Thank you for the opportunity to submit a revision of JoVE59153 "Detached leaf assays: A simplified approach to study gene expression in potato during infestation by the chewing insect Manduca sexta. Each editorial and reviewer comment is answered in blue text. For ease of reading and continuity of line numbering, all line numbers referenced in this document refer to the **‘clean’ version** of the manuscript.

**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.

The manuscript has been checked for spelling and grammar.

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].”

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3. Please revise the title to avoid punctuation.

Title has been changed from “Detached leaf assays: A simplified approach to study gene expression in potato during infestation by the chewing insect *Manduca sexta*” to “The use of detached leaf assays to simplify gene expression studies in potato during infestation by the chewing insect *Manduca sexta”*

The revised title has been added to the document. However, the authors feel that the original title conveyed the importance of the simplified protocol, namely the detached leaf assay, better with the colon.

4. Keywords: Please provide at least 6 keywords or phrases.

Additional keywords have been added to the manuscript.

5. Please define all abbreviations before use.

This has been done for the first use of each abbreviation throughout the manuscript.

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

All units have been changed to SI abbreviations.

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

The manuscript has been checked to make sure there is a space between values and their corresponding units.

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.

Detail has been added throughout the protocol and per editor’s specific requests in 9-14 below.

9. 1.1.1.3: Please specify the preservative/biocide used in the protocol.

The preservative/biocide is Plant Preservative Mixture (PPM) and was originally not included because it is a proprietary mixture. It has been added to the protocol in line 130.

10. 1.1.1.4: Please specify the sterile culture vessels used in the protocol.

The sterile culture vessels are Magenta or Plantcon and are trade names for this type of culture vessel. This has been added to the protocol in steps 1.1.1.2 (line 133), 1.1.2.2 (line 142), 1.2.1.2 (line 160), and 1.2.2.2 (line 170).

11. 1.1.2.4: What is the composition of 1:1 bleach?

1:1 bleach is 1 part RO (reverse osmosis) purified water and 1 part concentrated germicidal/commercial grade bleach. This information has been added to the protocol in step 1.1.2.1 (lines 139 and 140).

12. 1.2.1.1: Please specify the amount of nutrient agar powder added.

36.43 g

This has been added to the protocol in step 1.2.1.1 (line 154).

13. 3.1.1, 3.4.1: Please specify the size of the petri dish.

The size of the petri dish is dependent on the size of the leaf used for the infestation. An example size has been added to the protocol for clarity in the NOTE immediately after step 3.1.1 (line 238 and 239). Additionally, two size options are listed in the materials list.

14. 7: Please specify how to calculate % damage of leaves.

This detail has been added in step 8 (lines 314-317) of the protocol.

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that shorter steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

The protocol has been reorganized and essential steps for video production have been highlighted.

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Complete sentences have been highlighted and contain at least one action written in the imperative tense.

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Each step’s sub-steps have been included in the highlighted section of the protocol.

18. Figure 2, Figure 3 (I-IV): Please name these video clips as movie 1, 2, 3, 4, and 5, respectively.

Figure 2 has been changed to movie 1 and Figure 3 (I-IV) have been changed to movie 2-5 in both the legend section and the results section.

19. Figure 4: Please define error bars in the figure legend.

The error bars in Figure 4 (now Figure 3) are standard deviation (line 399).

20. Lines 458-461: Please remove the weblink and use a superscripted numbered reference instead.

The weblink references have been removed, replaced with the superscripted numbered references 40-42 (line 465), and properly referenced in the reference section (line 639-line 651).

21. References 3, 8: Please provide complete citation information including journal name, volume number, and page number.

Citation information has been added to complete reference 3 and 8.

22. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

The Materials list has been sorted alphabetically.  
  
**Reviewers' comments:**  
  
  
  
**Reviewer #1:**

No changes were suggested by this reviewer.  
  
**Reviewer #2:**

Major Concerns:  
none  
  
Minor Concerns:  
-Lines 129 and 161 "15psi" This is not an international unit for pressure. The United States is probably the only country in the world that still uses pounds and inches.

15 psi has been changed to 101.3 kPa (line 131 and 158).

-Line 185: change to "Fewer cuttings"

“Less cuttings” has been changed to “Fewer cuttings” (line 177).

-Line 278: Are sterile scissors really needed to feed a leaf to a non-sterile caterpillar in a relatively short feeding assay?

Yes. The introduction of microorganisms not inherent to the plant or insect may affect gene expression.

-Line 321: Please explain how percent damage is measured and calculated based on the images.

This was added to the protocol in step 8 (lines 314-317).

-Line 457: Please list commercial sources, for those who do not maintain a Manduca colony.

Reference 38 and 39 were added to specify sources of *Manduca sexta* and sources were also added to the materials list.

-Figure 1: For a controlled study, it would be better to randomize the positions of the leaves. Otherwise, there can be environmental effects, e.g. one side of the room is slightly darker or cooler.

Since only one tray/placement template/set of 6 leaves is set up at a time, and each set makes a relatively small footprint we didn’t consider randomizing the leaf positions. This can certainly be done however. A unique placement template could be created for each infestation/harvest time point. If before and after images are recorded, the template (and arrangement of the leaves) would need to be kept consistent.

-Figure 3B: How was the amount of damage quantified? Did this involve some sort of image processing, e.g. Image J, or is this a visual estimate by the researchers?

For panel A, visual estimates were made based on the damage in panel B. Panel B % damage was calculated using a leaf area calculation program called Phenophyte. Detail referring to these two options has been added to the protocol section in step 8 (lines 314-317).

-Figure 4: What statistical test was used to calculate significance, what P-value was used, what do the error bars mean (standard deviation or standard error)? Please provide GenBank ID numbers or some other record locator for the genes.

The following was added in lines 392-403, Figure 3 legend “Mean transcript level is, 2^− dCT with (dCT = CT of test gene-CT of exogenous control gene). Excised control leaves in (blue) and excised infested leaves in (red) are shown at each time point. Each value is the average of three biological replicates. Three-way ANOVA was conducted on the ΔCt values. Significant differences in control leaves over time are shown with capital letters. Significant differences in infested leaves over time are shown in lower case letters. Significant differences between control and infested leaves at the same time point is shown with an asterisk (\*). Pr> F values were all less than 0.001, except for StZFP6 control treatment with 0.0086. Error bars represent standard deviation. NCBI accession numbers are: StLOX3-X96406.1, StZFP2- BQ121105.2, StZFP4-CV500970.1, StZFP6-DN587601.1, StZFP7-DN590005.1. Spud DB accession numbers from <http://solanaceae.plantbiology.msu.edu> are StMYC2-PGSC0003DMT400045204, StZFP3-PGSC0003DMT400040144, StZFP5-PGSC0003DMT400040141”

-Table of Materials: Capitalize Fisher Scientific. Capitalize Eppendorf, if you are recommending use of this specific brand of microcentrifuge tube. Otherwise, call them 1.7 ml microcentrifuge tubes. Add Manduca to the list of required materials for this experiment.

Eppendorf has been changed to microcentrifuge in the materials list. *Manduca sexta* sources have also been added.

-It should be noted that the Manduca larvae used were raised on artificial diet rather than plants (judging by the pictures in Figure 3A). This will undoubtedly affect plant responses.

Yes, we agree. This was mentioned in the protocol (lines 210-212) and discussion (lines 465-469).

-It should be noted that the time of day (or time in the day-night cycle of the growth chamber) matters for these experiments. About half of all plant genes show significant diurnal variation. This is also a reason for analyzing the leaves as quickly as possible. If samples are processed over a two-hour time period, there is likely to be measurable variation in gene expression.

Thank you for pointing this out. The following statement has been added to the paragraph at the beginning of the protocol “Any additional repetitions of the experiment should be set up at the same time of day to eliminate possible diurnal influences on gene expression.” (line 110-111)

The comparison between detached and infested leaves at each time point allows the reader to see the response to infestation and how it compares to detachment. This also indicates how the gene may be affected diurnally.  
  
  
**Reviewer #3:**  
  
Major Concerns:  
1. For the analysis of gene expression in early response to herbivorous damage by the larvae of M. sexta in potato leaves, the authors randomly selected 6 transcription factors belonging to the same group C2H2. Analysis of the expression of genes encoding transcription factors belonging to different families or transcriptome analysis seems to be more interesting. Such global data would significantly increase the value of the entire manuscript.

Our lab is interested in C2H2 ZFPs, especially those that are infestation responsive. While data mining for ZFPs in potato we discovered five potato homologs to *StZFP2* on a 28,000 base stretch of chr 11. There were only 2 homologs in this region of chr 11 in the tomato genome (a close relative of potato). Since we have found that over-expression of *StZFP2* can enhance tolerance to *Manduca sexta* we were curious whether any of the 5 additional StZFP2-like genes were also responsive to infestation. In other words, the method was primarily developed to be a quick ‘screen’ of a subset of similar genes. You have a good point that perhaps a more global test of the assay would be interesting. However, we did examine, StLOX3 and StMYC2 as markers for wounding and infestation. These genes demonstrate that the leaves were responsive to detachment (wounding), but infestation did not significantly enhance their expression.

2. In this article, the authors point out that this simplified approach can be used for the analysis of gene expression profiles, although the aspect related to the description of the expression analysis is strongly overlooked. In this respect, the authors refer to the indicated reference (Lawrence & Novak, BMC Research Notes, 2018). Lawrence & Novak (2018) for analysis of gene expression used quantitative RT-PCR (RT-qPCR). Meanwhile, in this manuscript the authors use Semi-qRT-PCR (line 395). This experiment really needs quantitative real-time PCR. Please explain!!!

Thank you for bringing attention to this because it is an error in the current manuscript! Semi-quantitative has been changed to Real-Time qRT-PCR in the current manuscript (line 391). Both the 2018 publication and this one used the same methods.   
  
Minor Concerns:  
1. Please explain the shortcut RO (page 4 lines: 124, 157).

RO is reverse osmosis purified water. This detail has been added to the protocol in lines 126 and 154.