JoVE51204R1

'Optimization and Utilization of Agrobacterium-mediated Transient Protein Expression in Nicotiana'

Shamloul M. et al.

Dear Dr. Yusibov,  
  
Your manuscript JoVE51204R1 'Optimization and Utilization of Agrobacterium-mediated Transient Protein Expression in Nicotiana' has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.   
  
Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.  
  
**Editorial comments:**  
  
\* Please be sure to use the latest version of your manuscript which can be found on the Editorial Manager.

**Responses to Reviewers’ Comments**

**Reviewer #1**

*Manuscript Summary:*   
Agrobacterium-mediated transient expression system has been known for many years as a rapid and highly efficient method for protein expression in plants. Multiple modifications of this technique, including combining viral suppressors of gene silencing, have been made by a number of researchers to enhance protein expression. The article by M. Shamloul and co-authors adds to this pool of data by optimizing Agrobacterium cultivation procedure to achieve large-scale production of target proteins in plants. I think the paper falls within the mission and scope of the journal and is acceptable for publication in the JoVe because of the thorough and detailed presentation of this useful technology.  
  
*Major Concerns:*  
N/A  
  
*Minor Concerns:*  
On page 4, the authors stated that "The major drawback of industrial scaling of the agroinfiltration technique is centrifugation of harvested bacteria and resuspension of the bacterial pellet in medium containing acetosyringone...We have been able to overcome these problems by optimizing the Agrobacterium growth and infiltration conditions".  
  
It is not clear from the results and/or discussion what exactly has been made in overcoming these challenges: both the centrifugation and resuspension steps are present in the protocol and remain substantially unchanged.

*Author’s response:*

We believe that we have overcome the large-scale infiltration challenges by omitting the centrifugation and induction steps, which simplified the infiltration process and made it applicable for industrial scale. Also, we optimized vacuum infiltration pressure and duration, which allowed for enough *Agrobacteria* penetrating into plant intercellular spaces. Our data showed that infiltration of plants with non-induced *Agrobacteria* growing in AB medium and then diluted in Mill-Q water and with *Agrobacteria* induced in MMA media resulted in similar levels of reporter protein (GFP) production (Figure 1A). Therefore, *Agrobacteria* grown in AB medium and diluted in Mill-Q water to A600 of 0.5 were suitable for large and industrial scale plant infiltration.

*Additional Comments to Authors:*  
N/A

**Reviewer #2**

*Manuscript Summary:*   
This paper addresses several issues related to industrial-scale production of recombinant proteins using transient expression in Nicotiana plants. Authors have tested several aspects of transient expression. Authors have challenged the necessity of centrifugation and re-suspension steps in preparation of Agrobacterium prior to infiltration. Alternative strains of Agrobacterium and Nicotiana were tested to optimize the accumulation levels of the target protein. Authors have also investigated the effects of vacuum pressure and duration on the rate of infiltration, and of chemical induction of Agrobacterium vir genes for optimizing accumulation levels of recombinant proteins. However, there are inconsistencies in experiment descriptions, and some results interpretations are incorrect. No statistics were shown for any quantitative data presented, and even the high resolution images are not of good enough quality. This paper will be a good fit for the JoVE after addressing the following comments.   
  
*Major Concerns:*  
- For all western blots (figs 1, 4 and 5A), please provide the amount of TSP loaded per well, or the fresh weight equivalent that was loaded per well so the reader can assess the quantitation.

*Author’s response:*

The amount of fresh leaf weight equivalent loaded per well has been added to the legends of Figures 1, 4 and 5.

- For all graphs (Figs 2 and 5B), Please show error bars and show statistical significance by running a statistical test. It is not clear if any differences in both graphs are significant.

*Author’s response:*

The error bars have been included in Figure 2. In the Results section, we mention the statistical analysis used showing significant differences between *Agrobacterium* strains GV3101 and C58C1 in LicKM production.

- Effect of silencing suppressors on transient expression: The protocol mentions a ratio of 3:1 for the HAC1 construct, while in the results the authors say they used 4:1. The authors claim that p19 and p23 performed similarly in figure 5B, while the graph clearly shows p19 to be superior, especially in days 4, 5, and 6. It is not clear if the differences are significant at any day, especially at day 7. Error bars are needed, as well as statistical analysis of the data to be able to draw any conclusions. I don't agree with the authors conclusion that p19 and p23 resulted in a similar increase of HAC1, and that they are equally efficient in their system.

*Author’s response:*

We agree with the Reviewer, and the ratio has been corrected in the Protocol to be 4:1. The increase in target protein production in *N. benthamiana* co-infiltrated with the launch vector (TMV-based vector) and a silencing suppressor (approximately, 15-25%) was not significant compared to the 50-fold increase when the silencing suppressor was co-infiltrated with a binary vector (Reference #34). It has been claimed that TMV helicase has a suppressor of RNA silencing activity (References #64 and 65).

- Effect of chemical induction on protein expression: The authors describe the use of glucose and acetosyringone at various amounts to test their effect on induction of agrobacterium vir genes. They conclude from figure 4C that these compounds have no effect on GFP fluorescence or expression, and they hypothesize that the vir genes could be induced by plant phenolic compounds or monosaccharides present during replication of the launch vector in plant cells. However, Figure 4C legend states that the acetosyringone concentration experiment was done in the presence of 2% glucose, and the glucose concentration experiment was done in the presence of 200 uM acetosyringone. The authors don't show a negative control with no glucose and no acetosyringone. This means that an inducer is present in all their treatments, and therefore their conclusions are not valid.

*Author’s response:*

This perception by the Reviewer was because we did not state the reason for developing this experiment clearly enough. The purpose of the experiment was to evaluate the effect of chemicals (monosaccharide and acetosyringone) on *Agrobacterium* vir gene induction. We used different concentrations of chemicals added to MMA induction media as described in the protocol section 3.5. Also, we evaluated protein accumulation in the absence of these chemicals (0 µM of acetosyringone or 0% of glucose) from MMA induction media. Our results show no significant differences in protein accumulation in the presence or absence of these chemicals. In addition, comparison of *Agrobacteria* growing in LB, YEB or AB media followed by dilution in Mill-Q water (non-induced) and *Agrobacteria* centrifuged and re-suspended in MMA induction medium (induced) showed very similar levels of GFP production (Figure 1B). From this experiment we concluded that induction of *Agrobacterium* vir gene is not necessary for the launch vector activity.

- Alternative strains of Agrobacterium. Authors should introduce the LicKM (lichenase enzyme) and the importance of its production since this is the first time it is mentioned. Figure 2 legend does not describe the figure. The legend describes activity of lichenase, quantified by zymography while the graph's Y axis shows lichenase expression in mg/kg. This should be lickenase accumulation not expression if authors are referring to protein levels, and is the value calculated in terms of fresh or dry leaf weight? or is it per total soluble protein weight? Enzyme activity is usually assessed in units/weight. Please either correct the figure or the legend. Also, please explain how you quantify lichenase, is it by densitometry? what standard did you use on the zymogram? Showing the zymogram itself would be very informative.

*Author’s response:*

We agree with the Reviewer and added three sentences introducing the lichenase enzyme (LicKM) and the importance of its production. Also, we included Figure 2B showing the enzyme activity of lichenase produced using all *Agrobacterium* strains comparing it with bacteria-produced lichenase. Bacteria-produced lichenase was used as a standard when estimated LicKM protein production. The Figure and Figure legend have been corrected.

- Figure 1 legend describes lanes with undiluted YEB, AB and LB, but these are not shown on the figure. Please correct the legend.

*Author’s response:*

Figure 1 legend was corrected (YEB, AB and LB were deleted).

- Throughout the manuscript, protein expression should be changed to protein production or protein accumulation. Genes are expressed, not proteins.

*Author’s response:*

Per Reviewer’s request, “protein expression” was changed to “protein production” or “protein accumulation” throughout the manuscript.

- Page 7, paragraph 2, line 11. "In contrast, at 10 dpi no differences in GFP expression were observed among plants infiltrated with the four cell suspension densities (data not shown)". I believe showing this set of data is required, as it will clarify the amount of accumulation levels at 10 dpi compared to 4 and 7 dpi and therefore provides more proof for the argument on line 13: "This occurs because the pBID4 expression vector is able to move from cell to cell but not systemically and therefore, newly grown leaves at 10 dpi do not contain the vector and do not contribute to target expression". Also, the potential reduction of protein accumulation at 10 dpi might be due to degradation of recombinant protein over time. This is another reason why authors should provide information about in vivo targeting of their target proteins.

*Author’s response:*

As requested by the Reviewer, we added a Figure (Figure 1C) showing GFP accumulation at 10 dpi with all *Agrobacterium* densities. We included the suggested sentence “Also, the potential reduction of protein accumulation at 10 dpi might be due to degradation of recombinant protein over time” in the Discussion section.

Also, the last 3 lines of this paragraph can be moved to the discussion section of the manuscript.

*Author’s response:*

We thank the Reviewer for the comment, and the last 3 lines of paragraph 2 (page 7) were moved to the Discussion section.

- Page 11, paragraph 3, line 27-28. The phrase "(up to 50-60% of total soluble protein)" is not accurate and this value is mentioned for the first time by total soluble protein in the manuscript at the discussion. It should be mentioned specifically at the result section first for the reader to be able to accept the argument.

*Author’s response:*

The statement "(up to 50-60% of total soluble protein)” has been deleted from the Discussion section.

- Page 11, paragraph 4. These are results that should be described in the results section, not in the discussion section.

*Author’s response:*

Page 11 paragraph 4 has been deleted from the Discussion and described in the Results section.

- Page 12, paragraph 2. This is also a result to be described in the results section. This sentence is out of place here.

*Author’s response:*

Page 12 paragraph 2 has been removed from the Discussion. We added Figure 5C, to support the data showing stability of *Agrobacteria* harboring the launch vector for more than three years in the Result section.

- Page 12, paragraph 5, line 11. "?GFP at 6-7 dpi was equal?". No quantitative data about this argument are presented in the result section. Please show this data.

*Author’s response:*

We added Figure 3B to compare the estimated GFP accumulation at 7 dpi. We deleted 6 dpi from the text.

*Minor Concerns:*  
- In the long abstract, bacteria were diluted in "ddH2O". In the manuscript, water was referred to as "Mill-Q" most of the time and "H2O" some other time. It is suggested that authors choose one of the formats and follow that at all times.

*Author’s response:*

H2O was changed to Mill-Q water throughout the manuscript.

- In the introduction, first paragraph, line 8, (and throughout the manuscript) et al should change to et al.

*Author’s response:*

The indicated correction has been made.

- Under section 1.1, brand name, company and the formulation of "fertilizer solution" is missing.

*Author’s response:*

The company name, product and the catalog number were added in the Material and Equipment Table.

- Under section 3.2, indicate the scientific name of "acetosyringone".

*Author’s response:*

The scientific name of “acetosyringone” was added to the text.

- Figure 5. Figure legend should explain what abbreviations (W/O) and (W/) mean (without and with).

*Author’s response:*

The abbreviations (W/O) and (W) were deleted from Figure 5B.

- Paragraph 2, line 4. Need to explain what "cGMP" stands for since this is the first time it has been mentioned in the manuscript.

*Author’s response:*

The definition of “cGMP” has been added to the Discussion section.

*Additional Comments to Authors:*  
N/A

**Reviewer #3**

*Manuscript Summary:*   
The JoVE article by Shamloul et al. describes in detail the optimization of conditions for using agrobacterium-mediated protein expression in leaves of Nicotiana benthamiana plants. It is clearly and easily followed for the most part. The technique will be of considerable interest for those using agroinfiltration for the production of proteins in plants.  
  
*Major Concerns:*  
There are no major concerns.  
  
*Minor Concerns:*  
The description is straightforward, but there are a few places in the text that are unclear:  
1) Page 12, second paragraph "While the glycerol stock of GV3011?" sentence is not complete. The word 'while' suggests a comparison to something, but that something is missing.

*Author’s response:*

The sentence has been corrected and Figure 5C was added to the Results section to support the statement.

2) The authors list the vector pBID4 and when looking up the reference to that vector it is not clear what the base of that vector is - I assume it is TMV as reference is made later in the text to silencing suppressors and TMV. More detail about the launch vector should be given.

*Author’s response:*

We are a little confused by this comment. The vector had been fully described in our former publication (Musiychuk et al., 2007 [ref #18]). This reference has been mentioned in the Abstract, Introduction and Discussion.

3) On page 4, item 3.3 in the protocol, a description is made of diluting cultures grown in AB media with Mill-Q water prior to infiltration. However, in 3.2, the cells grown in LB or YEB media are centrifuged and resuspended in MMA. It is not clear if the AB cultures are centrifuged or just diluted directly in water.

*Author’s response:*

The sentence has been corrected to clarify the method of *Agrobacterium* dilution.

4) In 4.1, are the agrobacteria containing the silencing suppressor constructs treated in the same way as agrobacteria containing the pBID constructs described in 3.2 and 3.3?

*Author’s response:*

*Agrobacteria* harboring a silencing suppressor have been treated in the same way as *Agrobacteria* carrying the pBID constructs: both were diluted in Mill-Q water. This is described in Protocol sections 4.1 and 4.2.

**Reviewer #4**

The study by Shamloul et al described the optimization of Agrobacterium-mediated transient protein expression in Nicotiana. To achieve the goals, the effect of different culture conditions, stains of Agrobacterium, selecting host species, vacuum pressure and duration, concentrations of acetosyringone and glucose, and RNA silencing suppressors were evaluated. Species N. excelsiana was proven as the most promising host with the highest biomass generation under the same growth conditions. The experiments in the paper were well designed and carried out. The results of this manuscript were carefully analyzed and provide solid information to better understand the mechanism of Agrobacterium-mediated transient protein expression in Nicotiana. However, some experiments were not clearly described in the Protocol section, and some statements in the discussion were not correlated with the results.  
  
*Below are some comments to the manuscript:*   
  
1. More information should be summarized in the Abstract section, including the effect of different strains of Agrobacterium, vacuum pressures and concentrations of chemicals on protein expression.

*Author’s response:*

We have modified the Abstract and added more information about *Agrobacterium* strains, the chemicals used for induction, and vacuum pressure and duration.

2. In the Protocol section, the methods of all the experiments performed in the manuscripts should be clearly described.  
A) The authors mentioned that phosphorus in the nutrition solution was critical to plant germination. The information of nutrition solution should be supplied in 1.2.

*Author’s response:*

The company name, product and the catalog number were added in the Material and Equipment Table.

B) 2.2: More details need to be provided about how to introduce the launch vectors into GV3101 strain.

*Author’s response:*

The method of *Agrobacterium* transformation was added in the Protocol section 2.2. Also the company name, product and the catalog number of the equipment used were added in the Material and Equipment Table.

C) As shown in Figure 1A, cultures grown in YEB, LB or AB media were diluted with Mill-Q water (1:5, A600 of 0.6-0.8 or 1:10, A600 of 0.3-0.4). All of these should be mentioned in 3.3.

*Author’s response:*

The dilutions of *Agrobacteria* were mentioned in the Results section in detail. We also mentioned these in the Protocol section 3.2 (unless otherwise noted). Since this is a specific experiment conducted to study the effects of undiluted and diluted *Agrobacterium* broth on plant health, we would like to leave the sentence in the Results section unchanged.

D) 3.4: Change "50-100 mbar vacuum for 60 sec" to "50-400 mbar vacuum for 30s or 60s". Which tissue was submerged in Agrobacterium suspension?

*Author’s response:*

The sentence has been changed in the section 3.3.

E) 4.1 and 4.2 should be inverted.

*Author’s response:*

The sections 4.1 and 4.2 have been inverted.

F) 4.2: "?at a 3:1 ratio?" All the ratios (1:1, 2:1, 3:1 and 4:1) should be mentioned.

*Author’s response:*

All the ratios (1:1, 2:1, 3:1 and 4:1) have been added in the Protocol section 4.1.

G) 5.2: The ingredients of phosphate-based buffer should be provided?

*Author’s response:*

Since we used PBS buffer for protein extraction, we changed phosphate-based buffer to 1 x PBS buffer in the Protocol.

H) How to perform the infiltration with selecting Agrobacterium strains need to be included in the Protocol section.

*Author’s response:*

The method of *Agrobacterium* strains infiltration has been added to the Protocol section 3.6.

I) How to perform the chemical induction should be supplied in the Protocol section.

*Author’s response:*

The method of chemical induction has been added to the Protocol section 3.5.

3. Standard errors need to be supplied for Figure 2 and Figure 5B.

*Author’s response:*

Error bars have been included in Figures 2 and 5B.

4. Page 7: "at 10 dpi no differences in GFP expression were observed? therefore, newly grown leaves at 10 dpi do not contain the vector and do not contribute to target expression." This conclusion could not be drawn based on 'no differences in GFP expression were observed". Do you mean "no GFP expression was observed"?

*Author’s response:*

We observed no GFP expression in newly growing uninfiltrated leaves post infiltration, due to lack of systemic movement of the launch vector. This has been corrected and moved from the Results section to the Discussion section, as per request from Reviewer #2.

5. Page 8: How long would it take for N. excelsiana to reach infiltration readiness?

*Author’s response:*

*N. benthamina* and *N. excelsiana* reached the infiltration readiness at 4-5 weeks. We have added this information in the Protocol section 1.3.

6. Page 8: "Under the same growth conditions, the highest biomass can be generated from N. excelsiana: two to three fold higher than N. benthamiana and 50% higher than N. excelsior." It's better to have a table to summarize the amounts of biomass in different host species.

*Author’s response:*

A summary table (Table 1) has been added to show a comparison of leaf biomass of *N. benthamina* vs. *N. excelsiana* growing under same growth conditions for 5 weeks. We excluded *N. excelsior* because it required 7 weeks to reach infiltration readiness.

7. Page 9: The conclusion "co-infiltration of N. benthamiana with p19 or p23 resulted in a similar increase in HAC1 expression compared to using no silencing suppressor (~15-25%). This suggests that p19 and p23 are equally efficient in our system" is questionable. As shown in Figure 5B, the increases in HACL expression with p23 and p19 are not similar, especially before 5 dpi. Also, statistical analysis needs to be performed.

*Author’s response:*

We agree with the Reviewer, the increase (approximately, 15-25%) in target protein production in *N. benthamiana* co-infiltrated with the launch vector (TMV-based vector) and a silencing suppressor was not significant compared with a 50-fold increase observed when a silencing suppresso wasr co-infiltrated with a binary vector (Reference #34). It has been claimed that TMV helicase has an activity of suppressor of RNA silencing (References #64 and 65).

8. Page 11: "while some genes inserted into pBID4 and transformed into laboratory strains GV3101, C58C1 or LBA4404 elicited mild necrotic responses and leaf chlorosis/yellowing symptoms in infiltrated regions of leaves." Was this statement concluded from your observation? If yes, it should be mentioned in the Results section. If not, references need to be cited.

*Author’s response:*

We added Figure 2C showing the effect of *Agrobacterium* strain on plant phenotype post infiltration. We also included a statement in the Discussion section.

9. Page 12: "While the glycerol stock of GV3101 transformed with launch vector (cell bank) stored at -80°C has been very stable for three years without changes in transient protein expression in infiltrated plants." Data need to be provided in the Results section to support this statement.

*Author’s response:*

The sentence has been corrected and Figure 5C was added to support the statement.

10. Page 12: "N. benthamiana grown under our optimal conditions and between 35 and 42 days post sowing were optimal for vacuum infiltration-mediated transient gene expression." Data need to be provided in the Results section to support this conclusion.

*Author’s response:*

The citation for the indicated sentence has been added (reference #40).

11. Page 12: "GV3101 cultures harboring pBID4-GFP and diluted in H2O to an A600  
of 0.5 and infiltrated without the vir gene induction expressed the same amounts of GFP as those infiltrated with induced cultures." Was this statement concluded from your observation? If yes, it should be mentioned in the Results section. If not, references should be cited.

*Author’s response:*

As requested by the Reviewer, the data have been added in the Results section (Figure 1B). The comparison of *Agrobacteria* growing in LB, YEB or AB followed by dilution in Mill-Q water (non-induced) or centrifuged and re-suspended in MMA induction medium (induced) shows very similar levels of GFP production.