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Optimization and Utilization of Agrobacterium-mediated Transient Protein Production in Nicotiana

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Author Comments:	

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Question	Response

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“Optimization and Utilization of Agrobacterium-mediated Transient Protein Production in Nicotiana” by Shamloul M et al.

Attention of: Larissa A. Jarzylo, Ph.D.
Science Editor
JoVE

Dear Dr. Jarzylo,

Thank you for providing us the reviewers’ comments and for the opportunity to revise our manuscript to improve its editorial and scientific quality. We have accurately addressed the comments, and the revised manuscript (with tracked changes), figures and tables as well as the point-by-point response letter are submitted to JoVE. We hope our revisions and responses will make our manuscript suitable for publication in your journal.

Thank you very much for your attention.

Yours sincerely,

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Optimization and Utilization of *Agrobacterium*-mediated Transient Protein Production in *Nicotiana*

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Keywords:

Agroinfiltration, *Nicotiana benthamiana*, transient protein production, plant-based expression, viral vector

Short Abstract

Transient protein production in *Nicotiana* plants based on vacuum infiltration with *Agrobacteria* carrying launch vectors (*Tobacco mosaic virus*-based) is a rapid and economic approach to produce vaccine antigens and therapeutic proteins. We simplified the procedure and improved target accumulation by optimizing conditions of bacteria cultivation, selecting host species, and co-introducing RNA silencing suppressors.

Long Abstract

Agrobacterium-mediated transient protein production in plants is a promising approach to produce vaccine antigens and therapeutic proteins within a short period of time. However, this technology is only just beginning to be applied to large-scale production as many technological obstacles to scale up are now being overcome. Here, we demonstrate a simple and reproducible method for industrial-scale transient protein production based on vacuum infiltration of *Nicotiana* plants with *Agrobacteria* carrying launch vectors. Optimization of *Agrobacterium* cultivation in AB medium allows direct dilution of the bacterial culture in Mill-Q water, simplifying the infiltration process. Among three tested species of *Nicotiana*, *N. excelsiana* (*N. benthamiana* × *N. excelsior*) was selected as the most promising host due to the ease of infiltration, high level of reporter protein production, and about two-fold higher biomass production under controlled environmental conditions. Induction of *Agrobacterium* harboring pBID4-GFP (*Tobacco mosaic virus*-based) using chemicals such as acetosyringone and monosaccharide had no effect on the protein production level. Infiltrating plant under 50 to 100 mbar for 30 or 60 sec resulted in about 95% infiltration of plant leaf tissues. Infiltration with *Agrobacterium* laboratory strain GV3101 showed the highest protein production compared to *Agrobacteria* laboratory strains LBA4404 and C58C1 and wild-type *Agrobacteria* strains at6, at10, at77 and A4. Co-expression of a viral RNA silencing suppressor, p23 or p19, in *N. benthamiana* resulted in earlier accumulation and increased production (15–25%) of target protein (influenza virus hemagglutinin).

Introduction:

Plants are now recognized as a safe, reliable, scalable and inexpensive platform for producing heterologous recombinant biopharmaceuticals and industrial proteins¹⁻³ and have important advantages over microbial and animal cell expression systems⁴. Plants are able to express correctly folded proteins with post-translational modifications, including assembled multimeric antibodies⁵⁻⁷. Several plant-derived recombinant pharmaceutical proteins are undergoing clinical evaluation⁸. These include patient-specific recombinant idiotype vaccines (scFv) for the treatment of non-Hodgkin's lymphoma⁹, hemagglutinin-based pandemic and seasonal influenza vaccine candidates^{10,11} (Cummings et al., submitted to Vaccine), anti-Streptococcus surface antigen I/II antibody for the treatment of dental caries¹², and human insulin for the treatment of diabetes¹³. Furthermore, human recombinant glucocerebrosidase for enzyme replacement therapy in patients with Gaucher disease has been approved in Israel and the US and is provided under the Expanded Access Program outside of the US^{14,15}.

Heterologous proteins can be produced in stably transformed (transgenic or transplastomic) or transiently transformed plants. Transient protein production offers several advantages over production in transgenic plants, including short timeframe to achieve expression and accumulation¹⁶, and can be achieved by introducing bacterial binary vectors or recombinant plant viral vectors into plant tissues⁴. The most advanced transient expression system is based on the use of 'launch vectors' that combine components of plant viruses and binary plasmids, and are delivered by agroinfiltration^{17,18}. Agroinfiltration of a launch vector based on *Tobacco mosaic virus* (TMV) has been successfully applied at lab scale to produce vaccine antigens against pathogens such as human papilloma virus¹⁹, *Yersinia pestis*²⁰, influenza viruses A^{21,22}, *Bacillus anthracis*²³, and smallpox virus²⁴ in *N. benthamiana* leaves. *Agrobacterium*-mediated transient expression is also a promising method for the simultaneous production of multiple proteins^{2,25-27}. For example, plant transient expression systems have been used to produce tumor-specific recombinant antibodies^{28,29}, a glycosylated recombinant antibody against the epidermal growth factor receptor³⁰, and a monoclonal antibody specific for anthrax protective antigen^{31,32}. Co-infiltration of *Nicotiana benthamiana* plants with a target gene and a suppressor of gene silencing results in enhanced target protein expression^{33,34}.

Agroinfiltration is a common method for uniformly introducing bacteria harboring a gene of interest into plant tissues³⁵⁻³⁷. Vacuum infiltration of *Agrobacterium* for transient gene expression in intact plant leaves is a rapid, scalable, and useful method for production of foreign proteins without the need to generate transgenic plants³⁸⁻⁴¹. During vacuum agroinfiltration, plants are flipped upside down and aerial parts submerged in *Agrobacterium* suspension. Then vacuum is applied causing gases to evacuate from leaf intercellular spaces through stomata. Rapid re-pressurization following release of the vacuum results in the infusion of the *Agrobacterium* suspension into the leaf. Following vacuum infiltration of *Agrobacteria*, plants are further cultivated and target expression is monitored. The highest levels of target expression are typically observed 2-3 days post infiltration (dpi) with a binary vector and 4-7 dpi with a launch vector, after which the expression level typically decreases^{17,18,42-45}. *Agrobacterium tumefaciens* is the most widely used vehicle for delivering a gene of interest into a plant for protein production. Agroinfiltration works exceptionally well in *N. benthamiana* but relatively poorly in most other plants, including *Arabidopsis thaliana*⁴⁶.

In this study, we developed a simple, efficient, and economical method for transient protein production in 5–6 week-old *N. benthamiana* using *A. tumefaciens* infiltration. The major drawback of industrial scaling of the agroinfiltration technique is centrifugation of harvested bacteria and resuspension of the bacterial pellet in medium containing 4'-hydroxy-3',5'-dimethoxyacetophenone (acetosyringone), monosaccharides, and 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer for induction of the *vir* genes. We have been able to overcome these problems by optimizing the *Agrobacterium* growth in AB medium (minimal medium) followed by directly diluting in Mill-Q water and by controlling the infiltration duration and conditions. We have also compared target protein production in the wild-type tobacco host species *N. benthamiana* and *N. excelsior*, as well as in hybrid *N. excelsiana*.

Protocol:

1) Plant growing

For subsequent agroinfiltration we evaluated two wild-type *Nicotiana* species (*N. benthamiana* and *N. excelsior*) and a hybrid (*N. excelsiana*) grown hydroponically on rockwool in indoor facilities.

1.1) Soak rockwool slabs in a plant fertilizer solution.

1.2) Sow seeds of wild-type *N. benthamiana*, *N. excelsior* and *N. excelsiana* (hybrid of *N. benthamiana* × *N. excelsior*) on the nutrients soaked rockwool surface.

1.3) Grow plants from the seeds under controlled conditions (24°C and 40–65% relative humidity) and a long-day photoperiod (14 h light and 10 h dark, with illumination of 130–150 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 4–5 weeks for *N. benthamiana* and *N. excelsiana*, and 5–6 weeks for *N. excelsior*.

2) Construction of vectors for agroinfiltration

2.1) Insert a synthetic reporter gene (green fluorescent protein [GFP]), full-length hemagglutinin (HA) from the A/California/04/2009 strain of influenza virus (HAC1), and re-engineered lichenase enzyme (LicKM)¹⁸ separately into the launch vector pBID4¹⁸ (TMV-based vector) to obtain pBID4-GFP, pBID4-HAC1 and pBID4-LicKM, respectively^{18,32,41,47}.

2.2) Introduce 10–50 ng of pBID4 carrying GFP or HAC1 into electrocompetent cells of *A. tumefaciens* strain GV3101 and LicKM into electrocompetent cells of *A. tumefaciens* strains GV3101, C58C1, GLA4404, At06, At10, At77 and A4 with the gene electroporator.

2.3) Use the transformed *Agrobacteria* for infiltration experiments unless otherwise noted.

3) Vacuum infiltration of *Agrobacterium* into *Nicotiana* plants

3.1) Grow *A. tumefaciens* strains overnight (o/n) in LB medium, YEB medium or AB medium supplemented with 50 mg/L of Kanamycin at 28°C with shaking at 200–250 rpm.

3.2) Dilute *Agrobacteria* in Mill-Q water to an optical density at 600 nm (A_{600}) of 0.5 or centrifuge *Agrobacterium* cells grown in LB or YEB or AB at $4,000 \times g$ for 10 min at 4°C, re-

suspend in induction medium (1x MS salt, 10 mM MES, 200 μ M acetosyringone, 2% sucrose [MMA]) to A_{600} of 0.5, and stir at room temperature for 1-3 h, unless otherwise noted.

3.3) Infiltrate plants in a vacuum chamber by submerging *Nicotiana* plant aerial tissues in *Agrobacterium* suspension and applying a 50-400 mbar vacuum for 30 or 60 sec. The optimal infiltration is routinely applied at 50-100 mbar for 60 sec.

3.4) Once the vacuum is broken, remove plants from the vacuum chamber, rinse in water, and grow for 5–7 days under the same growth conditions used for pre-infiltration growth.

3. 5) To test the efficacy of chemicals inducing *Agrobacterium* vir gene, different concentrations of acetosyringone (0, 100, 200 or 400 μ M) were added to the *Agrobacteria* suspended in infiltration buffer (1x MS, 10 mM MES, 2% glucose). For the effect of monosaccharide on induction of vir gene, different percentages of glucose (0, 1, 2 or 4%) were added to *Agrobacteria* suspended in the infiltration buffer (1x MS, 10 mM MES, 200 μ M acetosyringone). *N. benthamiana* plants were infiltrated as mentioned above in steps 3.3 and 3.4).

3.6) *Agrobacterium* laboratory strains GV3101, C58C1 and LBA4404 and wild-type strains A4, At06, At10, and At77 harboring the pBID4-LicKM vector were diluted in Mill-Q water to A_{600} of 0.5. *N. benthamiana* plants were infiltrated with each particular strain as mentioned above in steps 3.3 and 3.4.

4) Co-agroinfiltration procedure for the viral silencing suppressor

4.1) Mix the Mill-Q water-diluted *Agrobacterium* GV3101 cultures carrying the GFP gene and the viral silencing suppressor p19 of *Tomato bushy stunt virus* (TBSV) at 1:1, 2:1, 3:1 and 4:1 ratios. Infiltrate *N. benthamiana* plants as described above.

4.2) Infiltrate *N. benthamiana* plants with a mixture of two Mill-Q water-diluted *Agrobacterium* GV3101 cultures: the first carrying the pBID4-HAC1 plasmid and the second carrying one of the silencing suppressors – p19 of TBSV or p23 of *Citrus tristeza virus*, in the pCassp plasmid (pCassp19) and in the pGR binary plasmid under the 35S promoter (pGR-P23), respectively, at the ratio 4:1.

5) Western blot analysis

5.1) Collect random leaf samples from *N. benthamiana*, *N. excelsior* or *N. excelsiana* plants at 4–7 dpi and pulverize in liquid nitrogen to a fine powder.

5.2) Add three volumes of 1x PBS buffer containing 0.5% Triton X-100 to each sample.

5.3) Gently shake the extracted samples for 15 min at 4°C.

5.4) Spin the extract for 5 min and collect total soluble protein into a clean Eppendorf tube.

5.5) Dilute the extracts to an appropriate dilution (1:50 1:100) in 1x PBS extraction buffer, and add 5x sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 0.5% bromophenol blue, 50% glycerol v/v, and 500 mM DTT) to a final 1x concentration.

5.6) Boil samples for 5 min.

5.7) Separate proteins by 10% SDS-PAGE, transfer onto Immobilon-P transfer membrane, and block with 0.5% I-block.

5.8) Detect GFP using rabbit polyclonal anti-GFP antiserum at 1:5,000 and HAC1 using mouse anti-poly-histidine monoclonal antibody at 1:1,000 in blocking solution for 1 h.

5.9) After primary antibody labeling, wash the membranes three times for 10 min each with 1x PBST-20 and incubate with a horseradish peroxidase (HRP)-conjugated anti-rabbit antibody at 1:5,000 or a HRP-conjugated anti-mouse antibody at 1:10,000 for 1 h, for GFP and HAC1 detection, respectively.

5.10) Process Western blots using the SuperSignal West Pico Chemiluminescent substrate or a similar reagent.

5.11) Use the GeneTools Software to analyze band intensity of the protein and obtain the band calibrated quantity.

Protein production: (Calibrated quantity * dilution of sample)/amount of sample loaded) * 4 = mg/kg.

$$P_{(\text{mg/kg})} = \frac{(C \times D)}{S} \times 4$$

Equation: Protein production (P), Calibrated quantity (C), Dilution of the sample (D) and amount of Sample loaded (S).

6) Zymogram assay

6.1) Collect pBID4-LicKM-infiltrated *N. benthamiana* random tissue samples.

6.2) Extract proteins using the same methods described above for Western blot analysis and then analyze by 10% SDS-PAGE with 0.1% lichenan included in the gels.

6.3) After electrophoresis, wash the gels two times for 10 min each in wash buffer (100 mM Tris-HCl [pH 8.0] and 0.1% Triton X-100) and then incubate in wash buffer at 65°C for 1 h.

6.4) After incubation, discard the wash buffer and stain the gels with 0.5% Congo Red for 5 min at room temperature.

6.5) Rinse the gels in Mill-Q water three times for 10 min each, and add 1 M NaCl to visualize lichenase activity. The purified bacterial lichenase protein was used as a positive control for enzyme activity.

7) GFP imaging

7.1) Perform visual detection of GFP fluorescence in whole transiently transformed plants using a hand-held long-wavelength UV lamp.

7.2) Photograph transiently transformed plants with a digital camera through a Yellow 8, ES 52 filter (exposure time, 15 sec).

7.3) Obtain images from Western blot analyses using the GeneSnap software on a GeneGnome and quantify the results using the GeneTools software, with a calibration curve based on purified GFP standard.

7.4) Quantify HAC1 protein using a calibration curve based on purified HAC protein standard from the A/Indonesia/05/05 strain of influenza virus.

7.5) Calculate mean values from 3-4 replicates for all experiments.

Representative Results:

Nutrient requirements for plant growth. The use of hydroponic plant growth medium (Rockwool) and nutrient solution ensures uniformity of *N. benthamiana* growth and eliminates complexities (mechanical, regulatory and efficiency) associated with using soil for plant cultivation. We grew *N. benthamiana* on rockwool slabs soaked in commercially available fertilizers to determine the optimal conditions for plant growth and biomass accumulation. We observed 95–100% seed germination. One should note that including phosphorus is critical to achieve germination, because we found that nutrient solution lacking phosphorus failed to support germination and growth of *N. benthamiana* seeds (**Figure 1A**).

Effects of *Agrobacterium* growth and infiltration media on plant health and protein production. We have tested several media conditions to optimize the efficiency of the agroinfiltration technique for large-scale production. Bacteria (*A. tumefaciens* GV3101 strain) harboring the pBID4-GFP construct were cultivated o/n in different media conditions (YEB, LB or AB), and either centrifuged and re-suspended in induction medium (MMA) (containing 1× Murashige & Skoog [MS] Basal Salt Mixture, 10 mM MES pH 5.6, 20 g/L sucrose and 200 µM acetosyringone) or diluted in Mill-Q water to A₆₀₀ of 0.5 before using for plant infiltration. We observed that vacuum infiltration of plants with bacteria diluted in water resulted in protein production comparable to those achieved with any infiltration media in previous reports^{42,48}. In contrast, infiltration with undiluted *Agrobacteria* grown in YEB or LB media resulted in complete wilting of *N. benthamiana* leaves in less than 24 h post infiltration, while undiluted *Agrobacteria* grown in AB medium had no effect on the health of infiltrated plants (data not shown). As illustrated in **Figure 1B**, plants infiltrated with *Agrobacterium* cultures grown in YEB, LB or AB media and diluted with Mill-Q water (1:5, A₆₀₀ of 0.6-0.8 or 1:10, A₆₀₀ of 0.3-0.4) showed no symptoms and exhibited an average GFP production of 1645, 1520 and 1839, respectively. *Agrobacteria* centrifuged and re-suspended in induction medium (MMA) showed no symptoms and no significant difference in protein production compared to *Agrobacteria* directly diluted in Mill-Q water (1671 ± 102 and 1667 ± 131 mg/kg, respectively). Therefore,

Mill-Q water is recommended for diluting *Agrobacterium* cultures for plant infiltration and was routinely used in our subsequent experiments to achieve an A_{600} of 0.5.

Effects of *Agrobacterium* suspension cell density and time course on target expression. We next examined if bacterial cell density affects the efficiency of infiltration and levels of target expression. For this purpose, we assessed four different cell suspension densities of *Agrobacterium* carrying pBID4-GFP, A_{600} of 1.0, 0.5, 0.1 and 0.05. Following infiltration, *N. benthamiana* plants were monitored for visible symptom development and time course of target expression by collecting samples at 4, 7 and 10 dpi. At 4 dpi, we observed noticeable differences in GFP fluorescence among plants infiltrated with different cell suspension densities of *Agrobacterium* (no GFP expression was observed at A_{600} of 0.05). At 7 dpi, GFP fluorescence was similar in plants infiltrated at cell suspension densities of A_{600} 1.0, 0.5 and 0.1, but was lower in plants infiltrated at an A_{600} of 0.05. As shown in **Figure 1C**, these data were confirmed by Western blot analyses of samples collected at 4 dpi, showing very low protein production at A_{600} of 0.05 (5 mg/kg) and highest at A_{600} of 1.0 (1739 mg/kg). At 7 dpi, plants showed no significant differences in estimated GFP production at A_{600} of 1.0, 0.5 and 0.1 (1662, 1870 and 1890, respectively), while A_{600} of 0.05 showed lower GFP production (1199 mg/kg). In contrast, at 10 dpi no differences in GFP production were observed among plants infiltrated with either of the four cell suspension densities (1218, 1181, 1197 and 1304).

Infiltration with alternative strains of *Agrobacterium*. To increase the diversity of *Agrobacterium* strains available for transient protein production, we tested wild-type isolates. These strains, isolated from the crown-gall of natural hosts, were kindly provided by Dr. Gelvin (Purdue University, West Lafayette, Indiana). To examine their utility in transient protein production, we infiltrated *N. benthamiana* with the following strains carrying pBID4-LicKM¹⁸: *A. rhizogenes* (A4) and *A. tumefaciens* wild-type Nester strains A348, A208, and A281 (named At6, At10, and At77, respectively), as well as engineered laboratory strains of *A. tumefaciens* GV3101, C58C1, and LBA4404. The infiltrated leaves were collected at 7 dpi and the level of LicKM expression was estimated by Western blot assay. As shown in **Figure 2A**, the highest level of LicKM production can be achieved with the strains GV3101, A4 and LBA4404 ($\sim 1750 \pm 163$, 1650 ± 26 and 1450 ± 117 mg/kg, respectively), with slight differences; the lowest level of expression ($\sim 900 \pm 102$ mg/kg) with C58C1; and intermediate production with At6, At10 and At77 ($\sim 1250 \pm 19$, 1100 ± 42 and 1200 ± 111 mg/kg, respectively). The lichenase enzymatic activity was demonstrated using Zymogram assay. **Figure 2B** shows that lichenase produced in infiltrated plant tissues using any of the *Agrobacterium* strains was enzymatically active. One should also note that *N. benthamiana* plants infiltrated with A4 and At77 strains showed pathological symptoms (stunting, petiole elongation and curling, and leaf curling), while with At10 strain the symptoms were mild. No symptoms were observed in *N. benthamiana* plants infiltrated with laboratory strain GV3101 (**Figure 2C**).

Infiltration of alternative *Nicotiana* species. We compared the rates of biomass generation and protein production in two wild-type species of the *Nicotiana* genus (*N. benthamiana* and *N. excelsior*) and in a hybrid species, *N. excelsiana* (*N. benthamiana* \times *N. excelsior*). Of the tested species, *N. benthamiana*, a widely used host for transient protein production using *Agrobacterium*-based or viral-based expression systems^{2,34,49}, reaches infiltration readiness within 4–5 weeks of germination. The necessary growth period to generate the optimal level of

biomass is also 4-5 weeks for *N. excelsiana* but is longer (6–7 weeks) for *N. excelsior*. In addition, the plant internodes are relatively short for *N. excelsior* compared to other *Nicotiana* species.

Furthermore, we observed that vacuum infiltration of *N. benthamiana* and *N. excelsiana* at 50–250 mbar for 60 sec is highly efficient for agroinfiltration of entire leaves, while *N. excelsior* is difficult to infiltrate due to their lower canopy and leathery leaves, even when a vacuum was applied three times for 1 min each in the presence of non-ionic surfactants such as Sillwet-77 or S240. Also, the germination rate of *N. excelsiana* and *N. excelsior* seeds was ~40–50%; in order to increase the germination rate to 90–100%, seeds must be treated with 10% bleach for 1 h before seeding. Under the same growth conditions, the highest leaf biomass that can be generated from *N. excelsiana* is approximately two-fold higher compared with *N. benthamiana* (**Table 1**).

Protein production was examined in *N. benthamiana*, *N. excelsior* and *N. excelsiana* infiltrated with the *Agrobacterium* strain GV3101 harboring pBID4-GFP. GFP accumulation was assessed at 7 dpi in whole infiltrated leaves using UV light followed by Western blot analysis. **Figure 3A** shows even distribution of GFP in *N. benthamiana* and *N. excelsiana* and uneven distribution in *N. excelsior* (due to a difficulty of infiltrating an entire leaf area of *N. excelsior*). **Figure 3B** shows the level of GFP production estimated by UV light illumination in infiltrated leaves collected from the three *Nicotiana* species at 7 dpi. The GFP accumulation level was higher in *N. benthamiana* (~2.23 g/kg) than in *N. excelsiana* and *N. excelsior* (~1.89 and 1.54 g/kg, respectively). The low level of protein production in *N. excelsior* is due to uneven infiltration and distribution of accumulated GFP in the collected leaf.

We observed that upper leaves directly exposed to light often exhibit the earliest and highest levels of transient GFP accumulation (at 2-4 dpi) than leaves under the canopy. However, in our studies, GFP accumulation was the highest at 7 dpi and was distributed evenly across most leaves, except in uninfiltrated newly growing leaves which show no GFP accumulation.

Effects of vacuum pressure and duration on transient protein production. Vacuum infiltration significantly increases transient expression levels comparing to pressure applied by hand injection with a needleless syringe⁴². The application of a vacuum causes gases to evacuate from submerged plant leaves through stomata. When the vacuum is broken and pressure rapidly increases, the suspension of *Agrobacterium* is driven into leaves to replace the evacuated gases⁵⁰.

To test the effect of vacuum pressure on the leaves of *N. benthamiana*, we infiltrated plants with the *Agrobacterium* strain GV3101 harboring pBID4-GFP under various vacuum pressures (50–400 mbar) for 30 or 60 sec. It was demonstrated that the stronger vacuum (below 50 mbar) applied for 30 or 60 sec results in mechanical damage of infiltrated leaves, leading to tissue wilting and plant death shortly after infiltration (24–48 h). On the other hand, application of the milder vacuum (400 mbar) results in infiltration of only 50% of the leaf area and a decreased level of GFP production (303 ± 90 mg/kg) (**Figure 4A**). Importantly, we observed no differences in GFP production under 50, 100 and 200 mbar (1651 ± 107 , 1688 ± 40 , 1594 ± 26 mg/kg, respectively) (**Figure 4A**) and mild to no, detrimental impacts on plant health when vacuum pressures from 50–200 mbar were applied for 30 or 60 sec. Therefore, 50–100 mbar of vacuum pressure is recommended for infiltration experiments.

The effect of duration of the vacuum on target expression was assessed by infiltrating one flat of *N. benthamiana* plants every hour with an A₆₀₀ of 0.5 of GV3101 harboring pBID4-GFP for 8 h in the same *Agrobacterium* culture. **Figure 4B** shows that the level of GFP production was similar at all-time points up to 8 h, suggesting that over this period of time the *Agrobacterium* maintains its ability to launch a single-stranded DNA.

Effect of chemical induction on protein production. Certain plant phenolic metabolites and sugars can induce virulence genes of *A. tumefaciens*^{1,52}. As a consequence, many chemicals and monosaccharaides have been reported to enhance transient protein production in various plant species. Acetosyringone is most commonly added to cultures of *A. tumefaciens* to induce the *vir* operon before agroinfiltration^{40,53-57}.

We have assessed the effect of different concentrations of acetosyringone (0, 100, 200 and 400 µM) and glucose (0–4%) on transient GFP protein production in *N. benthamiana* infiltrated with the *Agrobacterium* strain GV3101 harboring pBID4-GFP. For this purpose, we re-suspended *Agrobacterium* cells in MMA induction media containing different concentration of acetosyringone and glucose for 1–3 h before infiltration. According to the results of both visual observation (data not shown) and Western blot analysis (**Figure 4C**), none of the tested concentration of these compounds induced a significant increase in GFP fluorescence or protein production compared with control where induction media contained no acetosyringone or glucose.

Effect of co-infiltration of a silencing suppressor on transient production of GFP and HAC1 genes in *N. benthamiana* leaves. It has been previously demonstrated that co-expression of a silencing suppressor (p19 of *Tomato bushy stunt virus* [TBSV]) interferes with post-transcriptional gene silencing (PTGS), resulting in enhanced production of reporter proteins³⁴.

We have evaluated the effect of co-infiltration of *N. benthamiana* with the launch vector carrying the GFP reporter gene (pBID4-GFP) and p19. Prior to infiltration, an A₆₀₀ of 0.5 dilutions of *A. tumefaciens* GV3101 cultures harboring pBID4-GFP and p19 were respectively mixed at ratios of 1:1, 2:1, 3:1 and 4:1. Expression of the silencing suppressor was controlled by the *Cauliflower mosaic virus* 35S promoter. As indicated by the results of Western blot analysis at 7 dpi (**Figure 5A**), the presence of p19 did not increase or decrease GFP production in *N. benthamiana*, at any ratio of the two *Agrobacterium* suspensions.

We have also compared the effects of two viral gene silencing suppressors – p23 and p19 – on the prevention of PTGS for HAC1. Cultures of *Agrobacterium* carrying the launch vector pBID4-HAC1 (H1N1 A/California/04/2009) and one of the two viral silencing suppressor plasmids were diluted to an A₆₀₀ of 0.5, mixed at a ratio of 4:1, respectively, and co-infiltrated into 4-5-week old *N. benthamiana*. A suspension of *A. tumefaciens* carrying pBID4-HAC1 alone was infiltrated as a control. The infiltrated leaf samples were collected from 3 to 8 dpi. The experiment was repeated three times and average levels of the HAC1 expression determined by Western blot analysis.

As demonstrated in **Figure 5B**, co-infiltration of *N. benthamiana* with p23 or p19 resulted in (642 ± 157 and 764 ± 108 mg/kg, respectively) an increase in HAC1 production compared with using no silencing suppressor (approximately 15–25%, respectively) at 6 dpi. This suggests that p23 and p19 are efficient in our system. However, it should be noted that accumulation of HAC1 occurred a day earlier when pBID4-HAC1 was co-infiltrated with p19. Therefore, our results demonstrate that the effects of the silencing suppressor p19 on HAC1 and GFP accumulation are different, suggesting selective enhancement of transient expression and/or stability of some proteins in *N. benthamiana*.

We also observed that both in the presence and in the absence of a silencing suppressor the level of the HAC1 protein production started declining at 7 dpi. This indicates that the timing of the decline in the transient protein production in *N. benthamiana* infiltrated with the launch vector is target-specific.

The cell bank of *Agrobacterium* harboring the launch vector was evaluated every year for target gene stability, *Agrobacterium* viability and the level of protein accumulation. The glycerol stock of the cell bank of GV3101 strain transformed with pBID4-HAC1 that was stored at -80°C has been shown to be very stable for more than three years without changes in the level of transient protein production in infiltrated *N. benthamiana* plants. **Figure 5C** demonstrates that the HAC1 protein production estimated by Western blotting in the years of 2010, 2011, 2012 and 2013 was 670, 685, 566 and 683 mg/kg, respectively. The average HAC1 production in *N. benthamiana* plants was 651 ± 49.4 mg/kg.

Tables and Figures:

Table 1. Comparison of *N. benthamiana* and *N. excelsiana* plant biomass production.

Figure 1. Western blot analysis of transient gene expression in *N. benthamiana*. (A) Comparison of GFP production in plants vacuum infiltrated with pBID4-GFP-harboring *Agrobacterium* GV3101 cultures grown in three different media: YEB, AB and LB. GV3101 cultures grown o/n in YEB or LB media were centrifuged at low speed and re-suspended in induction medium (MMA) (lanes: MMA-1 and MMA-2, respectively), or grown o/n in YEB, LB or AB media and directly diluted to 1:5 or 1:10 (lanes: YEB/5 and YEB/10; AB/5 and AB/10; LB/5 and LB/10). (B) Comparison of GFP expression at 4 and 7 dpi following vacuum infiltration with different concentrations (A_{600} of 1.0, 0.5, 0.1 and 0.05) of *A. tumefaciens* GV3101 strain carrying pBID4-GFP. (C) Six-week-old *N. benthamiana*, 1) plants growing in a fertilizer solution containing 4.8% phosphorus, 2) plants growing in a fertilizer solution containing 0% phosphorus. Twenty five μg of fresh leaf weight equivalent was loaded per lane.

Figure 2. Comparison of transient lichenase production and activity following vacuum infiltration of *N. benthamiana* plants with different strains of *agrobacteria*. Cultures of *Agrobacteria* strains (GV3101, A4, At77, C58C1, At6, At10 and LBA4404) harboring the launch vector pBID4-LicKM were infiltrated individually into leaves of *N. benthamiana*. Infiltrated leaves were collected at 7 dpi. (A) Lichenase production quantified by Western blotting. (B) Zymogram assay demonstrating lichenase production through enzymatic activity.

(C) Effect of *Agrobacterium* (wild-type A4, At10, At77 and laboratory strain GV3101) infiltration on *N. benthamiana* plant health at 7 dpi. Twenty five μg of fresh leaf weight equivalent was loaded per lane.

Figure 3. Transient GFP expression in leaves of *N. benthamiana*, *N. excelsiana* and *N. excelsior* at 7 dpi after vacuum infiltration with *A. tumefaciens* harboring the launch vector pBID4-GFP. (A) Visual examination of GFP expression under UV light. (B) Western blot analysis of GFP accumulation.

Figure 4. (A) Effects of vacuum pressure on transient GFP expression and plant health. *N. benthamiana* plants were infiltrated with pBID4-GFP under vacuum pressures of 400, 200, 100 or 50 mbar, at vacuum holding time of 30 or 60 sec. (B) Stability and infectivity of *A. tumefaciens* in *N. benthamiana* infiltrated with *Agrobacterium* GV3101 harboring pBID4-GFP grown in AB medium and diluted to an A_{600} of 0.5. Agroinfiltration was performed by infiltrating one flat of *N. benthamiana* plants every hour in the same diluted *Agrobacterium* culture (lanes 0-8). (C) Effect of different concentrations of acetosyringone and glucose on transient expression of GFP. The *Agrobacterium* strain GV3101 harboring pBID4-GFP was grown o/n in YEB media, centrifuged and resuspended to an A_{600} of 0.5 either in MMA containing 2% glucose with acetosyringone at 0, 100, 200 or 400 μM , or in MMA containing 200 μM acetosyringone with glucose at 0, 1, 2 or 4%. The *Agrobacterium* suspensions were kept for 3 h at room temperature before infiltration.

Figure 5. Effects of silencing suppressors on transient protein production in *N. benthamiana* leaves. (A) Western blot analysis of GFP protein following co-infiltration of pBID4-GFP and p19 at different ratios. Samples collected at 7 dpi (25 μg of fresh leaf weight equivalent was loaded per lane). (B) A culture of *Agrobacterium* carrying pBID4-HAC1 was individually mixed at a ratio of 4:1 with a culture carrying the p19 or p23 silencing suppressor plasmids. The resulting combinations of *Agrobacterium* cultures were vacuum infiltrated into plants. Infiltrated tissues of HAC1 were collected daily up to 8 dpi for recombinant protein quantification. (C) Stability of *Agrobacterium* cell bank. Plants were infiltrated with the same batch of the *Agrobacterium* cell bank every year to evaluate protein accumulation. Fifty μg of fresh leaf weight equivalent was loaded per lane.

Discussion:

In this study, we have developed a simple agroinfiltration protocol for routine transient protein production in selected *Nicotiana* species using *Agrobacterium* strains carrying the launch vector. In addition, we have identified the optimum conditions to achieve the highest recombinant protein production level in our transient plant expression system.

Vacuum infiltration of the diluted *A. tumefaciens* strain GV3101 harboring the launch vector pBID4 into *N. benthamiana*, *N. excelsiana* and *N. excelsior* resulted in higher levels of target protein production within 7 dpi compared to other plant species, such as *Pisum sativum* infiltrated with GV3101 harboring *Alfalfa mosaic virus*- or *Cucumber mosaic virus*-based

vectors expressing the GFP reporter gene under the 35S promoter⁴¹, or *Lactuca sativa*, *Solanum lycopersicum* and *Arabidopsis thaliana* infiltrated with the C58C1 strain of *A. tumefaciens* carrying the beta-glucuronidase reporter gene⁵⁷. *N. benthamiana* and *N. excelsiana* were easy to vacuum infiltrate at 50 mbar for 30-60 sec, with 90-95% infiltration efficiency. The remaining 5-10% of leaf area was not infiltrated because of some floating of leaves on the surface of the *Agrobacterium* suspension during application of the vacuum. Since the launch vector has ability for cell-to-cell movement¹⁸, transient protein accumulation occurs in entire leaves as well as petioles at 7 dpi. At 10 dpi, the estimated GFP production was slightly lower because the pBID4 expression vector is able to move from cell to cell but not to move systemically¹⁸; therefore, newly grown leaves do not contain the vector and do not contribute to production of target. In addition, degradation of the recombinant protein over time may contribute to reduced protein level at 10 dpi. Our results showed that infiltration of the *A. tumefaciens* strain GV3101 mediated high levels of transient protein production in *N. benthamiana*. Furthermore, target protein can be engineered as N-terminal, C-terminal or internal fusions to lichenase (LicKM), β -1,3-1,4-glucanase, which is a thermostable enzyme from *Clostridium thermocellum* and confers thermostability to many target protein fusion¹⁸. Infiltration of *N. benthamiana* with *A. tumefaciens* wild-type strains (at6, at10 and at77) harboring the gene of interest elicited mild or severe symptoms: leaf curling, petiole elongation, and curling. No pathological symptoms were observed in *N. benthamiana* infiltrated with the laboratory strain GV3101 harboring empty pBID4 vector, 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. Necrotic symptoms caused by wild-type *Agrobacterium* or disarmed strains in solanaceous plants have been reported previously^{56,57}. The necrotic response could result from virulence factors of the Type III secretion system, bacterial proteins transferred to the plant cell by the Type IV secretion system, and/or sensitivity to flagellin⁵⁸⁻⁶⁰. We have found that transient production of heterologous proteins may also elicit pathogenicity and a hypersensitive response in infiltrated plant leaves. Many researchers reported that agroinfiltration of different plant species with plant binary vectors produced up to 5-20 times higher levels of transient protein production compared to stably transformed plants^{28,57}. Our data shows that *N. benthamiana* infiltrated with GV3101 harboring pBID4-GFP transiently expressed high levels of GFP, which is similar to the GFP yield reported for *N. benthamiana* infiltrated with *Agrobacteria* carrying the pICH-GFPSYS viral vector (up to 80% of total soluble protein)⁴⁴. Agroinfiltration using our launch vector resulted in high production of the thermostable protein LicKM, 50-fold higher than that observed using a standard binary plasmid¹⁸.

To test the infectivity of *A. tumefaciens* and the stability of the launch vector, we infiltrated one flat of *N. benthamiana* every hour for up to 8 h using the same Mill-Q water-diluted culture of GV3101 harboring the pBID4-GFP plasmid. Our data showed that the GV3101 strain is efficiently infective for at least 8 h and pBID4 (the launch vector) is very stable during the 8 h-long infiltration.

Glycerol stock of GV3101 strain harboring the launch vector pBID4-HAC1 (cell bank) stored at -80°C has been shown to be very stable for three years without changes in transient protein production in infiltrated plants.

N. benthamiana plants grown under optimal conditions and between 35 and 42 days post sowing were optimal for vacuum infiltration-mediated transient gene expression⁴⁰. Younger plants (3–4 weeks old) cannot be infiltrated entirely because of leaves floating on the cell suspension surface and tissue damage from the mechanical effects of applying a vacuum. In plants older than 45 days, *N. benthamiana* bolting stage, under the optimal light conditions used, the level of transient expression is low.

Low molecular weight phenolic compounds (acetosyringone) and monosaccharaides (glucose) are known to induce *vir* genes in *A. tumefaciens*^{55,61}. Moreover, infiltration of *N. benthamiana* with the binary vector pCambia(gfp) in the presence of acetosyringone at the concentrations of 50–600 μ M were shown to slightly increase transient gene expression⁴⁰. We studied the effect of different concentrations of acetosyringone and glucose in our system by adding these compounds to GV3101 cultures harboring pBID4-GFP in MMA for 3 h, and found no difference in GFP expression. Interestingly, GV3101 cultures harboring pBID4-GFP and diluted in Mill-Q water 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. The *A. tumefaciens vir* gene(s) could potentially be induced by plant phenolic compounds (acetosyringone and sinapinic acid) and plant monosaccharaides (glucose and fructose) present in leaf tissue. Therefore, we speculate that similar levels of GFP expression in the presence or absence of exogenous *vir* gene inducers may be a result of the effect of cytoplasmic *vir* gene inducers present during replication of the GFP-expressing launch vector in plant cells.

Wild-type *N. benthamiana* has been used as a model host for transient protein production⁴⁹. However, *N. benthamiana*'s relatively low biomass yield hinders its application for large-scale production of recombinant proteins. The optimal host should combine a high level of transient expression, easy growth in a greenhouse, and be susceptible to *Agrobacterium* infiltration². To select an alternative host, we infiltrated two different wild-type species of *Nicotiana* (*N. benthamiana* and *N. excelsior*) and a hybrid *N. excelsiana* (*N. benthamiana* \times *N. excelsior*) with the *A. tumefaciens* strain GV3101 harboring the pBID4-GFP plasmid. Among these three species, the level of GFP expression was slightly higher in *N. benthamiana*. *N. excelsior* plants showed difficulty in vacuum agroinfiltration due to their leathery leaves, and *N. excelsiana* produced approximately two-fold more biomass under the same growth conditions. The transient production of GFP at 7 dpi is relatively similar in *N. benthamiana* and *N. excelsiana*. Therefore, *N. excelsiana* may be a more suitable host for recombinant protein production.

Agrobacterium-mediated transient protein production is limited by PTGS²⁶, which can be overcome by co-expression of gene silencing suppressors of plant virus origin⁶². Transient protein production has been previously shown to be enhanced 50-fold in the presence of the p19 protein of TBSV, which inhibits PTGS in infiltrated tissues³⁴. In our study, we assessed the effect of two viral silencing suppressors (p19 and p23) separately co-infiltrated with the launch vector pBID4-HAC1. The co-infiltration of these silencing suppressors seemed to have little influence on transient expression of HAC1, with only a slight increase in HAC1 protein accumulation (15–25%) in the presence of co-infiltrated p23 or p19. To positively affect protein production, silencing suppressors may need to be specifically selected to be effective for the targeted plant species and viral vector⁶³. TMV helicase has a suppressor of RNA silencing

activity^{64,65}. Our data confirm this observation as co-infiltration of p23 or p19 with pBID4-HAC1 resulted in no increase in GFP or a slight increase in the transient HAC1 protein production.

In conclusion, we have modified and optimized plant and *Agrobacterium* growth conditions and improved the efficiency of vacuum infiltration. This technology enabled us to grow and infiltrate hundreds of kgs of plant material in a few hours. We successfully automated the plant transient expression technique for high-throughput vaccine production at industrial scales under current Good Manufacturing Practices (cGMP) conditions. For more information about automation and utilization of the plant transient protein production system for the production of recombinant proteins, including subunit vaccine candidates, under cGMP conditions, readers are referred to the website www.fraunhofer-cmb.org.

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Figure 1

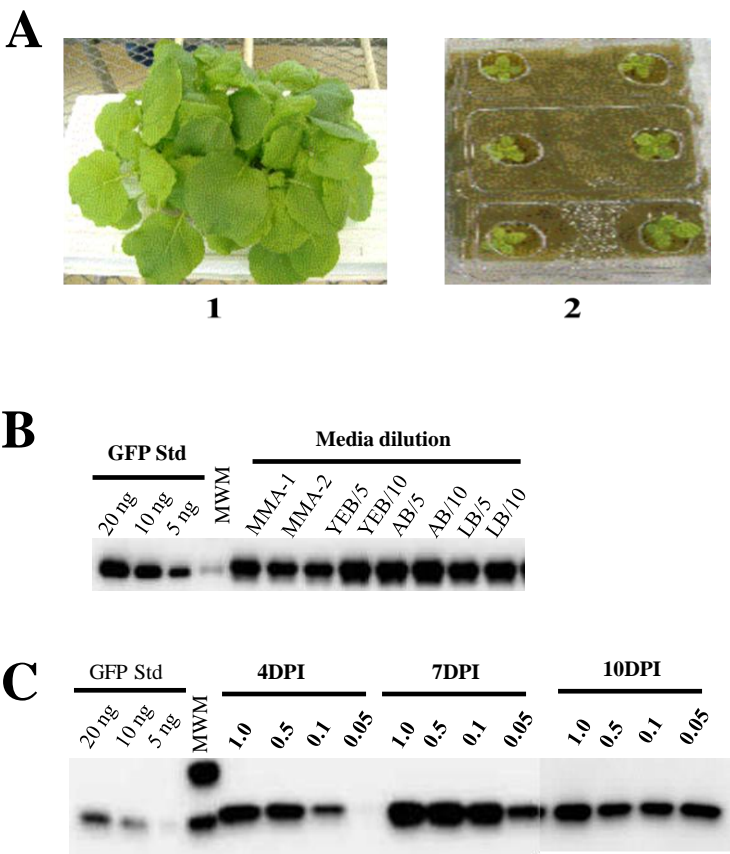


Figure 2

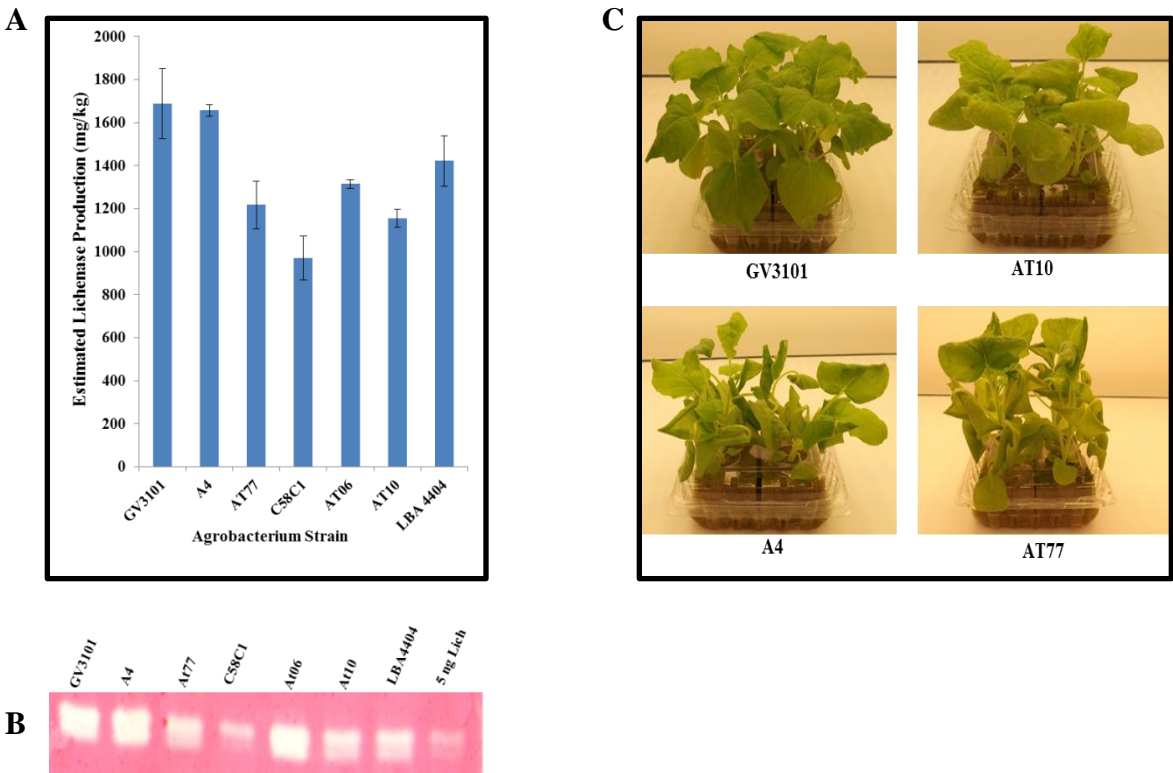


Figure 3

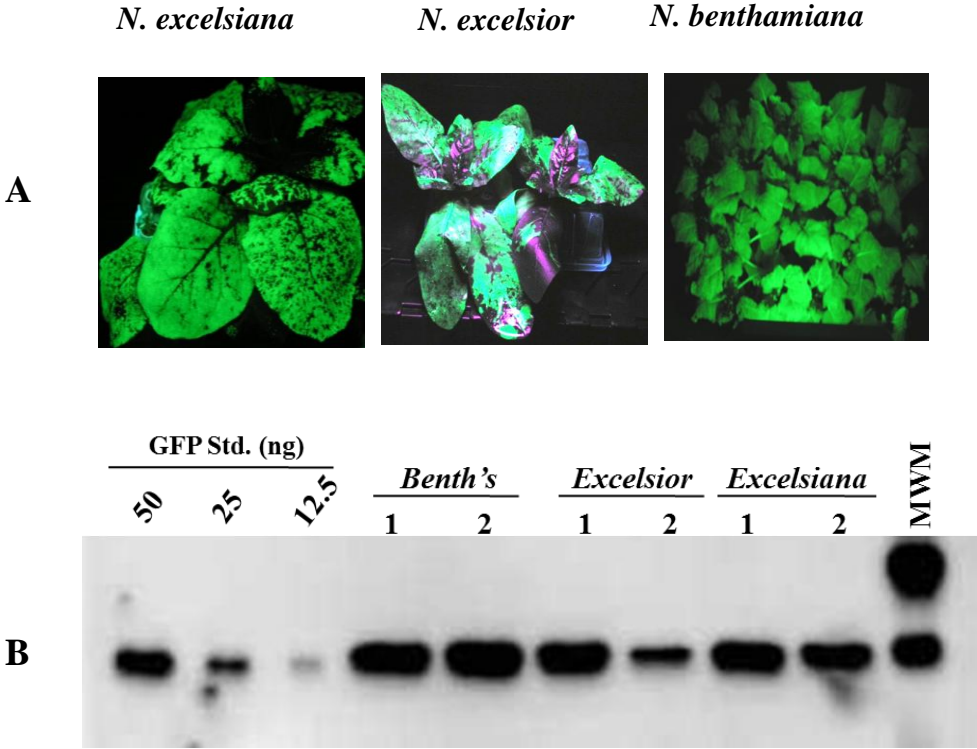
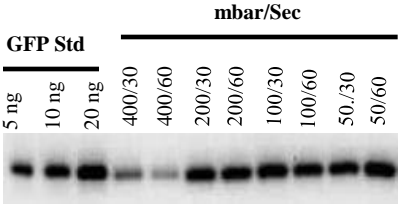
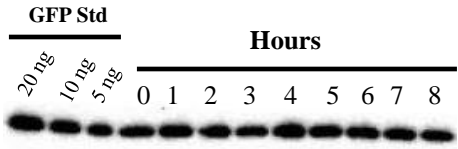


Figure 4

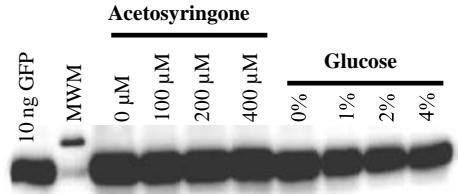
A



B



C



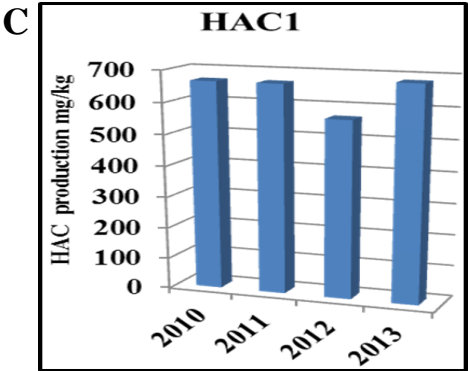
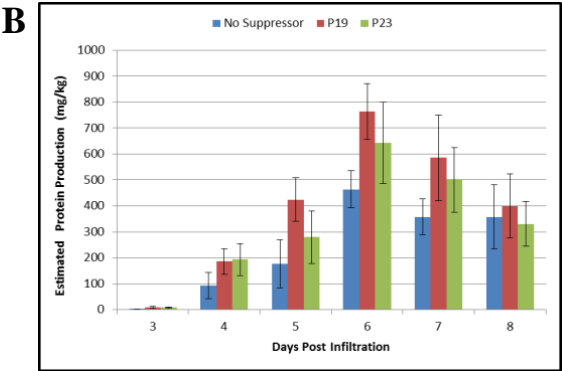
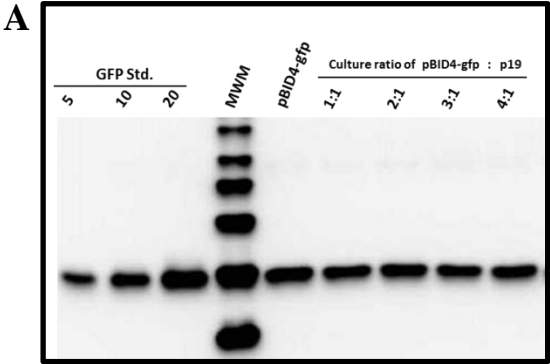


Table 1.

<i>Nicotiana</i> species	Fresh leaf mass (g)	Fresh stem mass (g)	Total fresh mass (g)	Leaf mass/ total mass (%)	Stem mass/ total mass (%)
Average ± standard deviation					
<i>N. benthamiana</i>	0.77 ± 0.12	0.35 ± 0.05	1.1 ± 0.2	66 ± 7	34 ± 7
<i>N. excelsiana</i>	1.17 ± 0.37	0.28 ± 0.04	1.45 ± 0.4	80 ± 4	20 ± 4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
<i>Nicotiana benthamiana</i>	Tobacco Germplasm Collection, Crop Science Dept., North Carolina State University	PI 555478 TW16	Infiltration
<i>Nicotiana excelsior</i>	Tobacco Germplasm Collection, Crop Science Dept., North Carolina State University	PI 555685 TW47	Infiltration
<i>Nicotiana excelsiana</i>	Dr. Wayne Fitzmaurice, Large Scale Biology Corporation, Vacaville, CA	LSBC EBA 042304.02	Infiltration
Vacuum skid	Abbas, Ashland, MA	Custom made	Plant infiltration
Rockwool	Grodan Inc., Ontario, Canada	AO 50/40	Hydroponic media for growing plant
2-(N-Morpholino) ethanesulfonic acid	Acros Organics, Thermo Fisher Scientific NJ	172595000	
Murashige & Skoog salt (MS salt)	Phyto Technology Lab	M524	Tissue culture media
Acetosyringone	Sigma-Aldrich, St. Louis, MO	D134406-5G	Agrobacterium induction
Immobilon-P transfer membrane	Millipore, Billerica, MA	IPVH00010	Western blotting
I-block	Applied Biosystems, Foster City, CA	T2015	Western blotting
Rabbit polyclonal anti-GFP antiserum	Washington Biotechnology, Baltimore, MD		Western blotting
Mouse anti–poly-histidine monoclonal antibody	Qiagen GmbH, Hilden	34670	Western blotting
Horseradish peroxidase- conjugated anti-rabbit antibody	Jackson ImmunoResearch, West Grove, PA	111-035-003	Western blotting
Horseradish peroxidase- conjugated anti-mouse antibody	Jackson ImmunoResearch, West Grove, PA	115-035-003	Western blotting

SuperSignal® West Pico chemiluminescent substrate	Thermo Scientific Pierce, Rockford, IL	34078	Western blotting
Lichenan (1-3: 1-4-beta-D-glucan)	Megazyme, Bray, Co. Wicklow, Ireland	P-LICHN	Lichenase Activity
Congo Red	Sigma-Aldrich, St. Louis, MO	C6277	Gel staining
Digital camera	Olympus, Center Valley, PA	C-8080	Chemiluminescence imaging
GeneGnome	Syngene, Frederick, MD		Chemiluminescence imaging
GFP standard	Made in house		Chemiluminescence imaging
Plant fertilizer solution	Griffin Greenhouse & Nursery Supplies, Newark, DE	67-23-20	Plant growing
Lichenase Standard	Purified in house		Western blotting
MicroPulser Electroporator	BioRad, Hercules, CA	165-2100	Agrobacterium transformation



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Vidadi Yusibov

Department:

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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 *Agrobacterium* penetrating into plant intercellular spaces. Our data showed that infiltration of plants with non-induced *Agrobacterium* growing in AB medium and then diluted in Mill-Q water and with *Agrobacterium* induced in MMA media resulted in similar levels of reporter protein (GFP) production (Figure 1A). Therefore, *Agrobacterium* grown in AB medium and diluted in Mill-Q water to A_{600} 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 μ M 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 μ M 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 lichenase 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, strains 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. benthamiana 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. benthamiana* 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 HAC1 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 suppressor was 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.