Differential effects of Viral Silencing Suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1

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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 06 February 2012

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, both referees find the manuscript interesting and support publication here pending adequate revisions. In particular referee #1 finds that further experiments are needed to support 1) the relevance of the p38 transgenic model using virus-infected non-transgenic plants and 2) that p38 doesn't function via sequestering siRNAs. Given the comments raise, I would like to ask you to submit a revised manuscript that addresses the concerns raised in full.

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.

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS

Referee #1

This paper is of interest. The authors present several pieces of evidence to argue for two main points: i) The majority of viral encoded RNA silencing suppressors (VSRs) function differently on siRNAs and miRNAs, which has not been demonstrated in previous reports, and ii) these VSRs do not act via sequestration of small RNA duplexes as have been suggested by previous in vitro studies. Using VSR-expressing transgenic plants as a model, they demonstrate clearly that all VSRs investigated prevent the loading of 21-22 nt siRNAs onto AGO1 but (except for P19) do not affect the loading of miRNAs, although both siRNA and miRNA-mediated silencing of target genes are inhibited. They show that companion cell-specific expression of P38 does not prevent local spread of silencing in the SUC-SUL system, suggesting that P38 does not physically bind and sequester the SUL siRNAs. They also show that while miRNAs are readily detectable in AGO1 immunoprecipitates, miRNA passenger strands (miRNA*) are not detectable in the AGO1 IP in the VSR transgenic plants despite their increased accumulation, suggesting that the VSRs do not bind miRNA duplexes to prevent their unwinding and subsequent loading onto AGO1. These findings are significant and interesting, shedding novel insight into the function of viral silencing suppressors.

However, the following points need to be considered:

1. In a previous paper by the group (Azevedo J et al, Genes & Development 2010), it was shown that P38 expressed from a transgene and P38 derived from infecting TCV affect siRNA loading onto AGO1 differently, with the latter having very little effect. This difference would raise questions over the biological relevance of the results obtained using the transgenic model in the current study. The authors should address this issue in the text, or preferably, perform AGO1 IP followed by siRNA/miRNA Northern using virus-infected (such as TCV-infected) non-transgenic plants. Could it be possible that transgenically expressed VSRs have different subcellular localization patterns to virally expressed VSRs and therefore have a different effect on siRNAs and miRNAs?

2. The experiment with companion cell-specific expression of P38 is nice and the result is consistent with a lack of siRNA binding by P38. However this only provides indirect evidence. It is not inconceivable that the lack of a clear effect on silencing spread is due to relatively weak siRNA binding by P38 in comparison to P19 or relatively low-level of P38 protein expression in the transgenic context tested (according to the authors the other 17% of the SUC-P38 plants do show an altered silencing pattern - do all the 17% plants give a non companion cell-specific ectopic P38 expression pattern?). To provide direct evidence that P38 does not function through sequestration of siRNAs, an immunoprecipitation experiment should be performed on transgenically expressed P38 (or the other VSRs) followed by siRNA and miRNA analysis of the P38 IP by Northern blot hybridization.

3. In Fig4C, no P38 hybridization signals were observed in the AGO1 IP of the SUC-P38 plants. Is this due to low expression level of P38 or does it indicate a lack of or weak P38/AGO1 association? This needs to be clarified.

Referee #2

In this manuscript, Schott et al. first investigated the effects of a viral RNA silencing suppression (VSR) P38 of TCV on siRNA and miRNA loading into AGO1. They introduce a 35S-P38 transgene into the SUC-SUL Arabidopsis system, in which an inverted-repeat (IR) construct driven by the phloem companion cell (CC)-specific SUC2 promoter directs silencing of the SULPHUR (SUL) endogenous mRNA. They found that P38 expression completely abolised the appearance of the SUL silencing phenotype. Further analysis showed P38 prevented loading of 21nt SUL siRNAs, but not the conserved endogenous miRNA, into AGO1 by immunoprecipitates assay. However, the conserved miRNA-mediated regulation of targets was also interfered in 35-P38 plants, suggesting P38 might impair miRNA-AGO1-RISC activity.
The authors further analyzed other VSRs and found that all of the VSRs tested (including PCV-encoded P15, TuMV-encoded Hc-Pro, and the tombusviral P19) compromised loading of siRNAs into AGO1, only P19 was found to concurrently prevent miRNA loading. Based on the results, the authors draw conclusion that the existence of at least two distinct AGO1 pools, of which one is specifically loaded with siRNAs, and the other specifically loaded with miRNAs, and they are targeted differently by VSRs. The authors further used SUC2 promoter-P38 transgenic system and confirmed that P38 did not bind directly the mobile 21-nt siRNA. This observation led to rule out a significant contribution of siRNA binding to the VSR activity of P38 in planta.

Having read this manuscript (that in vitro binding assays and in vivo miRNAs* stabilization are not reliable indicator of VSR action) and the published in this field recently (that in vitro interaction between AGO and the CMV-encoded 2b and the suppression of the slicer activity of AGO1 by the 2b are not accounted for the VSR activity of 2b in planta (Duan et al., 2012. Plant Cell), it might now prove possible and serious to investigate genuine in vivo suppression action of VSRs. Overall the work is well written, however, I will recommend the authors to cite the recent published (Duan et al., 2012. Plant Cell) to modify the introduction prior publication.

1st Revision - authors' response 21 February 2012

Point-by-point answers to reviewers’ comments

Referee #1

This paper is of interest. The authors present several pieces of evidence to argue for two main points: i) The majority of viral encoded RNA silencing suppressors (VSRs) function differently on siRNAs and miRNAs, which has not been demonstrated in previous reports, and ii) these VSRs do not act via sequestration of small RNA duplexes as have been suggested by previous in vitro studies. Using VSR-expressing transgenic plants as a model, they demonstrate clearly that all VSRs investigated prevent the loading of 21-22 nt siRNAs onto AGO1 but (except for P19) do not affect the loading of miRNAs, although both siRNA and miRNA-mediated silencing of target genes are inhibited. They show that companion cell-specific expression of P38 does not prevent local spread of silencing in the SUC-SUL system, suggesting that P38 does not physically bind and sequester the SUL siRNAs. They also show that while miRNAs are readily detectable in AGO1 immunoprecipitates, miRNA passenger strands (miRNA*) are not detectable in the AGO1 IP in the VSR transgenic plants despite their increased accumulation, suggesting that the VSRs do not bind miRNA duplexes to prevent their unwinding and subsequent loading onto AGO1. These findings are significant and interesting, shedding novel insight into the function of viral silencing suppressors. However, the following points need to be considered:

1. In a previous paper by the group (Azevedo J et al, Genes & Development 2010), it was shown that P38 expressed from a transgene and P38 derived from infecting TCV affect siRNA loading onto AGO1 differently, with the latter having very little effect. This difference would raise questions over the biological relevance of the results obtained using the transgenic model in the current study. The authors should address this issue in the text, or preferably, perform AGO1 IP followed by siRNA/miRNA Northern using virus-infected (such as TCV-infected) non-transgenic plants. Could it be possible that transgenically expressed VSRs have different subcellular localization patterns to virally expressed VSRs and therefore have a different effect on siRNAs and miRNAs?

> This is a good point raised by referee#1. However, we do believe the difference between transgenically- and virally-expressed P38 effects on AGO1 loading is likely to reflect more a difference in the pattern of P38 accumulation rather than a true caveat of the transgenic system used. Indeed, in transgenic plants, P38 is expressed from a ubiquitous promoter in many tissues of the plants and at very early developmental stages. By contrast, not all cells are infected during TCV infection, and in those cells, P38 accumulation has to build up form viral replication, which presumably allows a significant proportion of SUL siRNAs to incorporate into AGO1 before the onset of P38-mediated inhibition of siRNA loading. This likely explains the more modest effect of P38 observed in infected tissues. Moreover, as suggested by Azevedo and colleagues (2010), P38 may only be able to prevent siRNA incorporation into non-loaded RISC. Therefore, only siRNAs

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produced concomitantly with P38 expression will be prevented from incorporation into AGO1. We have now added this point in the result section.

For the reasons evoked above, together with the fact that TCV infection causes a dramatic and general decrease in miRNA accumulation and a complete change in Dicer homeostasis (Azevedo et al., 2010), it will be difficult, not to say impossible, to compare the impact of transgenically or virally-expressed P38 on small RNA loading into AGO1.

2. The experiment with companion cell-specific expression of P38 is nice and the result is consistent with a lack of siRNA binding by P38. However this only provides indirect evidence. It is not inconceivable that the lack of a clear effect on silencing spread is due to relatively weak siRNA binding by P38 in comparison to P19...

> This is true. But this is exactly what we said in the text when we state that “these observations rule out a significant contribution of sRNA binding to the VSR activity of P38 in planta” or “the results strongly argue against a prevalent contribution of sRNA binding to the VSR action of P38”. We are not arguing that P38 binds siRNA (it is a fact in vitro, at least), but rather, we argue that this binding, should it occur in vivo, is most likely insignificant in terms of its contribution to silencing suppression by P38.

... or relatively low-level of P38 protein expression in the transgenic context tested (according to the authors the other 17% of the SUC-P38 plants do show an altered silencing pattern - do all the 17% plants give a non companion cell-specific ectopic P38 expression pattern?).

> We do not think that P38 does not impact the spread of silencing due to low expression levels for several reasons. First, the SUC2 promoter-driven construct used to express P38 was previously used with two other VSRs, namely P19 and P21 (Dunoyer et al., 2010). In these previous experiments, both VSRs inhibited non cell-autonomous RNA silencing very efficiently. We do not see any particular reason that can explain why, in that case, P38 would be expressed at significantly lower levels than these two other suppressors, also taking into account that many distinct transgenic lines were used in parallel. Second, out of the 13 SUC-P38 transgenic lines exhibiting reduced SUL silencing, five have been randomly selected for in situ hybridization. All displayed the same expression pattern as the line depicted in figure 4 (SUC-P38/SS#5) strongly suggesting that this inhibition of SUL silencing directly results from ectopic P38 expression in the neighbouring (recipient) cells rather than a more elevated level of P38 accumulation in the companion (incipient) cells. Third, none of the lines exhibiting companion cells-specific P38 expression displayed a reduced SUL silencing phenotype. Yet, the accumulation of P38 in those lines, although variable, can reach relatively high levels based on the easy detection of the VSR (both by in situ hybridization and western blot analysis) even though it is expressed in a very small fraction of the total number of cells present in leaves. The same was actually true of P19 and P21 originally used in Dunoyer et al., 2010.

To provide direct evidence that P38 does not function through sequestration of siRNAs, an immunoprecipitation experiment should be performed on transgenically expressed P38 (or the other VSRs) followed by siRNA and miRNA analysis of the P38 IP by Northern blot hybridization.

> We have already tried to perform this experiment. Unfortunately, the P38 antibody available in the lab is not appropriate for immunoprecipitation experiments. To overcome this problem, we have generated FLAG-tagged version of P38 in both N-terminal and C-terminal orientation. However, so far none of our fusion constructs remained functional in terms of silencing suppression (we can provide the result of the patch-test assay if requested by referee#1). Moreover, based on the very strong association between P38 and AGO1 (Azevedo et al., 2010), P38 IPs (if they were achievable) would also very likely pull-down AGO1 and its associated small RNAs. As P38 does not affect miRNA loading into AGO1, analysis of this particular class of small RNAs will, therefore, not be very informative. For the other VSRs, we are also in the process of generating various functional FLAG-tagged versions, but this will take several months before the analysis requested can be achieved and will not bring much to the take-home message of the present paper, we believe.

3. In Fig4C, no P38 hybridization signals were observed in the AGO1 IP of the SUC-P38 plants. Is this due to low expression level of P38 or does it indicate a lack of or weak P38/AGO1 association?
This needs to be clarified.

> Another point well spotted by referee#1. Most likely, the absence of P38 signal in the AGO1 IP does not result from a weak P38-AGO1 interaction as it was shown before that this complex is unaltered by treatments of up to 800mM KCl (Azevedo et al., 2010) and is therefore highly stable. We believe it does not result neither from a low expression level per se but rather from the cell-specific expression of P38. Therefore, even when P38 is highly expressed in companion-cells (based on in situ hybridization signal and western blot analysis), its interaction with AGO1 is diluted away as IPs are performed on whole leaf samples, not on pre-dissected vascular tissue. We have now added this point in the result section.

Referee #2

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Having read this manuscript (that in vitro binding assays and in vivo miRNAs* stabilization are not reliable indicator of VSR action) and the published in this field recently (that in vitro interaction between AGO and the CMV-encoded 2b and the suppression of the slicer activity of AGO1 by the 2b are not accounted for the VSR activity of 2b in planta (Duan et al., 2012. Plant Cell), it might now prove possible and serious to investigate genuine in vivo suppression action of VSRs. Overall the work is well written, however, I will recommend the authors to cite the recent published (Duan et al., 2012. Plant Cell) to modify the introduction prior publication.

> This is true. This paper was published online, just after we submitted this manuscript. We have now modified the introduction to include this reference.

2nd Editorial Decision 06 March 2012

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 to look at the revised version and have now received the comments back. As you can see below, referee #1 is supportive of publication here. However, the referee also indicates that more caution might be needed when interpreting the results from the transgenic system. I think that I would be good to add a few lines to the discussion to address this concern. Once we receive the revised version, we will proceed with its acceptance here.

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS

Referee #1

The paper provides evidence to suggest that i) siRNA binding is not the predominant mode of action by viral suppressors of RNA silencing (VSRs) and ii) VSRs act differently on siRNA and miRNA-directed silencing, and hence two different AGO1 pools might exist in plants. These conclusions are novel and unorthodox and contradict the popular view that VSRs function primarily by binding and sequestering siRNAs. The revised version provides explanations about the seeming functional difference between virus and transgene-derived p38, which appear plausible. The authors also provide an explanation as to why p38 could not be detected in the AGO1 IP of the transgenic SUC-p38 plants, which is possible although p38 could be detected in the total input samples and even in the supernatant after IP (Fig 4C). The authors explain in their response letter that immunoprecipitation of p38 could not be done in the near future due to a lack of suitable p38, which is an acceptable reason. However, while the findings of the paper are novel and potentially provide a clearer picture on the function of VSRs, they also raise many questions. For instance, if miRNA loading to AGO1 is not affected by VSRs, how is miRNA-mediated target gene regulation affected? Also, if siRNAs are not loaded to AGO1 and not bound by VSRs, where and in what form do they exist in the plant cell? Isn't true that unbound siRNAs are unstable? Do most of the VSRs function like p38, i.e. by binding AGO1 and preventing siRNA loading? Furthermore, while the authors argue that the transgene system is more useful than in vitro assays or viral infections for investigating the function of VSRs, it is nevertheless an artificial system expressing only a single viral protein from the nuclear genome, which is different to real viral infections expressing multiple viral proteins/RNAs whose subcellular localization and interactions might be important. Therefore, while exciting and insightful, cautions might be needed in the interpretation of the results.

2nd Revision - authors' response 08 March 2012

As suggested by referee#1, we have now added a sentence at the beginning of our discussion to bring to the reader's attention that the results obtained in the transgenic systems used in this study should be used with some caution as they may provide an oversimplified view of authentic viral infections. We are hopeful that this amended version is now suitable for publication in the EMBO Journal.