Nov 09, 2018

Dear Dr. Steindel,

Thank you for your letter of October 22, 2018 with editorial and peer two reviews of our Manuscript No. JoVE59169 "Inhibition of *Aspergillus flavus* α-amylase in maize expressing the *Lablab purpureus* AILP gene: analysis of fungal growth and aflatoxin production", by Kanniah Rajasekaran, Ronald J. Sayler, Christine Sickler, Rajtilak Majumdar, and Jeffrey W. Cary. The title of the revised manuscript is "**Inhibition of *Aspergillus flavus* growth and aflatoxin production in transgenic maize expressing the *Lablab purpureus AILP* gene".** Thank you again for approving additional time to complete our revision.

We appreciate the editorial and reviewers' comments and have revised the manuscript in accordance with the suggestions. They have definitely helped us improve the paper. We took this opportunity to thoroughly proofread our manuscript to correct syntax and typographical errors. Our revision has been highlighted in Red in the manuscript text as well (no track editing to avoid clutter). The following is our item-by-item disposition of the comments and suggestions (in Red):

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
DONE

2. Please revise lines 319-321 to avoid previously published text.
DONE (lines 373-376)

3. Please revise the title to be more concise and avoid punctuation.
The title is revised to read "**Inhibition of *Aspergillus flavus* growth and aflatoxin production in transgenic maize expressing the *α*-amylase inhibitor from *Lablab purpureus* L."**

4. Please provide an email address for each author.
DONE

5. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Revised Summary is provided

6. Abstract: Please include an overview of the presented method and a summary of its advantages, limitations, and applications.
We have included an overview of the Kernel Screening Assay and highlighted its advantages, limitations and applications

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For

example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
DONE

8. 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. See examples below.
We have indeed provided additional details as pointed out

9. Line 100: Please also describe PCR conditions.
DONE (lines 113-118)

10. Line 111: Please specify growth conditions (temperature, light conditions).
DONE (lines 126-127)

11. Line 172: Please add more specific details about DNA isolation or provide relevant references.
DONE (lines 185-190)

12. Line 174: Please list PCR primers and conditions.
DONE (lines 192-195)

13. Line 182: Please add more specific details about RNA isolation or provide relevant references.
DONE (lines 202-205)

14. Line 187: Please add more specific details about preparing cDNA or provide relevant references.
DONE (line 206)

15. Lines 198-199: Please list an approximate volume of solutions to prepare.
DONE (line 215)

16. Line 223: Please provide more specific details.
We do not know the specific details of the proprietary information. However, we have given the information as available from the manufacturers, Romer Labs. (lines 240-242)

17. Please include single-line spaces between all paragraphs, headings, steps, etc.
DONE

18. Please remove commercial language (TriDye™).
DONE

19. Line 274: Please use a superscripted number for the reference.

DONE (line # 301 in the revision)

20. Figure 4: Please explain what different lanes are. Please consider combining Figure 4 and Figure 5 to reduce the total number of figures
DONE – see Figure Legends

21. Figure 6: Please explain what different bars represent. Different samples?
DONE

22. Figure 8 and Figure 9: Please consider combining Figure 8 and Figure 9 to reduce the total number of figures.
DONE - see Figure Legends

23. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the Name of Material/ Equipment.
DONE – sorted the list according to Name of Material/Equipment

24. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have added three paragraphs in the Discussion with citations to cover all the details suggested above.

25. References: Please do not abbreviate journal titles.
DONE

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript reports a protocol that should be of interest to research scientists interested resistance to Aspergillus flavus infection and aflatoxin contamination. It likely has applicability to other fungi as well with appropriate modifications.

Minor Concerns: The germplasm line Hi-II is highly susceptible to aflatoxin contamination. The effects of AILP would be better determined in lines better adapted to field conditions. I encourage the evaluation of the effects of AILP in other genetic backgrounds when future

experiments are undertaken.

Thanks to this reviewer for the comments. We agree that Hi-II hybrid is a susceptible to aflatoxin contamination. However, we worked with this variety for two reasons: 1) this is one of the few easily regenerable maize variety available when we started this work seven years ago; 2) any improvement in antifungal resistance can easily be measured in this susceptible line after prolonged selfing, as we have demonstrated in this paper. However, we agree that we will be using other promising inbred lines in our future experiments so that we could transfer the trait to commercial lines.

Reviewer #2:

Manuscript Summary:
Paper is interesting main results, well described and straightforward. I've some minor concerns and some point I'd be glad authors discuss further

Minor Concerns:
Please provide the data on af inhibitions normalized on fungal growth. The inhibition rate is meaningful but should be adjusted upon fungal growth inhibiting effect 'bias'.

The graph that we generated showing ng/g (ppb) levels of aflatoxin is one of the standard way of presentation in the scientific literature and also by the corn farming/marketing community. For these simple reasons, we would like to keep the graph as is; however, as per the reviewer's suggestions we have recalculated the inhibition rate devoid of bias due to fungal growth inhibition and is provided as a supplemental file. We have also provided a regression coefficient analysis, as a supplemental file, linking a close correlation between GFP values (=fungal growth) and aflatoxin levels.

Please provide a study on the omologues or orthologues presenting consistent match to your amylase inhibitor. The effect on maize growth - reduction - could fit better if you experience some cross inhibitory effects on maize omo/orthologues.

This is a relevant question. Production of α- amylase inhibitors targeting pathogen/pests are reported in a wide variety of plant species. This serves as an important natural defense component in plants. The substrate binding specificity of the plant derived inhibitors of pathogen's α- amylase is unique and only targets and inhibits α- amylases from pathogen/pests. Several investigators (Woloshuk and others cited in our manuscript) demonstrated previously the uniqueness of α-amylase inhibitor gene *AILP* from *Lablab purpureus* against *A. flavus* and other fungal pathogens. We also observed that transgenic expression of the *AILP* gene in maize did not negatively affect phenotype, growth or seed set in transgenic maize plants suggesting that the *AILP* gene does not affect maize native α- amylases.

If some protein sharing partly the same sequence and/or function exists, it could be possible to propose a genome editing (crisp cas9) approach instead of "classic" transgender strategy. It could

be more 'palatable' even for countries experiencing the AF problem but with strict legislation on the US of gmo. This point is solely intended for discussion.

We agree that CRISPR/Cas9 gene editing is favorable to transgenes from various sources. However, it is essential to first identify the gene(s) that we can go after for gene editing. As we know now, resistance to this saprophytic *A. flavus* is multigenic in maize other susceptible crops and it is difficult to identify native genes. Further transcriptomic/metagenomic analyses may provide clues to identifying suitable target genes for editing, which we are currently undertaking. For example, if there are specific maize α- amylases that are targeted by the pathogen for successful pathogenesis that would be a prime target for editing to increase resistance (without compromising agronomic traits). Hopefully all our and collaborators' research efforts will lead to selecting the most efficient, consumer-friendly tool to incorporate resistance to this toxic fungal species.

Hopefully I have answered all the comments or suggestions to your satisfaction and that the revised manuscript is now acceptable for publication in JoVE.

Thank you,

Best regards,

Kanniah Rajasekaran