EMBO reports

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PARENT-OF-ORIGIN CONTROL OF TRANSGENERATIONAL RETROTRANSPOSON PROLIFERATION IN ARABIDOPSIS

Jon Reinders, Marie Mirouze, Joel Nicolet, Jerzy Paszkowski

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Transaction Report:

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

Transfer Note:

Please note that this manuscript was originally submitted to The EMBO Journal, where it was peer-reviewed. It was then transferred to EMBO reports and the original editor's decision, as well as the referees' comments are shown below.

Original decision and referees' comments – EMBO Journal 24 January 2013

Thank you for submitting your manuscript to the EMBO Journal. I am very sorry for the delay in getting back to you with a decision, but due to the holiday break and referee availability it unfortunately delayed the process a bit. Two referees have now reviewed your manuscript and their comments are provided below.

As you can see below, both referees appreciate the interest of the topic. However as referee #1 also indicates, the analysis remains very descriptive and we gain limited molecular insight into how retrotransposon activity is regulated. Referee #2 raises a number of concerns regarding the assay

used. I have discussed the referee reports further with my colleagues and while we appreciate the interest in the findings, we also are in agreement with referee #1 that the analysis remains too descriptive for the EMBO Journal. I am therefore sorry to say that we can't offer publication here.

However give the interest of the findings reported and the concise nature of the findings, I have taken the opportunity to discuss the manuscript and referee comments further with my colleague Dr. Esther Schnapp at EMBO reports. EMBO reports is interested in considering the manuscript for publication should you be able to address the concerns raised by referee #2. If you submit the paper to EMBO reports, they would have the manuscript re-reviewed by referee #2. You can contact Dr Schnapp at esther.schnapp@embo.org should you have further questions regarding this.

For the EMBO Journal I am sorry that I can't be more positive on this occasion, but I hope that you will consider EMBO reports.

REFEREE REPORTS:

Referee #1:

The manuscript by Reinders et al. focuses on retrotransposition in plants. Active retrotransposition has only very recently been directly observed in plants using epigenetic recombinant inbred lines previously developed by this and other groups of researchers. The subject is of fundamental biological interest, given the importance of retrotransposons in genome evolution and gene regulation.

The study uses reciprocal crosses between plants harbouring active EVADE (EVD) retrotransposons and inactive EVD. EVD activity is assayed by quantification of EVD extrachromosomal DNA in progeny either by southern blotting, or in semiquantitative PCR assays that measure circularized extrachromosal DNA.

The results are clear: The progeny contains active retrotransposons only when it is transmitted through the pollen. These results are the basis for the main conclusion of the paper: active retrotransposons are inherited through the male, but not through the female gametophyte. They further use mutants in small RNA and histone modifying components of transcriptional silencing pathways previously shown to control retrotransposition to determine whether such mutant backgrounds break the maternal restriction of active retrotransposons. They cannot demonstrate any effect of the introduced mutations (nrpd2a, kyp, ago5).

In summary, the genetic analyses are well executed, and the conclusion of the paper is well supported by the data presented. It is a simple, descriptive result that does not offer any mechanistic insight, but it is, nonetheless, a result of substantial biological importance.

I only have minor criticisms on the manuscript.

1) In their discussion, the authors follow the temptation to speculate in some detail on the possible mechanistic bases for the parent-of-orgin effect they describe. I would prefer to resist this temptation given the lack of insight into this problem provided by their results. In my opinion, a simpler, less involved discussion, is better suited to a descriptive study such as the present one.

2) Please correct piwiRNA to piRNA (page 12).

3) I agree with the authors from Figures 3B and S4B that none of the mutations nrpd2a, kyp, ago5 break maternal restriction of active EVD (or inactive for that matter). However, the data for the nrpd2a series in Figure 3B were not quite clear to me. They seems to suggest that little or no active EVD is transmitted through the male in this series. Could the authors please comment on this result?

Referee #2:

This manuscript details experiments that demonstrate that epigenetically activated Arabidopisis EVD retrotransposons are suppressed when active elements are introduced via the female germ line, but not when they are introduced via the male germ line. Because this suppressive effect is not eliminated when the F2 gametes are mutant for genes encoding components of the siRNA pathway, they suggest that this effect is due to the production of small RNAs in the maternal sporophytic tissue, consistent with early observations. These results are interesting, but I had some significant problems that should be addressed. One big problem was the primary assay used by the authors, involving an assessment of copy number variation (CNV). It would appear that initially they measured only ecDNA copies of EVD (a measure of potential (but not proved) transposition intermediates (Figure S2A), but then switched (?) to qPCR of the overall copy number of LTRs (both integrated and extrachromosomal (Figure S2C). Thus, throughout the rest of the manuscript they conflate chromosomal and extra-chromosomal copies. This was a decidedly odd choice of assays, since it's a bit like combining expression levels of elements with copy number assays. This is particularly important since the authors suggest that they are examining "transgenerational propagation" of ERVs, but they may simply be tracking the production of non-productive transposition intermediates. I don't think this is necessarily a huge problem, but the use of "copy number" in this context, which almost invariably means the number of integrated copies of a given TE, is problematic. Further, they are not measuring "variation" in each sample, they are measuring an absolute number (that of LTRs).

I was also concerned with the mutant analysis. Given that they now have the lines available, wouldn't the test of their interesting theory be to use mutants with and without active EVDs crossed reciprocally to wild-types with and without active EVDs? Why take us to the brink of an actual test of the hypothesis without actually carrying the test out? To summarize, I found this to be a very interesting serious of experiments that used a questionable assay and stopped one experiment early.

Page 3: "However, the genome-wide abundance of TEs suggests that they are also associated with certain benefits". Debatable, since it assumes that TEs can't drive themselves into a population despite negative fitness effects on their hosts, but we know from P-elements in D. melanogaster that this can be the case.

Page 4. "..they have not defined host responses to actively mobilized TEs or shown how active TEs are transmitted or how their copy numbers increase from generation to generation." Actually, this process has been well documented in maize using Mutator as a model. The relevant literature should be cited.

Page 5. "but that epigenetic mechanisms involving siRNAs and/or H3K9me2 and acting in maternal sporophytic tissues of the flower prevent transgenerational proliferation of active EVDs." There is an obvious genetic test here. I hope the authors performed it.

Page 6. "...was crossed reciprocally to the Landsberg erecta (Ler) accession (EVD-silent)". Why was the reciprocal cross not made to Columbia, since then the only variable would have been epigenetic; by crossing of Ler, haven't you introduced a lot of additional genetic variation? I see that the authors address this later, but I can't actually see the simple reciprocal cross between epi12 and Col.

Figure 1). I'm not sure how the DNA here was quantified, but the ethidium staining for Ler makes it look like there is much less DNA (particularly low ML DNA than rF1(L/e). Similarly, why is the high molecular weight DNA for rF1(L/e) hybridizing so much better than the HMW DNA for F1(e/L). Are these samles really comparible?

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Page 6. "We observed not only EVD activation but also a clear parent-of-origin effect on the presence of EVD ecDNA in F1 progeny of the reciprocal crosses (Fig. 1B)". Maintenance of activity is not the same as "activation".

Page 6. "...,The qPCR reported all types of EVD elements, including linear ecDNA, circular ecDNA and chromosomal EVD insertions, thus providing a general readout for progressive EVD accumulation.." Or perhaps a conflation of a readout of copy number increases with increased amounts of ecDNA? What would you have seen if you had size selected the undigested DNA so see if the number of integrated copies were increasing? Seems like a pretty simple assay.

Page 7. "...EVD copy numbers were also determined in progeny from the self-fertilized parents (epi12 and Ler) to provide controls for relative changes in EVD copy number". I'm finding this somewhat difficult to follow, since no distinction is being made between integrated and extrachromosomal copies of the element. It's as if both transcript levels and DNA copies of an element were combined into a single assay. For instance, is there a difference between ep12 F4 and epi12F5 because of a increase in the copy number of integrated EVDs, or an increase in extra-chromosomal copies, or both? I can't tell from the data. However, it does seem that, since the "inactive" epi-rill lines have values that are similar to Ler and Col, perhaps there is no actual increase in copy number of integrated elements, in which case what is being measured may not be a reflection of active transposition of these elements.

Page 7. "In contrast, copy number levels in reciprocal F1 siblings from populations A and B, differing in the initial degree of the paternal transmission, increased by over eight- and threefold, respectively". I'm not seeing 8 fold here. F2 e/L was 2. F2 L/e was 8. Isn't this 4 fold? Where are these numbers coming from? A change in the ratios? Further, all of the numbers increase, including that of epi12 F5.

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Page 9. "We concluded, therefore, that the parent-of-origin effects on transgenerational propagation of EVD" Frankly, I'm not seeing evidence for or against transgenerational propagation of EVD, and plenty of evidence that there is no real increase in the number of integrated EVD in these lines.

Page 11. Rate of increase in a change over time. Are we seeing that here?

Page 11. "However, this was ruled out when the effect was found to be maintained in intra-accession Col x Col crosses (Fig. 4)." I don't see this in figure 3 or figure 4.

1st Revision – authors' response 25 May 2013

Please find enclosed a revised manuscript by Reinders *et al.* entitled "Parent-of-origin control of transgenerational retrotransposon proliferation in Arabidopsis" which we would like you to consider for publication in the *EMBO reports*.

Within the revised version we have provided new data to address the concerns raised by each reviewer. As described in our rebuttal, we have directly addressed the most frequent concerns raised

by the second reviewer, including new data shown in Supplemental Figure 5 demonstrating that we are not conflating "expression levels of elements with copy number assays" but are indeed reporting on *EVD*'s mobilization and transgenerational propagation. We have also cited new publications relevant to our conclusions and performed additional mutant experiments as suggested by Reviewer #2. We would like to thank you again for your patience while we were coping with the difficulty inherent to these mutants.

We hope you find this manuscript suitable for publication in the *EMBO reports*.

REFEREE #1:

The manuscript by Reinders et al. focuses on retrotransposition in plants. Active retrotransposition has only very recently been directly observed in plants using epigenetic recombinant inbred lines previously developed by this and other groups of researchers. The subject is of fundamental biological interest, given the importance of retrotransposons in genome evolution and gene regulation.

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1) In their discussion, the authors follow the temptation to speculate in some detail on the possible mechanistic bases for the parent-of-orgin effect they describe. I would prefer to resist this temptation given the lack of insight into this problem provided by their results. In my opinion, a simpler, less involved discussion, is better suited to a descriptive study such as the present one.

The discussion was revised to support our current figures and conclusions.

2) Please correct piwiRNA to piRNA (page 12).

This has been revised as suggested.

3) I agree with the authors from Figures 3B and S4B that none of the mutations nrpd2a, kyp, ago5 break maternal restriction of active *EVD* (or inactive for that matter). However, the data for the nrpd2a series in Figure 3B were not quite clear to me. They seems to suggest that little or no active *EVD* is transmitted through the male in this series. Could the authors please comment on this result?

We agree that low activity levels were transmitted in the nrpd2a x e12.38 population. This result shows that not all gametes confer identical EVD activity levels.
This point is now addressed in the Results section (Page 9, lines 12-15).

REFEREE #2:

Remark #1
[This manuscript details experiments that demonstrate that epigenetically activated Arabidopisis *EVD* retrotransposons are suppressed when active elements are introduced via the female germ line, but not when they are introduced via the male germ line. Because this suppressive effect is not eliminated when the F2 gametes are mutant for genes encoding components of the siRNA pathway, they suggest that this effect is due to the production of small RNAs in the maternal sporophytic tissue, consistent with early observations. These results are interesting, but I had some significant problems that should be addressed. One big problem was the primary assay used by the authors, involving an assessment of copy number variation (CNV). It would appear that initially they measured only ecDNA copies of *EVD* (a measure of potential (but not proved) transposition intermediates (Figure S2A), but then switched (?) to qPCR of the overall copy number of LTRs (both integrated and extrachromosomal (Figure S2C). Thus, throughout the rest of the manuscript they conflate chromosomal and extra-chromosomal copies. This was a decidedly odd choice of assays, since it's a bit like combining expression levels of elements with copy number assays. This is particularly important since the authors suggest that they are examining "transgenerational propagation" of ERVs, but they may simply be tracking the production of non-productive transposition intermediates. I don't think this is necessarily a huge problem, but the use of "copy number" in this context, which almost invariably means the number of integrated copies of a given TE, is problematic. Further, they are not measuring "variation" in each sample, they are measuring an absolute number (that of LTRs).]

Reply to remark #1:
We originally used the concept of "copy number" to describe the additional copies of an activated retrotransposons, in both extrachromosomal and integrated forms. Reviewer 2 points out that copy number almost invariably requires integration of the replicated retrotransposons to cause structural variation of the genome. Indeed, Hastings et al state, "A change in copy number requires a change in chromosome structure, joining two formerly separated DNA sequences." We agree this was not the scope of our experiments and that using "CNV" in this context was inappropriate. We revised the manuscript to state the qPCR results measure the "Relative EVD DNA content" (as used for the retrotransposon L1 qPCR quantification in Coufal et al., Nature, 2009, and Baillie et al., Nature, 2011).

We would like to explain that the semi-quantitative end-point analysis for circular intermediates of extrachromosomal EVD (ecDNA) in Fig S2A was only a diagnostic assay distinguishing between epi12 "EVD-active" and "EVD-Silent" lines. The relative EVD DNA content in these lines was only analyzed by qPCR. The two methods were not "switched". We have further clarified this by stating the circular assay is a "LTR-PCR" assay that detects the presence of "ecDNA" whereas the qPCR assay detects the "Relative EVD DNA content". Please note the LTR-PCR assay is only used as a diagnosis for EVD initial activation. The data from qPCR assays (Fig 2B-C, 3B) or DNA blots (Fig. 1B, 4B) reflect our main objective to quantify the total amount of EVD DNA level changing in the initial generations after EVD's activation. For this reason we continue to state we are examining "transgenerational propagation" of the EVD retrotransposon.

Remark #2
[I was also concerned with the mutant analysis. Given that they now have the lines available, wouldn't the test of their interesting theory be to use mutants with and without active *EVD*s crossed reciprocally to wild-types with and without active *EVD*s? Why take us to the brink of an actual test of the hypothesis without actually carrying the test out? To summarize, I found this to be a very interesting serious of experiments that used a questionable assay and stopped one experiment early.]

To address the concern regarding our hypothesis testing for EVD activity in the presence and absence of relevant mutations, we suggest the reviewer reconsider our experiment design and corresponding results in Fig 3, Fig 4 and Fig S4. To expand beyond what is stated within the manuscript, we would like to make the following points:

i) The primary factor required to monitor EVD transmission is an active EVD epiallele.
Here we used one F_3 family (epi12) in a Col x met1-3 biparental population selected due to its inheritance of an active, met1-derived EVD epiallele. Transgenerational propagation of EVD had

already been demonstrated for this family over multiple generations (see Mirouze et al., 2009), thereby satisfying this primary requirement.

ii) A secondary requirement is then to have the EVD-active donor source produce viable reproductive structures. Please note single mutants for KYP and Pol IV/PolV are EVD-silent (see Mirouze et al., 2009 and Fig 3 and Fig S5). Attempting to do intrapopulation-based comparisons between backcrossed-derived families segregating with and without the mutations that we used, but rather originating from met1-3/kyp7 or met1-3/nrpd2a double mutants as donor sources (as seemingly implied by the reviewer), is not feasible due to negative pleiotropic effects (see Mirouze et al, 2009 and Cell 130:851-62 for supporting phenotypic evidence). In contrast, the epi12 F_3 family satisfies this requirement, especially when KYP/kyp7 or NRPD2A/nrpd2a F_1 plants, either with our without EVD activity, are reciprocally crossed to both the wild type and mutant parents. This is what the reviewer is requesting and precisely what we describe in this manuscript.

iii) A tertiary concern was to use an EVD-active donor source that had the propensity to become active, yet still possessed a nearly nascent genome lacking newly inserted EVD copies. Again, the epi12 F_3 family satisfies this requirement since it had not had time to propagate over multiple generations (see Mirouze et al., 2009).

Please see our reply for Remark #11 in regards to the reviewer's concern about our "questionable" assay.

Remark #3
[Page 3: "However, the genome-wide abundance of TEs suggests that they are also associated with certain benefits". Debatable, since it assumes that TEs can't drive themselves into a population despite negative fitness effects on their hosts, but we know from P-elements in D. melanogaster that this can be the case.]

We agree with the reviewer's comment. The sentence has been replaced with : "…it was hypothesized that some TEs could also be associated with certain benefits…".

Remark #4
[Page 4. "..they have not defined host responses to actively mobilized TEs or shown how active TEs are transmitted or how their copy numbers increase from generation to generation." Actually, this process has been well documented in maize using Mutator as a model. The relevant literature should be cited.]

We have corrected this statement in the introduction to better reflect the main focus of the manuscript. We do agree with the reviewer that there is relevant literature, specifically in maize, and this has been cited in the Discussion (page 11).

Remark #5
[Page 5. "but that epigenetic mechanisms involving siRNAs and/or H3K9me2 and acting in maternal sporophytic tissues of the flower prevent transgenerational proliferation of active *EVD*s." There is an obvious genetic test here. I hope the authors performed it.]

The effect of the parental sporophyte on EVD initiation of proliferation is now analyzed in the new Figure 4C.

Remark #6
[Page 6. "...was crossed reciprocally to the Landsberg erecta (Ler) accession (*EVD*-silent)". Why was the reciprocal cross not made to Columbia, since then the only variable would have been epigenetic; by crossing of Ler, haven't you introduced a lot of additional genetic variation? I see that the authors address this later, but I can't actually see the simple reciprocal cross between epi12 and Col.]

Our initial finding based on the cross with Landsberg erecta (Ler) accession was an unexpected discovery. Although we describe our initial, critical observation as it occurred, we established this

was reproducible when crossed with lines isogenic for the Columbia genome (Fig 3) for the exact reason that the reviewer has stated here.

Remark #7
[Figure 1). I'm not sure how the DNA here was quantified, but the ethidium staining for Ler makes it look like there is much less DNA (particularly low ML DNA than rF1(L/e). Similarly, why is the high molecular weight DNA for rF1(L/e) hybridizing so much better than the HMW DNA for F1(e/L). Are these samles really comparible?]

It is true that the DNA loading was not optimal and therefore HMW signals vary. However, even with such unequal loading the absence or presence of the extrachromosomal DNA in F1 and rF1, respectively, is obvious. This is the main observation illustrated in this figure.

Remark #8
[Page 5. "...we developed a PCR assay based on the semi- quantitative detection of circular ecDNA forms (Fig. S2A)" I think this is fine, but calling this copy number variation (CNV) is a little confusing, since it is really a reflection of variation in ecDNA. Perhaps better to refer to it is ecCNV? I'm assuming that the authors don't want to give the impression that you are showing that the overall number of integrated *EVD*s is increasing, which is not actually demonstrated here.]

We agree and we have modified the manuscript accordingly. We now clarify this in the text where the circular assay is named "LTR-PCR" assay and detects the presence of "ecDNA".

Remark #9
[Page 6. "We observed not only *EVD* activation but also a clear parent-of-origin effect on the presence of *EVD* ecDNA in F1 progeny of the reciprocal crosses (Fig. 1B)". Maintenance of activity is not the same as "activation".]

As suggested, this statement was corrected.

Remark #10
[Page 6. "...,The qPCR reported all types of *EVD* elements, including linear ecDNA, circular ecDNA and chromosomal *EVD* insertions, thus providing a general readout for progressive *EVD* accumulation.." Or perhaps a conflation of a readout of copy number increases with increased amounts of ecDNA? What would you have seen if you had size selected the undigested DNA so see if the number of integrated copies were increasing? Seems like a pretty simple assay.]

We performed a similar assay as the one suggested by the reviewer (Fig S1): we quantified the proportion of EVD ecDNA versus integrated EVD DNA by quantifying the band intensity in non-digested DNA blots. Figure S1 shows that EVD ecDNA level represents around 10% of the total EVD DNA level (n=23) in EVD-active lines. No EVD ecDNA could be detected in EVD-silent lines.

Remark #11
[Page 7. "...*EVD* copy numbers were also determined in progeny from the self-fertilized parents (epi12 and Ler) to provide controls for relative changes in *EVD* copy number".
I'm finding this somewhat difficult to follow, since no distinction is being made between integrated and extrachromosomal copies of the element. It's as if both transcript levels and DNA copies of an element were combined into a single assay. For instance, is there a difference between ep12 F4 and epi12F5 because of a increase in the copy number of integrated *EVD*s, or an increase in extra-chromosomal copies, or both? I can't tell from the data. However, it does seem that, since the "inactive" epi-rill lines have values that are similar to Ler and Col, perhaps there is no actual increase in copy number of integrated elements, in which case what is being measured may not be a reflection of active transposition of these elements.]

To understand how retrotransposons have become so prevalent in eukaryotic genomes, more so than DNA transposons, we used qPCR results to measure the total "relative EVD DNA content" – meaning detecting EVD in all its forms. Although considered a "questionable" assay in this reviewer's second remark, we point the reviewer to previous experiments likewise using qPCR for similar reasons as precedence (e.g. Coufal et al., Nature, 2009, and Baillie et al., Nature, 2011). Since there is no basis to assume that ecDNA are not factors for germinal transmission of

retrotransposons, as implied by the reviewer, we continue to include the abundance of ecDNA for measuring the total "Relative EVD DNA content". Nonetheless, to address the reviewer's concerns we now include transposon display results and show that there are insertions and that these increase above the original epi12 donor level (Fig S5) demonstrating that EVD-active lines do have insertions in agreement with our qPCR assay results.

Remark #12
[Page 7. "In contrast, copy number levels in reciprocal F1 siblings from populations A and B, differing in the initial degree of the paternal transmission, increased by over eight- and threefold, respectively". I'm not seeing 8 fold here. F2 e/L was 2. F2 L/e was 8. Isn't this 4 fold? Where are these numbers coming from? A change in the ratios? Further, all of the numbers increase, including that of epi12 F5.]

We agree we did not originally state these results correctly and have revised this section for improved clarity as recommended. We agree that the epi12 F5 has increased and we view this as the positive control demonstrating the levels of transgenerational propagation specific to each different EVD donor line.

Remark #13
[Page 7. "We repeated the reciprocal crosses using the isogenic Col background to test whether the Ler genome itself influences *EVD* mobility (Fig. 3A)." I can't see in figure 3A where a simple reciprocal cross between epi12 and Col was performed. The control cross was between mM hets (derived from crosses between epi12 and various mutants, which obviously have their own unique epigenetic history) and WT Col.]

By identifying F1 siblings within the same cross and originating from the same parents that were EVD-active, we believe it is imperative to select the related sibling EVD-silent line is the appropriate negative control. This is the reason why the silent F1 plants were used to create the silent BC populations (with and without mutations as described, Fig. S4). Although a Col x e12.38 population would indeed be EVD-silent, this material would be inappropriate since it has never been hybridized with the met1-3 epigenome. Hence, for the same rationale this reviewer refers to in remark #6 (the need to eliminate any type of variation that may be unaccounted for within the experiment), we performed our epi12 x Col crosses using each respective isogenic mutant line.

Remark #14
[Page 8. "as well as the F2 generation, are shown in Fig. 3." This is a bit confusing, but I assume the authors mean section marked "F1 active"?]

We agree and have revised Fig 3 and Fig S4 using standard pedigree nomenclature to reflect the selection of an active F1 that was self-fertilized to produce the F2 generations.

Remark #15
[Page 8. "In agreement with the previous results, self- fertilization of *EVD*-active F1 individuals produced two- to over fourfold increases in *EVD* copy number," relative to what? The F1 active parent? Epi12.38? "...while copy numbers of *EVD*-silent classes did not differ from Col 8(Fig. 3B and S5B)." So does this mean there was no increase in the integrated copy number of *EVD* up to 12.38?]

To clarify, we have revised this statement to say, "In agreement with the previous results, self-fertilization of EVD-active F_1 individuals produced two- to over fourfold increases in EVD copies relative to the wild type level, while copies of EVD-silent classes did not differ from the wild type level (Fig. 3B and S5B)".

Remark #16
[Page 9. "We concluded, therefore, that the parent-of-origin effects on transgenerational propagation of *EVD*" Frankly, I'm not seeing evidence for or against transgenerational propagation of *EVD*, and plenty of evidence that there is no real increase in the number of integrated *EVD* in these lines.]

We believe the transposon display results provide clear evidence for EVD integration.
See Figure S5.

Remark #17
[Page 11. Rate of increase in a change over time. Are we seeing that here?]

It is unclear what precise statement on page 11 is disconcerting to the reviewer. Relative to our previous study with 6 generations of inbreeding, we acknowledge that we are analyzing very few generations here, and thus, we do not focus on how the rate of increases in a change over time. Our main focus is how the parent-of-origin effect alters the rate EVD increases. Only on page 11 does the phrase including "rate of increase" occur, stated as: "Our analysis of reciprocal EVD transmission during sexual reproduction has now shown that the rate of increase in EDV copy number is determined by a parent-of-origin effect."

To address this concern and to improve clarity, we currently state: "Our analysis of reciprocal EVD transmission during sexual reproduction in A. thaliana has likewise shown that the rate of increase in EDV copy levels is affected by a parent-of-origin effect."

Remark #18
[Page 11. "However, this was ruled out when the effect was found to be maintained in intra-accession Col x Col crosses (Fig. 4)." I don't see this in figure 3 or figure 4.]

Please see our reply to point #3 raised by reviewer #1 regarding the lack of activity in the nrpd2a x e12.38 population. This issue aside, in Fig 3B, the increased levels of EVD activity in "Active Paternal" progeny within intra-accession Col x Col crosses clearly supports our statement.

1st Editorial Decision 31 May 2013

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the enclosed report from referee 2 who still has a few suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Referee 2 points out that changes in the copy number of integrated EVD retrotransposons should be determined directly. I agree that it would be much better if the study distinguished between integrated and ecEVD. Please let me know if and why you strongly disagree here. The remaining comments mainly regard better explanations and changes to the text.

I noticed that the manuscript currently uses the wrong reference style. Can you please change the style to a numbered one, according to our journal's policy? Thank you very much. Changing the reference style will also help in shortening the text, as it is currently still 5700 characters too long. You can still combine the results and discussion section, which should help in shortening as well.

The legends for figure 2 and 3 and SF1 and 4 still need to define the number of experiments that were performed and the calculated statistics are based on. Please note that error bars can only be shown if 3 or more experiments were performed, and that no error bars should be shown for replicates of a single experiment.

Please let me know if you have any further questions or comments.
I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The authors appear to be unwilling accept my concerns about the assay they use for copy number increases. However, this is something that reasonable people can disagree about, and the authors

have made good-faith efforts to at least seriously address my concerns. My comments below are meant to be helpful, if only to illustrate points of confusion for readers.

Reply to remark #1: We originally used the concept of "copy number" to describe the additional copies of an activated retrotransposons, in both extrachromosomal and integrated forms. Reviewer 2 points out that copy number almost invariably requires integration of the replicated retrotransposons to cause structural variation of the genome. Indeed, Hastings et al state, "A change in copy number requires a change in chromosome structure, joining two formerly separated DNA sequences." We agree this was not the scope of our experiments and that using "CNV" in this context was inappropriate. We revised the manuscript to state the qPCR results measure the "Relative EVD DNA content" (as used for the retrotransposon L1 qPCR quantification in Coufal et al., Nature, 2009, and Baillie et al., Nature, 2011).
We would like to explain that the semi-quantitative end-point analysis for circular intermediates of extrachromosomal EVD (ecDNA) in Fig S2A was only a diagnostic assay distinguishing between epi12 "EVD-active" and "EVD-Silent" lines. The relative EVD DNA content in these lines was only analyzed by qPCR. The two methods were not "switched". We have further clarified this by stating the circular assay is a "LTR-PCR" assay that detects the presence of "ecDNA" whereas the qPCR assay detects the "Relative EVD DNA content". Please note the LTR-PCR assay is only used as a diagnosis for EVD initial activation. The data from qPCR assays (Fig 2B-C, 3B) or DNA blots (Fig. 1B, 4B) reflect our main objective to quantify the total amount of EVD DNA level changing in the initial generations after EVD's activation. For this reason we continue to state we are examining "transgenerational propagation" of the EVD retrotransposon.

Reviewers response: "The qPCR reported all types of EVD elements, including linear ecDNA, circular ecDNA and chromosomal EVD insertions, thus providing a general readout for progressive EVD accumulation." I appreciate the argument the authors have made in their rebuttal, but I'm going to try to make this argument one more time. By including both ecEVD and chromosomal EVD together in their assay, the authors are conflating two distinct processes, since we cannot assume that all of the ecEVDs are going to in fact reintegrate. This makes the argument about "progressive" copy number increases more problematic than it needs to be. Given the relatively low copy number of the element, all the authors needed to do was to perform Southern blots or use transposon display and look directly at the changes in copy number of integrated elements. The appear to be unwilling to do this.
Further, the authors state that, "...conversely, when EVD-silent Ler plants were fertilized with EVD-active pollen increases in EVD copies were observed in all three EVD-active F1 populations derived from epi12 siblings carrying active EVD..." If Ler only has a few copies of EVC, and L/e is the result of a backcross Ler, then one would expect that L/e would have half the copy number (chromosomal) of epi12, which is what one sees in Figure 2B. So how is this a "copy number increase?"

Remark #5
[Page 5. "but that epigenetic mechanisms involving siRNAs and/or H3K9me2 and acting in maternal sporophytic tissues of the flower prevent transgenerational proliferation of active EVDs." There is an obvious genetic test here. I hope the authors performed it.]
The effect of the parental sporophyte on EVD initiation of proliferation is now analyzed in the new Figure 4C.

Reviewers response: Forgive me, but I find this confusing. In Figure 4C you show that the copy number of ecEVD goes up in the mutant progeny of plants whose parent had been heterozygous for both mutations. So the mother was heterozygous for the mutants but you see activation in the mutant progeny. The test I was referring to would look at heterozygous progeny of a mutant mother to see whether or not what is important is the genotype of the mother (that is, het progeny of homos would show more ecEVD than het progeny of hets). What I see here is evidence for activation in mutant progeny of a heterozygous mother, and then even more activity in the next generation. According to the authors, "Therefore, NRPD2A and KYP most likely contribute to sporophytic maternal suppression of EVD replication prior to gametophyte development.", which would imply that the maternal control that the authors note is the result of processing and transmission from maternal tissue.

[Figure 1). I'm not sure how the DNA here was quantified, but the ethidium staining for Ler makes it

look like there is much less DNA (particularly low ML DNA than rF1(L/e).Similarly, why is the high molecular weight DNA for rF1(L/e) hybridizing so much better than the HMW DNA for F1(e/L). Are these samples really comparable?]
It is true that the DNA loading was not optimal and therefore HMW signals vary. However, even with such unequal loading the absence or presence of the extrachromosomal DNA in F1 and rF1, respectively, is obvious. This is the main observation illustrated in this figure
.
Reviewers response: No, it is not obvious. Southern blotting can exhibit a threshold effect, such that under loading can lead to a loss of low copy signal. Since this was, indeed, the entire point of this experiment, under loading of this particular sample was unfortunate.

Remark #11
[Page 7. "...EVD copy numbers were also determined in progeny from the self-fertilized parents (epi12 and Ler) to provide controls for relative changes in EVD copy number". I'm finding this somewhat difficult to follow, since no distinction is being made between integrated and extrachromosomal copies of the element. It's as if both transcript levels and DNA copies of an element were combined into a single assay. For instance, is there a difference between ep12 F4 and epi12F5 because of a increase in the copy number of integrated EVDs, or an increase in extra-chromosomal copies, or both? I can't tell from the data. However, it does seem that, since the "inactive" epi-rill lines have values that are similar to Ler and Col, perhaps there is no actual increase in copy number of integrated elements, in which case what is being measured may not be a reflection of active transposition of these elements.]
To understand how retrotransposons have become so prevalent in eukaryotic genomes, more so than DNA transposons, we used qPCR results to measure the total "relative EVD DNA content" - meaning detecting EVD in all its forms. Although considered a "questionable" assay in this reviewer's second remark, we point the reviewer to previous experiments likewise using qPCR for similar reasons as precedence (e.g. Coufal et al., Nature, 2009, and Baillie et al., Nature, 2011). Since there is no basis to assume that ecDNA are not factors for germinal transmission of retrotransposons, as implied by the reviewer, we continue to include the abundance of ecDNA for measuring the total "Relative EVD DNA content". Nonetheless, to address the reviewer's concerns we now include transposon display results and show that there are insertions and that these increase above the original epi12 donor level (Fig S5) demonstrating that EVD-active lines do have insertions in agreement with our qPCR assay results.

Reviewers response: Figure S5 suggests that there are a limited number of new insertions, but the number of these new insertions does not square with the big increases in copy number reported via the qPCR results. Indeed, most of the new insertions do not appear to be unique to any particular sample and therefore represent segregating older insertions.
Remark #15
[Page 8. "In agreement with the previous results, self- fertilization of EVD-active F1 individuals produced two- to over fourfold increases in EVD copy number," relative to what? The F1 active parent? Epi12.38? "...while copy numbers of EVD-silent classes did not differ from Col 8(Fig. 3B and S5B)." So does this mean there was no increase in the integrated copy number of EVD up to 12.38?]
To clarify, we have revised this statement to say, "In agreement with the previous results, self-fertilization of EVD-active F1 individuals produced two- to over fourfold increases in EVD copies relative to the wild type level, while copies of EVD-silent classes did not differ from the wild type level (Fig. 3B and S5B)".

Reviewer response: I don't see an S5B.

2nd Revision - authors' response 10 June 2013

Our approach to studying *EVADE*'s transmission focused on observing the earliest dynamic changes to genome content, allowing us to observe the parent-of-origin effect, as did the "minimal mutator line" in reference 31. We clearly state what we are detecting with the qPCR assay. We addressed the reviewer's concern with transposon display and revised the manuscript further acknowledging its

limitations since we believe it is not reasonable for transposon display to be expected to equal the qPCR result. The reviewer remains concerned that not all ecDNA will be inserted, but we do not make this claim. However, because insertions require completing cDNA synthesis, a definitive lifecycle event, this clearly relates to how retroelements ultimately alter genomes. Thus our aim was to detect, without bias, total *EVD* activity during sexual reproduction. Also, we have already demonstrated such *EVD* activity leads to *bona fide* copy number increases, specifically including the progressive accumulation of *EVD* in the epi12 lineage (see reference 19, Figure 2e), leading to dynamic changes in genome stability.

Additional comments:

- We believe our efforts demonstrated in Figure S1 directly looked at changes in copy number of integrated elements using Southern blots.
- In Figure 2B, a value of 1.0 is the relative genomic content of *EVD* midpoint between the two parents. We interpret values above a value of 1.0 to reflect progressive *EVD* accumulation. We previously revised the text for clarity and believe our figure will allow readers to evaluate our data and conclusions.
- As stated in the manuscript, we agree that maternal control seems to result from regulation within maternal tissues. However, we did not know this before performing our experiments designed to study gametophytic regulation, the leading model in the field. So yes, we agree with the reviewer's proposed experiment and hope readers find this suggestion inspiring. Nevertheless, in our revised version the analysis of *EVD* ecDNA (Figure 4C) revealed that the priming of *EVD* correlated with the sporophyte genotype.
- We conceded that our Figure 1 Southern blotting was not perfect and we show our imperfection. It is also understood that there are sensitivity limits to Southern blots, which is in fact the basis for our use of the diagnostic LTR-PCR and qPCR assays. However, our conclusions are not based solely on Figure 1.
- We agree there is a predominant band associated with the *EVD*-active donor in the transposon display results. Of course one could for example resequence the lines to reveal all *EVD* insertions, but we believe that what we have performed in response to the reviewers' concerns continues to support our claims.

We apologize for not initially correcting the style and size requirements of the manuscript. These issues have been corrected. Revisions were applied to the figures and legends as suggested. We greatly appreciate your prompt attention to our manuscript that we hope you find suitable for *EMBO reports*.

2nd Editorial Decision 14 June 2013

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

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