April 7, 2020

Dear Editor,

We are grateful for your timely evaluation of our manuscript (Plant Direct: 2020-00428: ENGINEERING *MAIZE RAYADO FINO VIRUS* FOR VIRUS-INDUCED GENE SILENCING). We also thank the reviewers for their critical comments. Our revisions to the manuscript in response to each suggestion are listed below. We believe that the changes and corrections made have greatly improved our manuscript.

Sincerely,

Lucy R. Stewart

Research Molecular Biologist
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Response to Reviewer Comments

Reviewer #1:

The authors describe development of an infectious clone of maize rayado fino virus (MRFV) and application of MRFV for virus-induced gene silencing in maize. Their results indicate that MRFV will be an important new tool for gene silencing applications in maize. The authors tested four different insertion sites for VIGS inserts in the virus genome, including one that was previously shown to work for the related tymovirus, Turnip yellow mosaic virus. However, they found that a cloning site at the junction of the coding sequences of the helicase and polymerase proteins could be used without disrupting virus replication and systemic movement. The use of this cloning site is novel for members of the Tymoviridae and may be informative to other groups interested in making vectors from these viruses. The silencing phenotypes are quite impressive in maize plants, and they correlate with a strong knockdown of target gene expression and accumulation of corresponding siRNAs. Surprisingly, the inserts are very stable in this virus over the course of infection and following as many as four serial passages. The authors also demonstrated that the MRFV carrying pds could be transmitted by the leaf hopper vector with infrequent loss of inserts. This aspect is interesting, because it further supports the stability of the inserts. The MRFV clone induced VIGS in sweet corn and inbred dent corn lines B73, Mo17, and Va35. The results presented in this manuscript suggest that MRFV carrying inserts at a novel position can be a very effective gene silencing tool for the maize research community.

We thank the reviewer for recognizing the novelty and utility of our approach and results.

1. I was curious about the cloning strategy to insert MRFV derivatives into the pJL89 binary vector, but then authors used PCR to amplify the cloned genomes with primers that added T7 promoter and polyA tail and then infectious transcripts were generated by in vitro transcription. Did authors attempt to use an agroinoculation approach in maize and it was unsuccessful?

Agroinoculation of maize has been tried and efforts to optimize protocols have yet to show if this approach may work with MRFV delivery into maize tissue or not. Very few viruses are effectively delivered to maize by agroinoculation methods currently available. (MSV, maize streak virus, and MCMV, maize chlorotic mottle virus, are two, both highly infectious viruses that are also readily mechanically transmitted by DNA/cDNA inoculation even without agrobacterium). Preliminary experiments indicated that MRFV agroinoculation may result in local replication in the nonhost plant *Nicotiana benthamiana,* but further confirmation is needed. Since this was not a robust delivery method and would still require vascular puncture to deliver to maize (it can be an effective clone scale-up for rub-transmissible viruses, but MRFV is not), we did not pursue this avenue further. VPI is very robust for launching MRFV infection with viral transcripts, and bypasses adding an artificial nuclear step that occurs when attempting to launch an RNA virus through DNA delivery to plants. We cannot rule out the future possibility of agroinoculation methods to launch MRFV but, for focus, chose not to put preliminary failures of that approach into this manuscript.

2. Line 344 - healthy
We have accordingly made the correction to the typo by changing health to healthy.

3. Line 368 - citations here are not given as numbers
We have made the correction, with citations now given as numbers.

4. Line 420 - This reviewer is not convinced by the "TRV (37)" paper that is cited here
We agree that the maize photobleaching shown in this paper is very unusual for VIGS. As we cannot validate the claims of this paper independently and have not seen replication of the reported results, we have removed this reference from the manuscript.

5. Line 471 - it would be interesting to know the upper size limit tolerated by this virus, but not essential for this manuscript. For example, could authors express a small ORF like iLOV or nanoLUC? Such an ability could be useful to researchers who want to express small fungal and bacterial effectors in maize or overexpress small maize proteins, etc.
We have yet to determine the upper insertion size limit tolerated by the virus. Efforts to express GFP have resulted in rapid loss of large portions of the sequence, indicating that this is above the carrying capacity for MRFV (although size limits for virus vectors also can be sequence-dependent). Future research to evaluate small peptides and further explore the

capacity and limits of this vector are certainly next steps. We thank the reviewer for the suggestions of iLOV and nanoLUC.

6. Line 657 – healthy
We have accordingly made the correction to the typo by changing health to healthy.

7. Line 665-666 - is leaf 9 always used for 30 dpi?
To clarify this, we have inserted the following text in Materials and Methods section: Virus was RT-PCR assayed in ninth leaves 30 days post VPI, and in last leaves preceding tassels (~16th leaf) 60 days post VPI. We have accordingly modified the subsection heading to "*Plant inoculation, growth conditions and virus detection).*

8. Figure 8d - this panel is not cited in the text or explained in the figure legend. Should be deleted?
The panel (Figure 8d) was included out of an editing error in the first submission and has now been deleted. We apologize for the confusion this caused.

9. The Northern blot data was very convincing, but it would have been interesting to see a percentage of down regulation, like would be obtained from qRT-PCR. It is not essential, but if authors have such data, it would be good to add.
We have used ImageQuant software to measure relative band intensities as a quantitative measure of down regulation. The values have been incorporated into northern blot figures (Figure 3a, 3b, 6d, 7d, 8c) and an explanatory line added to the respective figure legends. This being a well-established quantitation method for Northern blots, we believe that it markedly improves the presentation of our data. The manuscript text now includes fold changes in silencing of PDS and LSP mRNAs. We thank the reviewer for this input.

Reviewer #2:
I have gone through the manuscript entitled with 'ENGINEERING MAIZE RAYADO FINO VIRUS FOR VIRUS-INDUCED GENE SILENCING'. Please find my comments below.

The authors convincingly showed establishment of a virus-induced gene silencing tool using the MRF virus in maize. They identified a region in which silencing fragments between 120 and 231 bp can be integrated without loss of infectivity. They demonstrated the stable transmission to non-infected leaves and the stability during several passages. The transmission via the natural leafhopper D. maidis was also confirmed. In addition, they showed that several maize accessions can be used, so it seems no obvious genotype-dependency.

I have only some minor points, which might be considered before acceptance.

In general, keywords should not repeat the title. Please choose some different words describing the content of the manuscript.

We have addressed this by choosing keywords that are not part of the title. We have thus replaced 'virus-induced gene silencing' and '*Maize rayado fino virus*' with 'Marafivirus' and '*Dalbulus maidis'* as keywords

L79 Since sgRNA is commonly used for single-guided RNA in the CRISPR RNA/Cas endonuclease system I would avoid this abbreviation. Since it is only four times used within the manuscript I suggest to write the full name.

Although the abbreviation sgRNA has long been used in virology (preceding discovery of CRISPR/Cas), to avoid confusion to a broader plant biologist audience we have removed sgRNA from the text and used the full name (subgenomic RNA) instead.

Other Corrections

At the end of the results subsection entitled "*Northern blot analysis of the chlorophyll photobleaching phenotype induced by MRFV-PDS$_{120}$ in maize,"* we have added a sentence for Northern data of MRFV-PDS$_{120}$ VIGS in maize tassels and silks (this was an unintended omission from the initial submission). Residual inconsistencies in nomenclature usage and format were also corrected ('noninoculated' replaced wherever interchangeable use of 'un-inoculated' remained, and 'photobleaching' replacing residual instances of 'photo-bleaching', etc.).