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Peer Review Report for 10.1105/tpc.15.01056

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This report is published as part of a pilot program, including a small set of articles, with the approval of all respective authors and reviewers, to introduce readers and authors to the concept and test the format.

tRNA-Related Sequences Trigger Systemic mRNA Transport in Plants

Wenna Zhang, Christoph Thieme, Gregor Kollwig, Federico Apelt, Lei Yang, Winter Winter, Nadine Andresen, Dirk Walther, and Friedrich Kragler *Plant Cell. Advance Publication June 7, 2-16; doi: 10.1105/tpc.15.01056.*

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Review timeline:

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	1st Decision:	Jan. 29, 2016 *revision requested*
TPC2015-01056-BRR1	1st Revision received:	Apr. 15, 2016
	2nd Decision:	May 10, 2016 *accept with minor revision*
TPC2015-01056-BRR2	2nd Revision received:	May 25, 2016
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REPORT:

(Note: The report shows the major requests for revision and author responses to major reviewer comments. Miscellaneous correspondence and confidential comments for the editors are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2015-01056-BR 1st Editorial decision – *revision requested* Jan. 29, 2015

We ask you to pay attention to the following points in preparing your revision.

As you will see from the reviewers' individual comments, they are excited and agree that the data are matching the expectation for the new format of a breakthrough report in Plant Cell. However, there are a few points to consider in a revised version. Reviewer 1 and 3 pointed out rightly that, other than mRNA mobility, there might be alternative explanations for the developmental phenotype that should be discussed and excluded. Considering potential effect on bicistronic transcripts or transcripts from neighboring genes is a valuable suggestion. They also request adding better control experiments to substantiate the statement that the tRNA is necessary for ck1 mobility. While we think that reviewer 2 is too critical about the content of the abstract, we agree with the reviewers that the manuscript in total could be improved in grammar and style, perhaps by a professional writer. We hope that you will also find the minor comments helpful, and we look forward to receive a revised manuscript.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-001056-BRR1 1st Revision received Apr. 15, 2016

We would like to thank you and the three reviewers for their efforts and the many helpful and encouraging comments. Upon revising our manuscript, we have carefully considered all points raised and hope to have addressed them appropriately. Most importantly, we added new data indicating the occurrences of di-cistronic tRNA sequences in the mobile mRNA dataset (new Figure 4 panels and Supplemental figure 5). These data are based on RT-PCR assays and RNAseq data and suggest that di-cistronic tRNAs and tRNA like structures (TLS) are relatively abundant in plants and significantly overrepresented in the mobile mRNA population.

Reviewer comments and author responses:

Reviewer #1 (Comments for author):

This manuscript describes an exciting discovery - that a tRNA-like RNA structure is necessary and sufficient to direct long-distance RNA movement. The experiments, which primarily rely on grafting, are appropriate and well-controlled. While there are a number of outstanding questions remaining, this is appropriate for a Breakthrough Report and I am confident that this will be an impactful story. I have a few suggestions for minor revisions to improve the manuscript:

Point 1. Does tRNA-Ile have a TSL? If so, this would seem to contradict the proposed model. The obvious experiment is to test whether the addition of this structure onto an RNA causes mobility, but for a breakthrough report I think it is sufficient to include discussion of what distinguishes tRNA-Ile from tRNA-Met.

RESPONSE: Thank you for raising this question. In short: we do not think that there is a contradiction. We have tested the tRNA-Ile core sequence as a GUS fusion in transgenic A. thaliana plants which shows that it is not graft mobile (see Figure 3). Also the predictor of tRNA-like structures (TLS) does not identify Ile-tRNA. This is mentioned in the results, methods and legends text. In addition, we added a short paragraph in the discussion referring to the structural differences shown in Figure 4).

Point 2. Were multiple transgenic events assessed for each construct? This is an important point, as the authors seem to invoke the idea that location near a tRNA gene increases the chance of mobility.

RESPONSE: Thank you for highlighting the importance of this aspect. Indeed, we forgot to include this important information in the manuscript. Of course, we tested multiple transgenics for each construct and only considered data, which could be repeated with independent transgenic plants (and with additional 5' and 3' fusion constructs not mentioned in the manuscript as they are intended for a follow up publication).

To address this point, we added a list to the Supplemental Data (Excel file) indicating how many independent transgenic lines were used for GUS and DNDMC1 fusion constructs and how many grafts were performed and positive/negative movement was observed after grafting. For the DNDMC1 fusions, we used at least three independent N. tabacum lines (see Supplemental Table 1 and Supplemental Data) pre-selected such that they show similarly high or inducible expression levels. In all cases, we observed similar mobility rates or non-mobility (control) as in the presented examples shown in the manuscript. Note that we also indicate for all experiments the number of grafts and the number of positive signals we obtained e.g. for PCR and GUS assays, which is normally not done in this detail in other manuscripts describing mobility.

Point 3. Line 224: It is not clear why there would be a phenotype due to loss of mobility in ck1.2 when there is no change in transcript level. This observation suggests that the mobile transcripts do something different than the transcripts expressed in situ. (I assume the RT-qPCR is detecting both mobile and non-mobile transcripts.) This is a critical idea and should be discussed.

RESPONSE: Thank you for raising this interesting question. Here the notion is that mobility from tissues with high expression levels to tissues with no or low expression is necessary to provide a robust growth rate. As the reviewer suggests, we propose that non-mobile CK1 results in an equivalent growth delay as seen with the ck1.1 KO line that does not or barely produce CK1 transcripts (determined by qRT-PCR shown in Figure 4). In the ck1.2 mutants, CK1

is only present in these tissues/cells where the promoter is active. Thus, there is a lack of CK1 activity in distant/neighboring tissues/cells which do not produce CK1 mRNA. The quantitative RT-PCR assays indicate that the produced non-mobile, truncated ck1.2 transcript is stable and present in similar amounts as in the wild type. Thus, we conclude that the ck1.2 mutant growth phenotype is not a result of attenuated expression levels or instability of the non-mobile CK1 mRNA. As outlined by the reviewer, this observation is very interesting and suggests that transcript mobility is equally important as the expression in the cells/tissue where the promoter is active. To meet the reviewer's suggestion, this aspect is now mentioned in more detail in the discussion.

Regarding the question of the RT-qPCR. The quantitative PCR was performed with two independent primer pairs (the result of one primer pair is shown in Figure 4) recognizing both, the wt and the ck1.2 poly(A)-RNA in samples from non-grafted plants to determine to which extent the SALK insertions affect CK1 mRNA production in the mutant ck1.1 and ck1.2 lines. Note also that both primer pairs resulted in equivalent results in such that in contrast to ck1.2 mutants, in ck1.1 mutants no or minor amounts of CK1 transcript could be detected.

Point 4. How does being in proximity to a gene with a TSL cause mobility of an independently transcribed RNA? Did the authors attempt any RT-PCR to see if bicistronic transcripts are ever produced? This finding should be integrated into their model and discussed in the discussion.

RESPONSE: To address this we performed RT-PCR assays and analyzed the presence of di-cistronic mRNA-tRNA fusions as poly(A) transcripts (new data and figure panels in the Supplement and Figure 4). In brief: of seven tested transcripts four show tRNA fusions to neighboring mobile transcripts (beside CK1). Also, more than 100 additional di-cistronic mRNA-tRNA fusions were found in our and external RNA-Seq data (see new Supplemental Data and new Supplemental Figure 5 and new panel now shown as Figure 4C). A statistical analysis taking these data into account showed an enrichment by a factor of 1.6 of mRNA-tRNA fusions in the mobile mRNA population. However, please note that a statistical significance could not be established as the sample size was small.

Point 5. If necessary for space, discussion of the evolutionary generation of mobile transcripts through gene duplication is purely speculative and could be removed from the discussion.

RESPONSE: Here we did not follow the reviewers suggestion as we feel that this is giving the reader a lead towards the observation that although we see very often mobile transcripts that are homologous between various species, sets of mobile transcripts have not been observed to overlap by more than 30 percent between distant species (See Walther and Kragler 2016, Nature Plants).

Point 6. It is not clear why there is no misshapen pollen in figure 1bc (rightmost panel). Later in the manuscript I gather that this construct was used because it is susceptible to RNA silencing and can be a marker for siRNA-mediated silencing. This should be explained earlier so as not to confuse the reader.

RESPONSE: We now mention this construct earlier in the results text describing Figure 1.

Reviewer #2 (Comments for author):

This paper describes the significant importance of tRNA-like structures (TLS) for mRNA moving via the phloem vasculature using grafting of transgenic tobacco and Arabidopsis carrying the several versions of reporter transcripts fused to TLS sequence at 3' or 5'. They claimed that mRNA fused to TLS can be graft-transmissible by phenotypic/enzymatic assays and RT-PCR and that TLS with predicted stem-bulge-stem-loop structures are sufficient for mRNA movement through the phloem vasculature across graft junctions using deletion mutants. Their results based on the various different systems are significantly novel in this field. Thus I recommend the manuscript for publication in Plant Cell.

However the manuscript is not well written and needs improvements including more descriptions and many English corrections. Basically the manuscript should follow the guidelines carefully. The main points are described below.

RESPONSE: We apologize for the grammar mistakes and formatting errors and we now made sure that the manuscript follows TPC guidelines.

Reviewer #3 (Comments for author):

Point 1. Figure 1 B,C; 2A,D, Table 1: The authors use a YFP-DNDMC-1 fusion transcript as a control to show that DNDMC-1 itself is non-mobile. I suggest to also test DNDMC-1 alone without YFP-tag to prove it is not mobile by using the pollen phenotype assay and RT-PCR. By now, it cannot be ruled out that the YFP-tag might cause immobility of the DNDMC-1. A tag-free DNDMC-1 would also show, if the peptide is not mobile. Alternatively, the YFP-DNDMC-1 could be fused to a tRNA element to prove mobility.

RESPONSE: We thank the reviewer for this comment! We had very similar concerns when we presented the data in the manuscript. However, we know that the DNDMC1 RNA is not mobile. One reason is that similar to the YFP fusion not all DNDMC1 RNA fusions are graft mobile. For example, we have performed mobility assays with additional fusions not mentioned in the manuscript (in total more than 12 RNA and protein fusion constructs). We did not include these data in the manuscript as they are intended for another study on alternative RNA mobility motifs (distinct from TLS). I hope that the reviewer considers this and accepts that neither AtDMC1 mRNA nor the corresponding protein contain sequences that renders them mobile.

Point 2. Figure 1 D,E: the data indicate that the 3´ end StBEL5 fusion induces higher mobility to DNDMC-1 than tRNAMet. The authors should state this observation and take it into account in their discussion. Are tRNA-like structures really an important feature of RNA mobility?

RESPONSE: Again, we thank the reviewer for this comment! The reason that we did not discuss this interesting aspect is that StBEL5 fusion construct serves as a positive control and because the focus of this manuscript is on the identified/predicted TLS structure (which seems not to be present in the StBEL5 RNA). Furthermore, we did not discuss the differences as the rate of sterility in wt flowers induced by the mobile transcripts after grafting was in the same order and not significantly different between the two constructs (DNDMC1::StBEL5 / wt with 19.7 ±14.3 % vs. DNDMC1::tRNAMet / wt with 14.2 ±7.6 %; see Figure 2 and Supplemental Table 1).

Point 3. Figure 2A (middle figure): Why does the scion-expressing YFP-DNDMC-1 not show any pollen phenotype? Is the transgene under the estradiol promoter? If yes, the authors should include a control of estradiol-inducible pollen phenotype.

RESPONSE: The intention here was to establish a control showing that we have no interference with mobile siRNA. Thus, we tested many transgenic grafts with YFP-DNDMC1 expressing transgenic lines as a “wt control” to have a readout for siRNA phenomena in CLSM assays. This allowed us to confirm that we have no aberrant production of graft-mobile siRNA by the used DNDMC1 transgenics giving a similar sterility readout. Thus, we have chosen a fusion not acting in a dominant negative (DN) fashion as the fused YFP sequence at the N-terminus interferes with its DN activity. As mentioned above, we have tested many more additional constructs including three N-terminal protein fusions (other than YFP) that show similar suppression of the DN activity due to N-terminally fused protein sequences.

Point 4. Figure 2B,C; 3 B,C: The pollen phenotype and partly RT-PCR results indicate expression of transgenes also without estradiol treatment, which indicates that this promoter leaks under the experimental conditions chosen.

RESPONSE: That is exactly what we observed. All tested lines displayed leaky expression and thus we decided to perform grafts with wild type lines to confirm mobility. Please also consider that we do not simply present the best inducible lines and consider that the expression as well as the phenotypic readout was increased by addition of estradiol in all used lines. (see PCR assays in Figure 1 and 2 and in Supplemental Figure 1).

Point 5. Figure 3C: the data indicate tRNA-mediated mobility of GUS in approx. 20% of cases. It stays a bit shady, if tRNA-structures are really an important feature in RNA mobility. Otherwise, there might be better reporter systems than GUS to evaluate RNA mobility. Interestingly, reducing the tRNA structure to a simple stem-loop structure (ΔDT-loop) obviously increases mobility of the reporter. It would be interestingly to see, if only the single A-loop of tRNAGly (minimal functional element) can switch tRNAIle or any other non-mobile transcript into a mobile one.

RESPONSE: Here, we respectfully disagree regarding the “shady”: The deletions clearly show two aspects. One is that part of the tRNA structure is providing mobility and not the tRNA function per se or a secondary modifications by tRNA editing enzymes producing mature tRNAs. The other aspect is that we went a step further and deleted subsequences to evaluate what would be the sequence part of the tRNA providing mobility. We were also surprised that one particular deletion combination actually worked better than the “wt” tRNA Met or Gly fusions and discuss

this in the text. Regarding the domain shuffling suggestions: That is exactly what we are currently planning to do. This will take at least one to two years because we have to establish stable transgenic lines and make hundreds of grafts and analyze also single base variants. Regarding the use of another reporter: This is a good suggestion indeed and we will be considering this in future assays. Nevertheless, please consider that we decided to use GUS as many of our colleagues are using this enzyme to test cell-autonomous expression patterns in e.g. promoter and genomic fusions and it is well established in the community that the used GUS reporter protein and mRNA is not mobile.

Point 6. Figure 4D: The RT-PCR analysis proves that the wt CK1 transcript is mobile, because a PCR product using primers that span C-terminal end of CK1 and the tRNAGly only give signals in ck1.2 mutants grafted with wt plants (indicated by asterisks). The fact that the truncated CK1.2 transcript cannot be detected in the WT plants that are grafted with ck1.2 mutant indicate that this transcript version is not mobile. However, this might be, because the mRNA became simply too big to be mobile (+1122 nucleotides). A transgene version of ck1 with (di-cistronic) or without the tRNA element expressed in the ck1.2 background would proof, if tRNA is necessary for ck1 to be mobile.

RESPONSE: We thank reviewer 3 for this insightful comment. That is exactly one of the reasons why we used the large GUS mRNA::tRNAGly fusion to provide additional evidence that this tRNA sequence found in in CK1 transcripts also mediates mRNA transport. Our data show that this particular tRNA sequence found in CK1 transcripts is sufficient to trigger GUS mRNA mobility. Regarding the potential length changes: As discussed in a number of reviews, we do not see a bias in movement due to the length when dealing with actively transported macromolecules via plasmodesmata. Of course, small protein (e.g. GFP) diffusion occurs also over graft junctions, but we do not see the GFP transcript or BASTA resistance encoding mRNAs to be mobile in A. thaliana (see Thieme et al. 2015). A GFP transcript (GFP cDNA length is ~ 1000 bases) or a non-fused GUS transcript is not graft-mobile. However, CK1 cDNA which has >1600 bases, or the significantly larger GUS::tRNA fusions (>2000 bases) are mobile. Also the long DNDMC1::StBEL mRNA fusion (> 3000 bases) used in N. tabacum grafts is mobile. Thus, we conclude that active mRNA transport mediated by a TLS or other potential motif(s) e.g. found in small viroids (<200 bases) whose mutant versions are non-mobile (see published work by Biao Ding) is not related to the size of a particular mobile RNA.

Point 7. Figure 4E: The authors should provide a Western blot of CK1 in WT and ck1.2 mutant. Although the mRNA levels of CK1 might be comparable in both lines, translational efficiencies might be affected in ck1.2 that would explain the plant morphological phenotype, rather than mRNA mobility.

Moreover, GUS-promoter assays in WT plants might reveal spatio-temporal expression of CK1 that would support CK1 mobility and function.

RESPONSE: We agree with both statements. An analysis of the CK1 protein presence and CK1 promoter activity would be highly desirable. However, due to the lack of an available CK1 antibody or a GUS genomic fusion, we feel that this is beyond the scope of the given time frame.

TPC2015-01056-BRR1 2nd Editorial decision – *accept with minor revision* May 10, 2016

In particular, please consider the following:

- all grammar, style, and formatting corrections as appropriate;

- questions concerning interpretations and/or discrepancies

- additional control data if they exist already.

We do not expect the additional experiments suggested by reviewer 3 but her/his open questions or alternative explanations should be discussed.

TPC2015-01056-BRR2 2nd Revision received May 25, 2016

Reviewer comments and author responses:

Reviewer #1 (Comments for author):

The authors have addressed all of my concerns and I find the publication suitable as a Breakthrough Report. The addition of evidence for bicistronic transcripts that include a tRNA sequence is a particularly nice addition to this revised manuscript.

RESPONSE: We thank the reviewer for his/her thoughtfulness.

Reviewer #2 (Comments for author):

The revised manuscript has been improved, including some modifications and corrections according to the comments by the reviewers. However it still needs some more modifications and corrections.

Point 1. Fig. 3: In ΔD and ΔDA tRNAmet there are apparently no transcripts by RT-PCR in wild type roots while GUS activity shown by arrows was detected. Is this contradiction because of a problem of detection level by RT-PCR or the samples used? Some explanation for that is needed in the results.

RESPONSE: Here a misunderstanding seemed to have happened: The level of GUS mRNA is very low and barely detectable in wild-type shoot/leaves as limited GUS-tRNA mRNA seems to be transported from hypocotyl grafted transgenic roots into wild type and not vice versa (into wild-type roots grafted with transgenic shoots). Please note that by using more than 45 RT-PCR cycles (e.g. 50 cycles), we could observe specific GUS mRNA in a small number of grafted plants, however, we prefer to show data obtained under the same experimental RT-PCR conditions (45 cycles for all grafts) for reasons of consistency and comparability.

Point 2. Fig. 4 E: There is no description on the phenotypes (like those in F) of grafted plants. It needs to mention what happened to the mutants (ck1-1 and ck1-2) grafted onto the wild type rootstocks and its interpretation in the results.

RESPONSE: Please note that we did not perform a phenotypic analysis of the grafted ck1 mutant plants. We tried this approach but realized that ck1 mutant growth (size) differences cannot be measured reliably after grafting as both wild type and mutant auto-grafted plants show high fluctuations in growth rate due to variant graft junction healing times. In our hands grafted plants show a highly variable growth rate not allowing us to measure a statistically significant difference of size or growth rate.

Point 3. Page 11, line 282: Calderwood et al. (2016) mentioned that "mRNA mobility can be explained by transcript abundance and half-life". It may possible that by inserting TLS in mRNA the stability, contributing to "transcript abundance and half-life", of mRNA can be increased. Can the experiments here exclude the possibility that "half-life" of mRNA is an important factor for the mobility? In this context some more discussion is needed here.

RESPONSE: Please note that, while we agree that the argument that half-life may play a role in facilitating mobility, at this point it is a theoretical concept only without being backed by experimental assays. Calderwood et al. also acknowledge that there is no stringent correlation between stability and mobility as a high number of analyzed mobile mRNAs deviate from the expected mRNA mobility predicted based on stability and abundance. Also, we performed a comparable expression-to-mobility correlation analysis and detected only a weak correlation between expression levels of mobile versus supposedly non-mobile transcripts, when we account for the chance of detecting a mobile transcript in RNAseq analysis (discussed in Thieme et al. 2015). In addition, in the submitted manuscript, we show by quantitative RT-PCR that the stability of the CK1 transcript lacking the tRNA-Gly did not change compared to the wild-type transcript (Figure 4), however, the ck1.2 - produced transcript was still not mobile. Furthermore, we did not notice a visual correlation between the GUS (blue color) stained transgenics and the mobility of the GUS mRNA fusions; and, as discussed (see page 12-13; Lines 334 to 341), we propose that stability is not a major determinant of mobility.

Reviewer #3 (Comments for author):

[The points correspond to the points and author responses provided by Reviewer 3 for previous verion].

Point 1: The answer of the authors is not convincing. Firstly, they should include data that support non-mobile character of DN-DMC1 either by additional fusion constructs, or as the reviewer suggested by testing an untagged DN-DMC-1, or an YFP-DN-DMC-1 fused to a mobility tag. Furthermore, what is the proof that the DN-DMC1 peptide is not mobile?

RESPONSE: We hope that the reviewer can now be convinced by the inclusion of new data in Supplemental Figure 1, new panel E, F, and G, supporting our claim that DNDMC1 RNA or protein is not mobile and do not contain a motif triggering mobility. These figures show data with plants expressing NtCET2::DNDMC1 RNA fusion constructs. Here it is obvious that the NtCET2::DNDMC1 RNA fusion is fully functional by inducing pollen sterility in transgenic plants or scions (>50% pollen sterility). As with tRNAMet::DNDMC1, this NtCET2 RNA fusion construct produces a truncated dominant negative acting (DN) DMC1 protein. Thus, if the DNDMC1 RNA or encoded protein were mobile by themselves, they should induce a pollen sterility phenotype in wild-type flower after grafting. However, we did not detect statistically significant appearance of misshaped (sterile) pollen in flowers on wild-type scions grafted on NtCET2::DNDMC1 stocks (Supplemental Figure 1, new panel F). In addition, note that the vector backbone was the same as used for the tRNAMet::DNDMC1 RNA fusion construct which is both functional and mobile (Supplemental Figure 1, new panel E). Furthermore, we present new RT-PCR data showing that the YFP-DNDMC1 fusion mRNA is not detected in the scion flowers (Supplemental Figure 1, new panel G).

Taken together, these new data suggest that neither the DNDMC1 protein nor DNDMC1 RNA contain mobility triggering motifs, nor does the DNDMC1 protein itself diffuse into wild-type flowers. Furthermore, as mentioned in our previous answer, please note that comparable results were obtained with transgenics expressing StBEL5::DNDMC1 RNA fusions or DNDMC1-GFP/YFP protein fusions, which we prefer not to present for the reasons already stated above.

Point 2: I disagree with the answer. The data of Fig.2 are less informative due to high data variability (>50-70%), than of Fig.1 (<40%). StBEL5 does not carry a TLS, but provide mRNA mobility, which should be discussed.

RESPONSE: Please note that mobility of StBEL5 mRNA, which we use as a positive control, was published by the Hannapel lab in more than three manuscripts and that we cite this work. Thus, we do not agree that it is necessary to include a lengthy discussion on its mobility in the presented context. We already provide a short discussion on the StBEL5 mobility in the text (see discussion text Lines 326-334). As proposed by Hannapel, StBEL5 seems to carry a PTB binding motif. This motif is distinct from a TLS-related sequences, which is mentioned in the discussion text. Such a PTB binding motif seems not to be present in the used tRNA fusions, and tRNA motif(s) seem not to be present in StBEL5 mRNA.

Another aspect mentioned by the reviewer relates to high variability of the sterility readout. This is due to expression differences between the individual transgenic lines and due to the fact that we show pooled/summarized data from different grafted transgenic lines and from biological replicates. Regarding the question whether tRNA-like sequence motifs (TLS) are important in providing mobility: Here, the reviewer may want to consider that we fused tRNAs to GUS and observe GUS mobility only in the presence of the TLS. Also, we observe CK1 transcript mobility only in the presence of the endogenous tRNA sequence. Furthermore, we found TLS motifs to be significantly enriched in the mobile mRNA population. In a nutshell, we do not rely on one line of evidence based on one construct or one model system. Rather, we employ two independent biological systems (N. tabacum and A. thaliana) and two RNA fusion constructs (DNDMC1 and GUS) to prove that TLS provide mobility.

Point 3: The authors give here [in response to reviewer comments] important information that is missing in the text. The authors should include this and their intention why they use YFP-DN-DMC1 as a control for transgene-induced silencing, otherwise the Fig.2A stays very confusing and not understandable. For instance, it is nowhere mentioned that an N-terminal tag abolishes the DN effect in DMC-1.

RESPONSE: To meet the reviewer's suggestion, the text was changed (Lines 114 to 133) as follows:

"To implement an mRNA mobility reporter system, we produced following transgenic Nicotiana tabacum plants: lines expressing YFP-DNDMC1 fusion proteins as a fluorescent reporter (Figure 1B, C) to test for DMC1 silencing signals (Zhang et al. 2014) potentially induced by the employed transgenic DNDMC1 constructs; lines expressing DNDMC1 mRNA fused to the known mobile full-length StBEL5 transcript (Cho et al., 2015) (DNDMC1::StBEL5) as a positive control (Figure 1A); lines expressing DNDMC1 mRNA 5' fused to the vegetative growth regulator CENTRORADIALIS-like 2 (Amaya et al. 1999) (CEN2::DNDMC1) (Supplemental Figure 1) as a negative control; and lines expressing DNDMC1 mRNA fused to full-length tRNAMet (AT5G57885; DNDMC1::tRNAMet; tRNAMet::DNDMC1) (Figure 1A), which was detected in the phloem sap of pumpkin (Zhang et al., 2009). Independent transgenic plants expressing DNDMC1 mRNA fusion constructs were verified to show a pollen sterility phenotype (Figure 1D, E) and used in grafting experiments (Figure 1F, G) to evaluate transcript mobility from transgenic source tissue to wild-type flowers. Transgenic lines expressing the YFP-DNDMC1 fusion did neither exhibit a dominant-negative effect on endogenous NtDMC1 function (Figure 1B), nor was the fusion transcript graft mobile

(Supplemental Figure 1). Thus, these plants could be used to evaluate grafted DNDMC1 transgenic plants for their potential production of mobile DMC1 siRNA targeting endogenous NtDMC1 in wild-type flowers triggering sterility (Zhang et al., 2009). "

and in Line 163 - 171:

" To exclude the possibility that the grafted chimeric plants produce a mobile DMC1 siRNA silencing signal moving into wild-type flower tissues and triggering a pollen sterility phenotype (Zhang et al., 2014), we grafted the DNDMC1::tRNAMet plants with the YFP-DNDMC1 fusion reporter line, which can be easily detected by fluorescence microscopy. In contrast to the DMC1 siRNA control lines, no systemic siRNA mediated silencing of the YFP-DNDMC1 reporter construct could be detected in sepals (Figure 2F). Thus, the DNDMC1::tRNAMet fusion transcript does not induce systemic silencing and the observed defects in pollen formation in grafted plants (Figure 2G) can be attributed to the systemic delivery of the DNDMC1 fusion transcripts.

Point 4: OK

RESPONSE: We are pleased that the reviewer agrees.

Point 5: The reviewer`s concerns are not satisfactory addressed here. The authors seem to agree that the GUS assay might not be appropriate, but they don`t attempt to verify their data with a second reporter system (e.g. GFP, YFP). Secondly, I consider exchanging the A loop in tRNA-Ile with the one of tRNA-Met as a not very time-consuming experiment (1-2 months), of course single nucleotide analysis would go beyond the reviewers suggestion.

RESPONSE: Please note that we did not agree that GUS assay might not be appropriate. We mentioned that it is a good idea to include additional constructs (besides the already used DNDMC1 and GUS), which we will consider for future experiments. Anyway, the reviewer may consider that we already published that transgenically produced YFP RNA and BastaR RNA is not mobile (Thieme et al. 2015) and, as previously mentioned, we are currently making the transgenic constructs and plants with point mutations in the tRNA sequences. In our opinion, we consider establishing new deletion constructs and stably expressing transgenic lines and to perform >50 grafts and all RT-PCR work in just 1-2 months not possible. In our hands, this takes > 8 months (by an experienced postdoc), which involves approx. 50 grafts for one construct (note that this should be multiplied by three, as negative and positive controls have to be included) and establishing independent, stably expressing, and confirmed transgenics, and the necessary RT-PCR assays and biological replicates.

Point 6: I agree with the author`s answer.

RESPONSE: We are pleased that agreement was reached.

Point 7: Time limitation is not a scientific argument. However, I understand that making a CK1 AB or a CK1-tagged line as well as a GUS-reporter line for CK1 is time consuming. Nevertheless the authors should at least state that they cannot rule out that observed phenotypes might be caused by effects other than mRNA mobility, as stated by the reviewer.

RESPONSE: Following the suggestion of the reviewer, we changed the wording in the text mentioning that translational activity of the ck1.2 mutant transcript might be an alternative explanation. Lines 274-277:

" This implies that either ck1.2 plants produce less functional CK1 enzyme due to the lack of the 3' tRNA sequence or that CK1 mRNA presence in expressing cells as well as CK1 mRNA mobility is equivalently essential for normal growth behavior of Arabidopsis."

TPC2015-01056-BRR2 3rd Editorial decision – *acceptance pending* May 30, 2016

We are pleased to inform you that your paper entitled "tRNA-related sequences trigger systemic mRNA transport via the phloem" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor June 7, 2016