? Are the 8 Hi transgene-free progenies shown?

(Our response) Figure 3A shows results for T1 plants of 14 independent transgenic lines, as described in the bottom of page 8 (in previous version) as "In all of multiple transgenic lines examined, the transgene induced somatic excision of the original copy (Figure 3A)." The results of T2 plants have not been shown in the previous manuscript. We repeated the experiment for T2 with the remaining DNA samples and the results were essentially the same. The results for the T2 family were added as a new Supplementary Fig S6.

4. Figure 5: Does the McrBC assay report on methylation at the original locus (not obvious from Figure 1)? In this epiRIL experiment Hi mobility is detected in half of the wt-originating Hi lines. Was this expected for this population?

(Our response) The McrBC assay relies on primers that only amplify the original Hi locus. The region analysed (L2) is now properly positioned in the revised Figure 1A. Proportion of epiRILs with the trans-acting active Hi would be determined by both Hi mobility and segregation. Therefore, the proportion does not contradict our expectation.

5. Page 12 line 10. The authors conclude that HiC plays a key role in Hi mobility. However in the transgenic plants lacking the HiC transgene Hi mobility is also detected (as mentioned in the discussion). The Figure S5 shows important evidence relative to the functional role of HiC in guiding precise excision and should be included in the main Figures.

(Our response) As suggested by this Referee, we moved the results in the previous Figure S5 to the main Figure. In addition, we repeated the experiments with additional Δ*hiC*-TG lines (new Supplementary Figure S9). The results indicate that HiC is not essential for the excision but can affect excision efficiency.

6. Figure S4 and S6: could the authors clarify if the transcript detection is specific for the endogenous or transgenic locus?

(Our response) For transcripts shown in Figure S4, we examined their origin using sequence polymorphisms in the Hi ORFs between the endogenous copy and the transgene. The results indicate that transcripts form endogenous copies are undetectably low amount compared to those from the transgene. That is consistent with the results shown in Fig S6, indicating that transcript levels from Hi transgene are much higher. The results were added as new Supplementary Figure S4B.

Minor points

7. Page 3. In the introduction the authors mention examples of TE that could "counteract" DNA methylation. Of note, in snapdragon the temperature dependent fixation of the Tam3 transposase to the corresponding genomic TE locus seems to prevent its DNA methylation (Hashida et al, 2006). In the case of Hi the proposed mechanism is independent of the transposase, yet the direct binding of HiC to the Hi locus could explain the effect on DNA methylation. Could the authors comment on this?

(Our response) We added discussion for that possibility with citing Hashida et al (2006).

8. Page 4: Please note that not all class II TEs have long TIRs (Wicker et al., 2007, Nat Rev Genetics).
(Our response) We added the explanation in the Introduction and cited Wicker et al (2007).

9. Please correct a few typos (e.g. p3 L17, p9 L14, p12 L18, p25 L10).
(Our response) We could not find a typo in p3 L17. We corrected other typos.

Referee #3
We appreciate that this referee regard that “the science is this manuscript is sound and well controlled.” Again, the referee’s comments were very constructive and helped us to improve the manuscript.

In this manuscript "Anti-silencing and mobilization of a Mutator-like element without terminal inverted repeats" by Fu et al., the activity of a Vandal-class transposable element is investigated in the model plant Arabidopsis. The data in this manuscript demonstrates that this silenced element can be activated in a ddm1 mutant that undergoes loss of control of heterochromatin and transcriptional silencing. More importantly, the endogenous element can be trans-activated and trans-de-methylated by a transgenic TE copy. The authors perform a deletion analysis of this

transposable element and demonstrate that one of the three proteins is specifically responsible for the trans-de-methylation of silenced copies, and this protein is not the transposase. The authors argue that this protein has likely been acquired during plant evolution and now is associated with successful element activity.

The science is this manuscript is sound and well controlled.

(Our response) We appreciate that comment.

I have specific minor comments that I have made below. However, my major comments are:

1. This manuscript does not go far enough in the analysis of the HiC protein to warrant publication in a journal as good as EMBO. This manuscript leaves very easy to test experiments on the table. These experiments need to be completed.

(Our response)
As seen in the major comment 2 below, we beg to differ from referee 3 on this point. Specifically, in addition to the HiC protein, we believe that control and evolution of non-TIR MULEs is also very important and interesting for both general and specialist readers, especially because of the link we made with HiC function (see our response to the major comment 2). Furthermore, we believe that the additional experiments and analyses greatly strengthened the manuscript, as described below.

a. Does HiC act as a general silencing suppressor? Test if other TEs or endogenous sequences undergo de-methylation in the plant with transgenic HiC.
(Our response)
We performed DNA methylation analysis genome-wide at single-base resolution. DNA methylation was affected not only in the Hi but also in the other VANDAL21 members, despite the divergence in their sequences (new Figure 9, new Supplementary Figure S12, S13, S14 and new Supplementary Table S3). As is the case for the Hi locus, methylation was affected more at non-CpG sites than at CpG sites. Interestingly, the genome-wide analyses also revealed that not only terminal regions but also internal regions of Hi and other VANDAL21 members are affected (new Figure 9, new Supplementary Figure S14). In addition, the results indicate that the effect of the Hi transgene is highly specific to VANDAL 21 members, as DNA methylation remained unchanged for most other sequences of the genome, including most TEs and genes. These results are discussed in the context of anti-silencing strategy of a TE in the Discussion section.

b. Are plants expressing a HiC transgene hyper-sensitive to viral infection? I am reminded of the following paper that demonstrated that a virus was carrying a protein that interferes with DNA and histone methylation: Buchmann RC, Asad S, Wolf JN, Mohannath G, Bisaro DM (2009) Geminivirus AL2 and L2 Proteins Suppress Transcriptional Gene Silencing and Cause Genome-Wide Reductions in Cytosine Methylation. Journal of Virology 83: 4717-4717
(Our response)
Hi does not seem to affect DNA methylation globally (new Figure 9). Instead, the anti-silencing functions specifically to VANDAL21 members. Therefore, we do not think it very promising to examine sensitivity of the transgenic plant to virus infection. We added discussion of the anti-silencing strategy of viruses with comparison to that of TEs (the second paragraph of Discussion).

c. Do HiA and HiC interact, for example in a yeast 2 hybrid assay?
(Our response)
We are currently generating tagged HiC transgene to examine its localization genome-wide by ChIP seq. We are also setting up a system to search for factors interacting with HiC. We would like to characterize HiC protein thoroughly in future works, rather than performing 2 hybrid assay between HiA and HiC for this paper. We do not feel that 2 hybrid assay between HiA and HiC alone can advance our understanding of these proteins significantly, even if the result were positive, especially because we now know that DNA methylation in internal regions of TEs are also affected (new Figure 9).

d. When integrating these experiments, I think much of the first few sections of the Results (Identification, Structure, Integration & Excision in ddm1), can all be very much condensed into one

Figure / section with one supplemental figure. This paper got off to a very slow start, and I think all of this can be greatly condensed.
(Our response)
As suggested by this Referee, we combined the previous Figure 1 and 2 into one Figure. That enabled us to add the new Figure 9.

2. I don't understand why the authors focus so much on the fact that this element has no TIRs. In fact, this point is made throughout the text, and is even in the title.
(Our response)
We have been trying to propose that HiC function and the unique terminal structures can be related. In the previous manuscript, we showed that every VANDAL member encodes a protein related to HiC (Supplemental Figure S7 in previous manuscript), and we suggested that the HiC function may be related to the non-TIR feature. In order to see if that is really the case, we extended the analyses in the revised manuscript and examined distribution of the HiC-related proteins in every *A. thaliana* MULE group of both non-TIR and TIR types. HiC-related proteins were found not only in VANDAL members but also in ARNOLD members (Fig 7A). Importantly, ARNOLD is also non-TIR MULE. In contrast, the HiC-related protein was not found in any of TIR MULEs. Therefore, the link between HiC-related proteins and unique terminal structures turned out to be very tight; HiC-related proteins are widespread in every non-TIR MULE groups, while not found in any of TIR MULE members. These new analyses have strengthened our proposal.

Supplemental Figure 1A shows that this element does indeed have TIRs, they are just highly degenerated and short. I have no problem with the fact that these elements have short degraded TIRs, but the language of them having NO TIRs should be removed from throughout the manuscript and title.
(Our response)
As suggested, we have modified the text throughout to reduce potential misunderstanding. Still, we would like to use the term “non-TIR MULE”. It is a term defined and used in previous publications by Thomas Bureau’s group (Le et al 2000 PNAS; Yu et al 2000 Genetics ; Hoen et al 2006 Mol Biol Evol). They extensively characterized MULEs of *A. thaliana* and very convincingly demonstrate that the “TIR-MULEs” and “non-TIR MULEs” are very different in the terminal structures. Even though “non-TIR MULEs” often have complementary terminal sequences (for example, Hi has 2 bases of TIR, followed by 3 bases of mismatch), they are much shorter than TIRs of typical MULEs, which are generally 100-300 bp of almost identical sequences (Table I of Yu et al 2000 Genetics). We would like to keep the term “non-TIR MULE”, because that would be less confusing, rather than using a new term or expression. We do not think the term misleads readers, because we introduced non-TIR MULEs as MULEs “with extensively degenerated TIR”, and showed the specific structure of terminal regions of Hi in Supplementary Figure S1.

I didn't see what this added to the main story, which was all about HiC.
(Our response)
As we explained above (in our first response to the major comment 2 by this referee), we believe the link between the HiC-related proteins and “non-TIR” feature is very tight. That would be especially interesting in the context of TE evolution.

3. I dislike the data presentation in Figure 8 and 9. In Figure 8A, if the authors are arguing that Vandal elements are only successful when they acquire HiC or the Ulp1 protease, then there must be a more statistical way to demonstrate that, rather than just showing a tree. Please use numbers and statistics to make this argument, rather than a picture. In fact, my dislike of Figure 8 and 9 both have to do with the fact that the authors are using pictures to demonstrate large amounts of data. I think its OK for Figure 8B, but for Figure 8A and Figure 9, I don't see what ever it is that the authors want me to see. I don't think the data or the analysis is bad, I just think the display of the information is poor. For example, in Figure 8A, could you calculate the number of elements/copy number per family for Vandal families that have the ULP1 protease, DUF287 or neither, and then create 3 box plots to demonstrate that the element families that integrate these protein domains are more evolutionarily successful?

We agree with the referee in that we should compare TIR MULEs and non-TIR MULEs, in order to discuss that the latter is more successful than the former. We therefore extended the analysis to all MULEs of *A. thaliana* and *A. lyrata* (Figure 7). What we wanted to show in the Figure is that the

non-TIR MULEs have many terminal short branches in the phylogenetic tree, suggesting their recent proliferations. The referee's suggestion was to "calculate the number of elements/copy number per family", but we feel it less accurate, because definition and range of TE "family" is arbitrary. For statistics, we compared copy numbers in clusters of TEs within a defined genetic distance (0.1 p-distance). As shown in new Table I, copy numbers of non-TIR MULE clusters are significantly higher than those of TIR-MULE clusters, statistically supporting our proposal that the former have been proliferating more successfully.

Other Comments:
1. Does the name "Hi" refer to one element at one locus, the TE family, or the group of just very closely related elements? Please be clear on the definition of the nomenclature.
(Our response) Hi refers to one element at one locus. That was explained in the second paragraph of the Results section, where the term Hi was first used, in both the original and revised manuscripts.

2. In the last line of the Results section, the authors state that HiC plays a role in the trans-activation and mobilization of Hi. I agree with trans-activation. However, I see mobilization as an indirect consequence of the trans-activation, and I don't see evidence for HiC playing a direct role in mobilization. I would remove this claim.
(Our response) We removed that part as suggested.

3. It's been a long time since I've seen so many agarose gels in a paper. Perhaps instead of showing a band as a binary measure of excision, the authors could perform qPCR and show a quantitative measure of excision frequency. The same is true for the expression analysis in Figure 6-7.
(Our response) In most cases, Hi movement occurred in an all-or-none manner. We therefore think it reasonable to show that by presence/absence of band. Transcriptional responses of the Hi-encoded genes are sometimes different. We therefore characterized them by quantitative RT-PCR (Figure S8).

4. I felt that the explanation of Figures 8 & 9 belong in the Results section, and not in the Discussion. I suggest moving what you have written into a new Results section topic of "HiC evolution", and discuss your analysis there, instead of the discussion section.
(Our response) As suggested, we moved the analyses from Discussion to Results.

5. In Figure 4A, why does ddm1 have a low copy number? I though Hi transposes in ddm1 mutants (Figure 2). Please elaborate on the reason behind this discrepancy.
(Our response) In both epiRIL and self-pollinated *ddm1*, the active Hi was kept multiple generations, causing accumulation of Hi copies. The control *ddm1* plants in the previous Figure 4A are not so extensively self-pollinated. Still, increases in the copy numbers are detectable in them (the values are bigger than 1).

Does HiC excise in ddm1, but not re-integrate?
(Our response) We assume that the Referee means "Hi" rather than "HiC". As shown in previous Figure 2A, Figure S1B, and Figure S2, Hi integrates into many different loci in *ddm1*.

6. The Figure legend of Figure 6 describes a molecular weight marker, but none is seen in the Figure.
(Our response) We do not know the reason why the Referee could not see any of the molecular weight markers. In the files we have, they are in left side of gels in previous Figure 6 (Figure 5 in the revised manuscript).

2nd Editorial Decision 25 June 2013

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers. I am happy to inform you that we are ready to proceed with acceptance of the paper, pending modification of a few minor points.

- Please indicate the number of biological replicates performed to generate all data to the Figure

legends. In addition, in order to avoid confusion, please label the size of the molecular weight markers in the Figures.

- The splicing of the agarose gel in Figure 5B (bottom part between GAP-C (RT+) and GAP-C (RT-) is not overly apparent and should be made more obvious.

- Please remember to add the data accession numbers for all high-throughput data sets.

- I would like to suggest changing the current title to "Mobilization of the plant DNA transposon Hiun by expression of the anti-silencing protein HiC" as well as some minor alterations in the Abstract.

Transposable elements (TEs) have a major impact on genome evolution, but they are potentially deleterious, and most of them are silenced by epigenetic mechanisms such as DNA methylation. Here we report the characterization of a TE encoding an activity to counteract epigenetic silencing by the host. In Arabidopsis thaliana, we identified a mobile copy of the Mutator-like element (MULE) with degenerated terminal inverted repeats (TIRs). This TE, named Hiun (Hi), is silent in wild type plants, but it transposes when DNA methylation is abolished. When a Hi transgene was introduced into the wild type background, it induced excision of the endogenous Hi copy, suggesting that Hi is the autonomously mobile copy. In addition, the transgene induced loss of DNA methylation and transcriptional activation of the endogenous Hi. Most importantly, the trans-activation of Hi depends on a Hi-encoded protein different from the conserved transposase. Proteins related to this novel anti-silencing factor, which we named HiC, are tightly associated with non-TIR MULEs and may have contributed to their recent success in natural populations of A. thaliana and A. lyrata lineages.

- Finally, we 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. I would therefore like to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I will now return your manuscript to you for one additional round of minor revision. After that we should be able to swiftly proceed with formal acceptance and production of the manuscript!

2nd Revision - authors' response 02 July 2013

In the revised manuscript, we changed the names of the TE-encoded genes from hiA/hiB/hiC to vanA/vanB/vanC. That is because an Arabidopsis protein named HIC (HI CARBON DIOXIDE) has already been reported (Nature 408, 713-) and using the same name can be confusing. Other modifications are basically following your comments (shown by red).

Please indicate the number of biological replicates performed to generate all data to the Figure legends. In addition, in order to avoid confusion, please label the size of the molecular weight markers in the Figures.

We moved the number of replicates in Figure S7 and S15 to the Figure legend. We labeled the size of the molecular weight markers in the Fiugres, as suggested.

The splicing of the agarose gel in Figure 5B (bottom part between GAP-C (RT+) and GAP-C (RT-) is not overly apparent and should be made more obvious.

We separated the panels for GAP-C (RT+) and GAP-C (RT-) in Figure 5B.

Please remember to add the data accession numbers for all high-throughput data sets.

We added data accession numbers for the data sets of genome re-sequencing and genome-wide bisulfite reads in Material and methods section.

I would like to suggest changing the current title to "Mobilization of the plant DNA transposon Hiun by expression of the anti-silencing protein HiC" as well as some minor alterations in the Abstract.

We changed the title to “Mobilization of a plant transposon by expression of the transposon-encoded anti-silencing factor”. We changed the Abstract as suggested, except for the change in the name of the anti-silencing factor.

Finally, we 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. I would therefore like to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

We uploaded the Figure Source Data as suggested.

I hope that the manuscript is now suitable for publication in the EMBO Journal. Thank you again for your help.

