Regensburg, 09.09.2018

Dear Dr. Steindel,

We are pleased to resubmit the manuscript “Guidelines for using *Ustilago maydis* as a Trojan horse for in situ delivery of maize proteins” and would like to thank the editors and reviewers for their time and helpful comments, which allowed us to improve the manuscript quality.

We revised our manuscript according to the editorial instructions (e.g., adding “Notes” and splitting some of the steps) and added further information on issues which seem to have been unclear before and which were kindly highlighted by you and the reviewers. This involves adding more detail to the protocol (e.g., regarding the preparation of *U. maydis* inoculum or recommended time frames for the respective assays) as well as including more information in the discussion as was requested by reviewers 2 and 3 (e.g. regarding the size limit of candidate proteins, possible uptake of the protein by the plant cell, or use of crossed, compatible *U. maydis* strains).

Please find a detailed response to all editor and reviewer comments on the next pages. Major changes in figures, supplemental figures, and the manuscript are highlighted in red.

Best regards,



**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response:

We performed a final proofreading of the manuscript and removed all remaining issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Author response:

We did not reuse any figures from previous publications for this manuscript.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Author response:

We made corrections throughout the manuscript.

4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Author response:

We changed the manuscript accordingly.

5. Please use centrifugal force (x g) for centrifuge speeds.

Author response:

We changed this throughout the manuscript.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., “we”, “you”, our” etc.).

Author response:

We changed the manuscript accordingly.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

Author response:

Regarding the phrase “should be”, we altered the manuscript.

8. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Author response:

We added notes at multiple steps throughout the manuscript.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Author response:

We altered the manuscript accordingly in steps 1.1, 1.4, and 1.5.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:2.3.2: Please add more details here about how to investigate the culture. What is observed?What type of water is used, deionized/distilled? What volume of water is used to wash? Please specify throughout.2.3.7: What volume of water is used to resuspend the cells?

Author response:

Regarding step 2.3.2 (now step 2.3.4), please see the newly added Figure 2 and line

145.

Regarding the type of water, we replaced H2O by ddH2O.

Regarding step 2.3.7/2.3.8: please see new steps 2.3.8 and 2.3.9.

Moreover, further details were added to the protocol for steps 2.3.1, 2.3.3 (formerly part of 2.3.1), 2.3.4 (formerly 2.3.2), 2.3.8 (formerly 2.3.6), 2.3.9 (formerly 2.3.7), 2.4.2, 2.5.3 and 2.8.1. Steps 2.3.2, 2.5.6, 2.6.6 and 2.7.6 were newly added (see also response to reviewer #2, point 5).

11. Please reference Table 1 in the manuscript.

Author response:

Please see line 95.

12. Discussion: Please discuss critical steps within the protocol and any limitations of the technique.

Author response:

For discussion of critical steps within the protocol, please see lines 358-363 and 368-373.

Possible limitations are addressed in lines 339-345 and 356-368.

13. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Author response:

We changed references throughout the manuscript.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Author response:

We changed the references to the above-mentioned style.

15. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Author response:

We include full journal titles, volumes, and issue numbers in all references.

**Reviewer #1:**

1. I wonder if the method can be used for the introduction of genes from an origin distinct to plants.

Author response:

As mentioned in line 71 and line 345-350 of the manuscript, theoretically any protein of interest can be

secreted into the apoplast, thus including non-plant proteins.

**Reviewer #2:**

Major concerns:

1. Determining protein function(s) in situ is critical to advancing our understanding of the roles they play. But this method seems tailored to the analysis of small, secreted maize proteins that exert their function from the apoplast on a cellular process that must be measured with a microscope. Can the Trojan horse strategy be used to study the function of other types of proteins like an enzyme, transcription factor, signaling molecule, receptor or structural protein? If so, please give examples where this as worked.

Author response:

We now added some information on this issue within the discussion (lines 340-350); see also Editorial comments #11 and Reviewer #1.

2. The abstract states the strategy can be used to transiently complement a loss-of-function phenotype, characterize the function of protein domains, and study off-target or over-accumulation effects. This was done quite elegantly for the ZmMAC1 protein but have the authors shown this will work for any other protein, even another small secreted peptide (e.g., any of the CLE-related peptides)?

Author response:

As mentioned in line 65-67, the method was also successfully applied to the investigation of the maize peptide ZmZIP1.

3. Do the secreted proteins remain in the apoplast where they exert their effects at the plasma membrane? Or are they taken up by the adjacent cells where they might also function in the cytoplasm or nucleus? This might be mentioned as it would be important for choosing what type of protein to use with this strategy.

Author response:

We added information about this to the discussion (lines 344–366).

Minor concerns:

1. Is there a size limit or recommended maximum on the size of the protein that can be expressed and secreted (especially since it is fused to mCherry already)?

Author response:

We now added information on this issue in lines 345-350.

2. Smut tumors appear within 7, 10 or 12 days depending on tissue and maize cultivar used. I assume tumor formation might affect the analysis of the protein being studied and so is there a recommended time frame for when protein effects should be assessed (e.g., between 1 - 3 days after inoculation but not past 5 days)?

Author response:

We added Note 2.1, which gives instructions on the proper timing for different experiments.

3. In line 113, three independent U. maydis transformants should be used to assess protein function. Does this mean, each independent transformant should be used in replicated inoculations? Or can the transformants bulked prior to inoculation? Or should this be avoided?

Author response:

A more detailed explanation towards this issue is now added to this step (lines 110-

112). We thank the reviewer for highlighting any misleading phrasings.

4. For the negative control, you suggest using the untransformed SG200 strain but also you suggest using a "no signal peptide" version of the studied protein. Are both needed? It seems, the "not secreted" version of the studied protein would be the best negative control. If there is another compelling reason to also use the untransformed SG200 strain, please state why.

Author response:

We now added further information on this issue within the protocol (see 2.3.1).

5. The two tables are split on multiple pages making them difficult to read.

Author response:

We thank the reviewer for its thorough revision. Both tables have been changed to fit on one page.

6. Some of the grammar and word choices are not correct. Copy editing would help. For example:Line 32 "offside"….. "onside". Perhaps "off-target" and "target (on-target?)" are better.Line 85 The Trojan horse strategy enables "the secretion of" any heterologous…..There are others too.

Author response:

Regarding general grammar/word choices, please view our response to the Editorial comments (#1). We applied the reviewer’s suggestion for “off-target” instead of “offside” (line 32).

**Reviewer #3:**

1. Therefore, the authors need to describe in the introduction the following papers:The Corn Smut ('Huitlacoche') as a New Platform for Oral Vaccines. PLoS ONE 10(7): e0133535. https://doi.org/10.1371/journal.pone.0133535; and The corn smut-made cholera oral vaccine is thermostable and induces long-lasting immunity in mouse. Journal of Biotechnology (2016), Pages 1-6. https://doi.org/10.1016/j.jbiotec.2016.04.047; in this papers the production of an oral vaccine using the Ustilago maydis expression system has been successfully achieved.

Author response:

We thank the reviewer for the suggestion and added the respective literature to our citations in the introduction as well as in the discussion (see lines 56 and 348).

2. Another important point is that the authors propose to use the strain SG200, this strain being only pathogenic would have the advantage of not requiring the crossing of 2 compatible strains; however, in my experience the infection efficiency is low with the SG200 strain, compared to using compatible strains such as FB1 and FB2, so the authors could mention the possibility of using compatible strains in their system.

Author response:

We now addressed this issue in the discussion (see line 358-366).