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An endogenous, systemic RNAi pathway in plants

Patrice Dunoyer, Christopher Brosnan, Gregory Schott, Yu Wang, Florence Jay, Abdelmalek Alioua, Christophe Himber and Olivier Voinnet

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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.)

1st Editorial Decision 09 March 2010

Thank you for submitting your manuscript for consideration at the EMBO Journal. It has been reviewed by two referees whose comments are listed below. Both referees express interest in the study and recommend publication pending satisfactory minor text revision. Given this strong support for the study I am happy to inform you that we would be happy to publish your manuscript in the EMBO Journal once these issues are addressed, it will make a great contribution to the journal.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is an excellent study that will be of great interest for plant and animal researchers interested in RNAi, endogenous loci supporting RNAi, and systemic RNAi silencing signals. The experiments are well designed and convincing data are presented. The discussion is interesting and provocative.

Referee #2 (Remarks to the Author):

In this manuscript, the authors provide compelling evidence that experimental RNAi has an endogenous counterpart in plants, and could therefore play a role in intra- as well as intercellular and systemic regulatory processes. The results presented, which are of the highest quality, follow an equally impressive series of results obtained by the same group concerning the identification of factors required in Arabidopsis for experimental RNAi and its spread (Dunoyer et al, Nature Genet 2007). While in their previous work the authors used a transgene harboring an artificial inverted repeat driven by the phloem specific SUC2 promoter to target the SULPHUR endogenous mRNA in a non-cell autonomous manner, they have now turned their attention to the study of two expressed, endogenous large inverted repeats (IR71 and IR2039) present in the Arabidopsis Columbia accession. In essence, it is shown that these two IRs are almost undistinguishable genetically from artificially constructed IRs. Furthermore, the authors provide evidence that, as expected from such structures, IR 2039 arose recently, and that processing of IR71 long dsRNA differs in two ecotypes as a result of the presence of a loss of function DCL2 variant in these. Finally, the authors demonstrate through an elegant set of micro-grafting experiments that 24nt-long siRNAs (and maybe others) derived from the two endogenous IR loci can move through the vasculature to direct DNA methylation of cognate sequences in a systemic fashion. Biolistic delivery of fluorescently labeled 21 and 24 nt-long siRNA duplexes was also used to demonstrate mobility of these two size classes. The latter results are particularly interesting in light of recent evidence suggesting that siRNAs may be specifically produced from non-reproductive cells that surround the gametes and the developing embryo and transported into these to reinforce silencing of repeat elements and hence stabilize the genome at the onset of plant development (reviewed in Mosher & Melnik, TIPS 2010; Martienssen, New Phyt 2010).
Minor points:
1) P3&4 The sentences "In C.elegans and Drosophila... (reviewed in Okamura and Lai, 2008)" and "We recently demonstrated that, of the SUL... gene silencing of SUL (Dunoyer et al. 2010)" are awkward.
2) P6 To my knowledge, there has been no rigorous assessment of the prevalence of long inverted duplications in the Arabidopsis genome. In particular, Lindow et al. 2007 refer at best to unpublished data.
3) P6 Could the authors speculate on why SUC-SUL, IR71, IR2039 and presumably other IRs produce different proportions of 21nt, 22nt and 24nt sRNAs?
4) P9 The authors fail to discuss properly the discrepancy between their observations and those of Park et al., 2005 concerning the impact of hst1 on 24nt siRNA accumulation.
5) P11 & 18 Loading of 22nt-long siRNAs onto AGO1 should be expressed with reference to the total number of reads (1683581?).
6) P11 What is the DNA methylation status of IR2039 as well as that of putative trans targets of both IR71 and IR2039 (if any, as defined by sequence complementarity)?
7) P11 I recommend replacing "The results thus strongly suggest..." with "The results thus suggest that IR71 produces siRNAs that direct its DNA methylation upon their incorporation into AGO4.
8) P11 Replace "significant drift mutations" by "many mutations"
9) P12 The part on variant DCL2 could be shortened.
10) P12 I recommend that "depending on specific ecological niches" should be omitted, given that there is no evidence that the contrasted output of IR71 long dsRNA processing on gene regulation has adaptive value. Similarly, there is no evidence that the evolution of endogenous IRs is driven by the environment and results from natural selection rather than genetic drift.
11) P13 the predicted transcription start site of IR71 and IR2039 should be indicated in Fig 1A.
12) P13 Delete the sentence "Rapidly evolving IRs may sense the environment..." for the same reason as in point 10.
13) P13 The last paragraph should be modified as only one direction of grafting (shoot:donor, root:recipient) is shown in Figure 5. Why not show the data for the other grafting direction, given that the results are statistically significant?

14) Shouldn't a proper control have been to perform grafts of roots and shoots of the same stock, to account for possible variations in siRNA abundance caused by the grafting procedure per se? This is particularly important given that small RNA production from IR71 (and IR2039) is exquisitely sensitive to a variety of stresses, as documented by the authors (Page 12).
15) P17 The argument about NRPD1, RDR2 and CSY1 being downstream of DCL4 and AGO1 is difficult to follow, and is based on a highly speculative interpretation of contradictory data obtained with another transgenic IR by Smith et al (2007). Please simplify.
16) P17&18 The part on the molecular sensors of the environment suffers from similar weaknesses to those indicated in points 10 and 12. The sentence "IR loci arise at an ecotype-based scale" is not very meaningful. By definition, any mutation that arise in a species, first concerns one individual, then a population, before being fixed (or not).
17) P18 In the section on epiallelism the authors need to acknowledge the huge body of literature on transposable elements in maize, starting from B. McClintock and which illustrates ways by which epigenetic states may be established, maintained and erased, with profound phenotypic and molecular consequences (see Banks et al, Genes & Dev, 1988 and related work by the group of N. Fedoroff in the 1990s, or a series of papers by D. Lisch in the last ten years for examples involving the Spm and Mu transposons, respectively). After all, most DNA transposons are characterized by inverted terminal repeats, and it is likely that readthrough transcription of transposable elements is the main source of long dsRNAs in eukaryotes with large genomes.
18) P19 The authors should also refer to a recent paper indicating that in Arabidopsis DNA methylation over most repeat elements can be stably maintained in the absence of siRNAs (Teixeira et al, Science 2009).

1st Revision - authors' response 22 March 2010

Referee #1 (Remarks to the Author):

This is an excellent study that will be of great interest for plant and animal researchers interested in RNAi, endogenous loci supporting RNAi, and systemic RNAi silencing signals. The experiments are well designed and convincing data are presented. The discussion is interesting and provocative.

We acknowledge the fact that Referee #1 is overall satisfied of our work,

Referee #2 (Remarks to the Author):

In this manuscript, the authors provide compelling evidence that experimental RNAi has an endogenous counterpart in plants, and could therefore play a role in intra- as well as intercellular and systemic regulatory processes. The results presented, which are of the highest quality, follow an equally impressive series of results obtained by the same group concerning the identification of factors required in Arabidopsis for experimental RNAi and its spread (Dunoyer et al, Nature Genet 2007). While in their previous work the authors used a transgene harboring an artificial inverted repeat driven by the phloem specific SUC2 promoter to target the SULPHUR endogenous mRNA in a non-cell autonomous manner, they have now turned their attention to the study of two expressed, endogenous large inverted repeats (IR71 and IR2039) present in the Arabidopsis Columbia accession. In essence, it is shown that these two IRs are almost undistinguishable genetically from artificially constructed IRs.
Furthermore, the authors provide evidence that, as expected from such structures, IR 2039 arose recently, and that processing of IR71 long dsRNA differs in two ecotypes as a result of the presence of a loss of function DCL2 variant in these. Finally, the authors demonstrate through an elegant set of micro-grafting experiments that 24nt-long siRNAs (and maybe others) derived from the two endogenous IR loci can move through the vasculature to direct DNA methylation of cognate sequences in a systemic fashion. Biolistic delivery of fluorescently labeled 21 and 24 nt-long siRNA duplexes was also used to demonstrate mobility of these two size classes. The latter results are particularly interesting in light of recent evidence suggesting that siRNAs may be specifically produced from non-reproductive cells that surround the gametes and the developing embryo and transported into these to reinforce silencing of repeat elements and hence stabilize the genome at

the onset of plant development (reviewed in Mosher & Melnik, TIPS 2010; Martienssen, New Phyt 2010).

Minor points:
1)P3&4 The sentences "In C.elegans and Drosophila... (reviewed in Okamura and Lai, 2008)" and "We recently demonstrated that, of the SUL... gene silencing of SUL (Dunoyer et al. 2010)" are awkward.
This has been changed now.

2) P6 To my knowledge, there has been no rigorous assessment of the prevalence of long inverted duplications in the Arabidopsis genome. In particular, Lindow et al. 2007 refer at best to unpublished data.
The Lindow publication referes to hairins that are not necessarily extensively folded. We have now reworded the statement to take this into account.

3) P6 Could the authors speculate on why SUC-SUL, IR71, IR2039 and presumably other IRs produce different proportions of 21nt, 22nt and 24nt sRNAs?
Nothing based on experimental data can explain these variations; however, one can certainly hypothesize that changes in tertiary structure of the various IRs (unpredictable) will influence the affinity or access of specific DCL isoforms to the dsRNA.

4) P9 The authors fail to discuss properly the discrepancy between their observations and those of Park et al., 2005 concerning the impact of hst1 on 24nt siRNA accumulation.
A good point from the referee. The text has now been re-worded to take this into account.

5) P11 & 18 Loading of 22nt-long siRNAs onto AGO1 should be expressed with reference to the total number of reads (1683581?).
Yes, this is true. It has been added at the adequate positions.

6) P11 What is the DNA methylation status of IR2039 as well as that of putative trans targets of both IR71 and IR2039 (if any, as defined by sequence complementarity)?
We are now doing these experiments so will not be able to add them to the amended manuscript in time; but this is certainly interesting.

7) P11 I recommend replacing "The results thus strongly suggest..." with "The results thus suggest that IR71 produces siRNAs that direct its DNA methylation upon their incorporation into AGO4.
Done

8) P11 Replace "significant drift mutations" by "many mutations"
Done

9) P12 The part on variant DCL2 could be shortened.
We have now considerably shortened this part.

10) P12 I recommend that "depending on specific ecological niches" should be omitted, given that there is no evidence that the contrasted output of IR71 long dsRNA processing on gene regulation has adaptive value. Similarly, there is no evidence that the evolution of endogenous IRs is driven by the environment and results from natural selection rather than genetic drift.
It is a valid point, though we did not intend to convey the idea that the IRs do evolve by natural selection; We have now reworded several sections of the manuscript to make it clear that it is just one possibility and that more work would be required to state on this.

11) P13 the predicted transcription start site of IR71 and IR2039 should be indicated in Fig 1A.
It is now done

12) P13 Delete the sentence "Rapidly evolving IRs may sense the environment..." for the same reason as in point 10.
We have modified also this section accordingly.

13) P13 The last paragraph should be modified as only one direction of grafting (shoot:donor,

root:recipient) is shown in Figure 5. Why not show the data for the other grafting direction, given that the results are statistically significant?
This is now modified, but we still think there is no need to add the second data set.

14) Shouldn't a proper control have been to perform grafts of roots and shoots of the same stock, to account for possible variations in siRNA abundance caused by the grafting procedure per se? This is particularly important given that small RNA production from IR71 (and IR2039) is exquisitely sensitive to a variety of stresses, as documented by the authors (Page 12).
Well spotted from the referee, who is entirely right on this. In any case this argument is only valid for small RNAs from the non-disrupted part of the IR71. It cannot apply the deleted part because siRNAs cannot be generated out of nowhere. Nonetheless, the argument is valid for the other parts, and so we have removed the sentences that pertained to the gain of small RNAs over the non disrupted region of IR71.

15) P17 The argument about NRPD1, RDR2 and CSY1 being downstream of DCL4 and AGO1 is difficult to follow, and is based on a highly speculative interpretation of contradictory data obtained with another transgenic IR by Smith et al (2007). Please simplify.
We have discussed this issue with the editor and would rather keep this section as it is. The value of the endogenous IRs is that it now allows us to draw conclusions about an otherwise very poorly understood process, which has confused the field for several years.

16) P17&18 The part on the molecular sensors of the environment suffers from similar weaknesses to those indicated in points 10 and 12. The sentence "IR loci arise at an ecotype-based scale" is not very meaningful. By definition, any mutation that arise in a species, first concerns one individual, then a population, before being fixed (or not).
This has been changed.

17) P18 In the section on epiallelism the authors need to acknowledge the huge body of literature on transposable elements in maize, starting from B. McClintock and which illustrates ways by which epigenetic states may be established, maintained and erased, with profound phenotypic and molecular consequences (see Banks et al, Genes & Dev, 1988 and related work by the group of N. Fedoroff in the 1990s, or a series of papers by D. Lisch in the last ten years for examples involving the Spm and Mu transposons, respectively). After all, most DNA transposons are characterized by inverted terminal repeats, and it is likely that readthrough transcription of transposable elements is the main source of long dsRNAs in eukaryotes with large genomes.
These are all very good points and it is certainly true that prior work has to be acknowledged. This is now done.

18) P19 The authors should also refer to a recent paper indicating that in Arabidopsis DNA methylation over most repeat elements can be stably maintained in the absence of siRNAs (Teixeira et al, Science 2009).
Done.

2nd Editorial Decision 22 March 2010

I have read through your response to the referees and find that you have satisfactorily addressed all the concerns and therefore I am pleased to let you know that your manuscript has been accepted for publication in the EMBO Journal.

Thanks and best wishes,

Editor
The EMBO Journal

