Video Article

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

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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 Milli-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).

Video Link

The video component of this article can be found at https://www.jove.com/video/51204/

Introduction

Plants are now recognized as a safe, reliable, scalable and inexpensive platform for producing heterologous recombinant biopharmaceuticals and industrial proteins[1-3] and have important advantages over microbial and animal cell expression systems[4]. Plants are able to express correctly folded proteins with post-translational modifications, including assembled multimeric antibodies[5-7]. Several plant-derived recombinant pharmaceutical proteins are undergoing clinical evaluation[8]. These include patient-specific recombinant idiotype vaccines (scFv) for the treatment of non-Hodgkin's lymphoma[9], 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 caries[12], and human insulin for the treatment of diabetes[13]. 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 accumulation[16], and can be achieved by introducing bacterial binary vectors or recombinant plant viral vectors into plant tissues[4]. 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 virus[19], *Yersinia pestis*[20], influenza viruses A[21,22], *Bacillus anthracis*[23], and smallpox virus[24] 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 receptor[30], 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 tissues[35-37]. 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 plants[38-41]. 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 Milli-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. Soak rockwool slabs in a plant fertilizer solution.
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.
3. Grow plants from the seeds under controlled conditions (24 °C and 40-65% relative humidity) and a long-day photoperiod (14 hr light and 10 hr dark, with illumination of 130-150 µE m^{-2} sec^{-1}) for 4-5 weeks for *N. benthamiana* and *N. excelsiana*, and 5-6 weeks for *N. excelsior*.

2. Construction of Vectors for Agroinfiltration

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 pBID4[18] (TMV-based vector) to obtain pBID4-GFP, pBID4-HAC1 and pBID4-LicKM, respectively[18,32,41,47].
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 MicroPulser electroporator.
3. Use the transformed *Agrobacteria* for infiltration experiments unless otherwise noted.

3. Vacuum Infiltration of *Agrobacterium* into *Nicotiana* Plants

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.
2. Dilute *Agrobacteria* in Milli-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 × g for 10 min at 4 °C, re-suspend in induction medium (1x MS salt, 10 mM MES, 200 µM acetosyringone, 2% sucrose [MMA]) to A_{600} of 0.5, and stir at room temperature for 1-3 hr, unless otherwise noted.
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.
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.
5. To test the efficacy of chemicals inducing *Agrobacterium vir* gene, different concentrations of acetosyringone (0, 100, 200 or 400 µM) 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 µM acetosyringone). *N. benthamiana* plants were infiltrated as mentioned above in steps 3.3 and 3.4).
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 Milli-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

1. Mix the Milli-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.
2. Infiltrate *N. benthamiana* plants with a mixture of two Milli-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

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.
2. Add three volumes of 1x PBS buffer containing 0.5% Triton X-100 to each sample.
3. Gently shake the extracted samples for 15 min at 4 °C.
4. Spin the extract for 5 min and collect total soluble protein into a clean Eppendorf tube.
5. Dilute the extracts to an appropriate dilution (1:50 1:100) in 1x PBS extraction buffer, and add 5× 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 1× concentration.
6. Boil samples for 5 min.
7. Separate proteins by 10% SDS-PAGE, transfer onto Immobilon-P transfer membrane, and block with 0.5% I-block.
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 hr.
9. After primary antibody labeling, wash the membranes three times for 10 min each with 1× 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 hr, for GFP and HAC1 detection, respectively.
10. Process Western blots using the SuperSignal West Pico Chemiluminescent substrate.
11. Use the GeneTools Software to analyze band intensity of the protein and obtain the band calibrated quantity.

Protein production: (Calibrated quantity x dilution of sample)/amount of sample loaded) x 4 = mg/kg.
Equation: Protein production (P), Calibrated quantity (C), Dilution of the sample (D) and amount of Sample loaded (S).

6. Zymogram Assay

1. Collect pBID4-LicKM-infiltrated *N. benthamiana* random tissue samples.
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.
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 hr.
4. After incubation, discard the wash buffer and stain the gels with 0.5% Congo Red for 5 min at room temperature.
5. Rinse the gels in Milli-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

1. Perform visual detection of GFP fluorescence in whole transiently transformed plants using a hand-held long-wavelength UV lamp.
2. Photograph transiently transformed plants with a digital camera through a Yellow 8, ES 52 filter (exposure time, 15 sec).
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.
4. Quantify HAC1 protein using a calibration curve based on purified HAC protein standard from the A/Indonesia/05/05 strain of influenza virus.
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 Milli-Q water to A_{600} 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 hr 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 Milli-Q water (1:5, A_{600} of 0.6-0.8 or 1:10, A_{600} 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 Milli-Q water (1671 ± 102 and 1667 ± 131 mg/kg, respectively). Therefore, Milli-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 (1,662, 1,870 and 1,890, respectively), while A_{600} of 0.05 showed lower GFP production (1,199 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 (1,218, 1,181, 1,197 and 1,304).

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-LicKM[18]: *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 (~1,750 ± 163, 1,650 ± 26 and 1,450 ± 117 mg/kg, respectively), with slight differences; the lowest level of expression (~900 ± 102 mg/kg) with C58C1; and intermediate production with At6, At10 and At77 (~1,250 ± 19, 1,100 ± 42 and 1,200 ± 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* × *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 hr 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 syringe[42]. 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 gases[50].

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 hr). 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 (1,651 ± 107, 1,688 ± 40, 1,594 ± 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 A_{600} of 0.5 of GV3101 harboring pBID4-GFP for 8 hr in the same *Agrobacterium* culture. **Figure 4B** shows that the level of GFP production was similar at all-time points up to 8 hr, 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 monosaccharides 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 hr 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 proteins[34].

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 A_{600} of 0.5 dilutions of *A. tumefaciens* GV3101 cultures harboring pBID4-GFP and p19 were respectively mixed at ratios of 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 A_{600} 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.

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

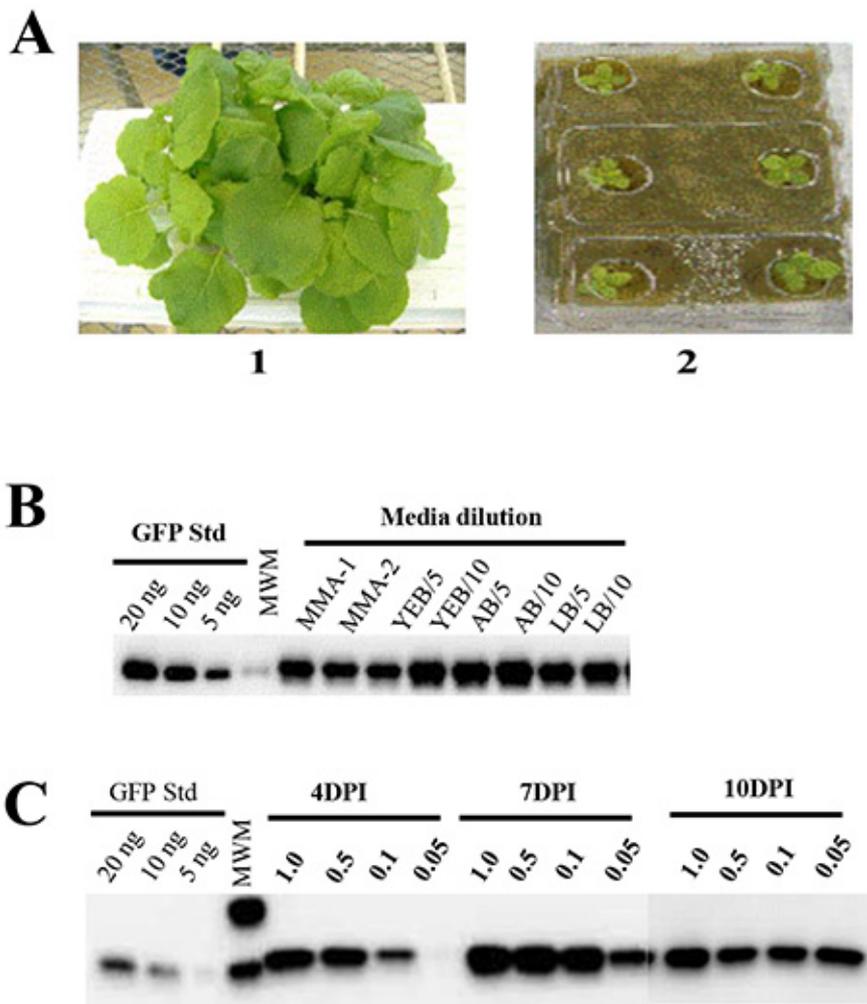**Table 1. Comparison of *N. benthamiana* and *N. excelsiana* plant biomass production.**

Figure 1. Western blot analysis of transient gene expression in *N. benthamiana*. (A) Six-week-old *N. benthamiana* **1**) plants growing in a fertilizer solution containing 4.8% phosphorus and **2**) plants growing in a fertilizer solution containing 0% phosphorus. Twenty five µg of fresh leaf weight equivalent was loaded per lane. **(B)** 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 with Milli-Q water (lanes: YEB/5 and YEB/10; AB/5 and AB/10; LB/5 and LB/10). **(C)** Comparison of GFP expression at 4, 7 and 10 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.
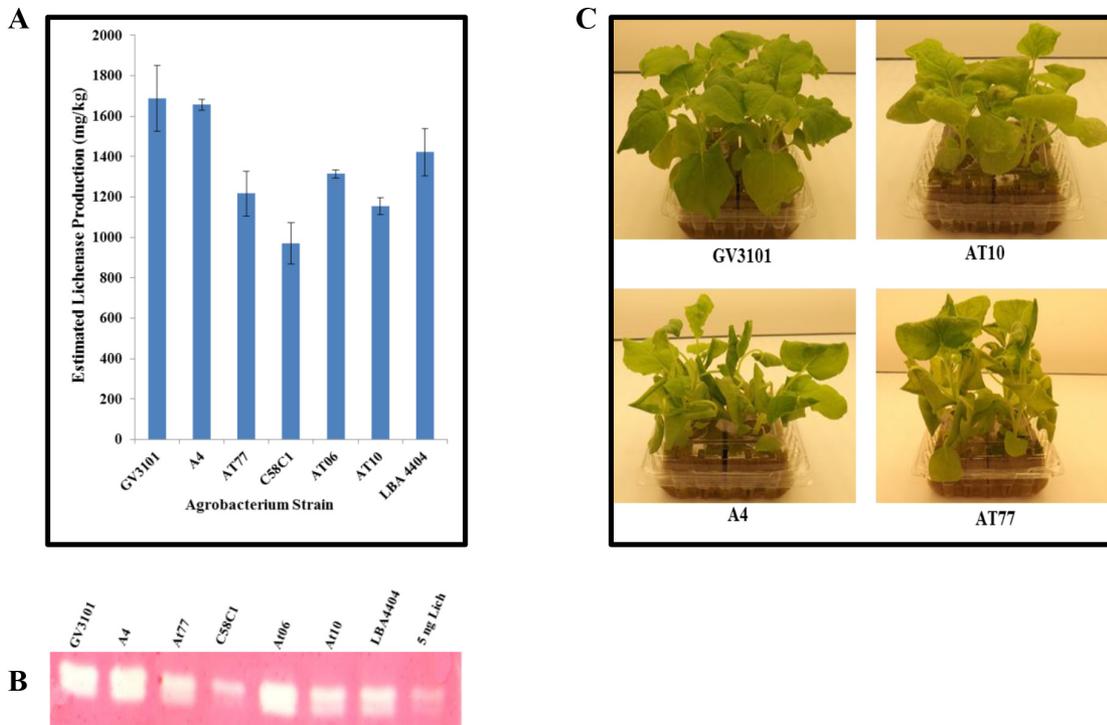
Figure 2. **Comparison of transient lichenase production and activity following vacuum infiltration of *N. benthamiana* plants with different strains of *Agrobacteria*.** Cultures of *Agrobacteria* strains (GV3101, A4, At77, C58C1, At6, At10 and LBA4404) harboring the launch vector pBID4-LicKM were infiltrated 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.
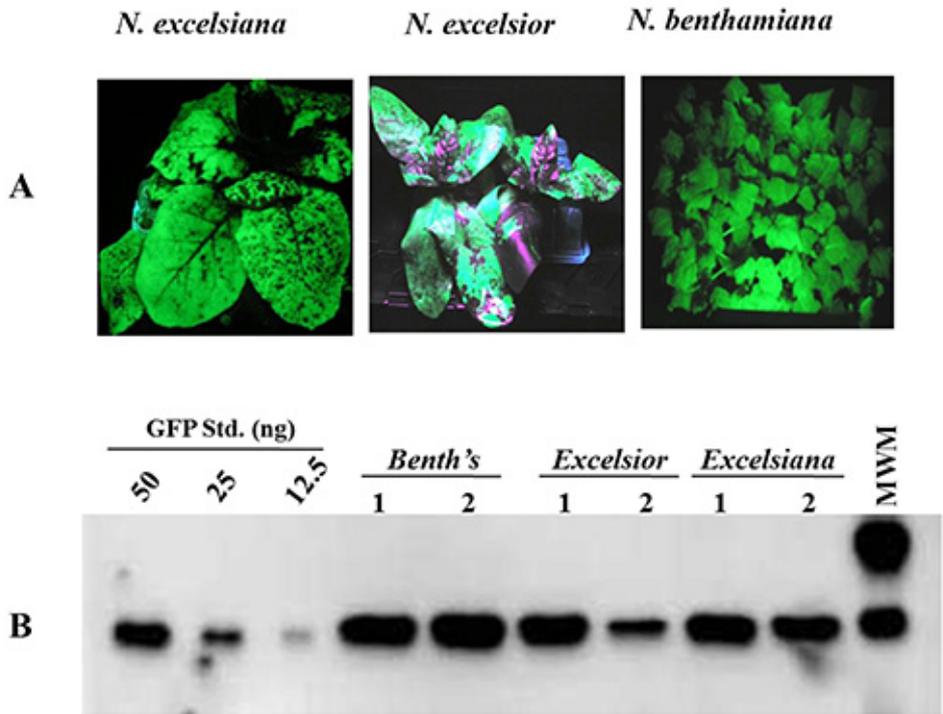
Figure 3. Transient GFP expression in leaves of *N. benthamiana, N. excelsiana and N. excelsior* at 7 dpi after vacuum infiltration with *A. tumefaciens* harboring the launch vector pBID4-GFP. (A) Visual examination of GFP expression under UV light. **(B)** Western blot analysis of GFP accumulation.
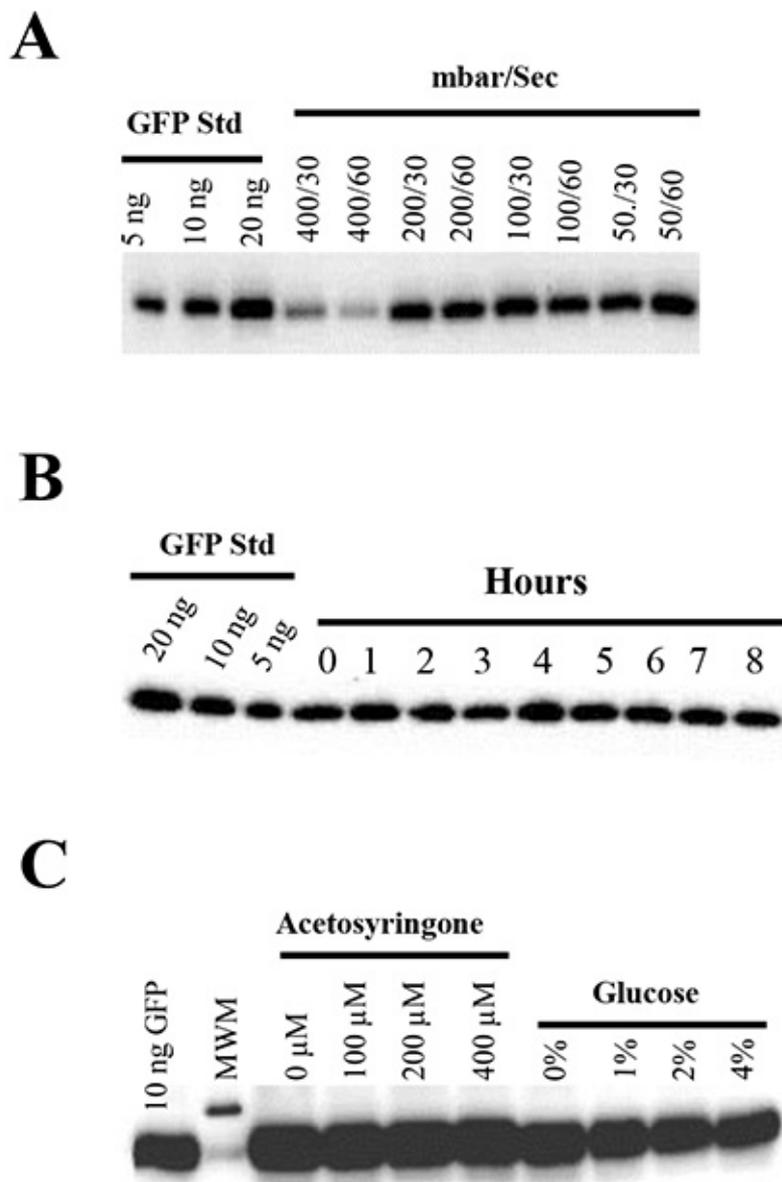
Figure 4. (A) Effects of vacuum pressure on transient GFP expression and plant health. *N. benthamiana* plants were infiltrated with pBID4-GFP under vacuum pressures of 400, 200, 100 or 50 mbar, at vacuum holding time of 30 or 60 sec. **(B)** Stability and infectivity of *A. tumefaciens* in *N. benthamiana* 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 hr at room temperature before infiltration.
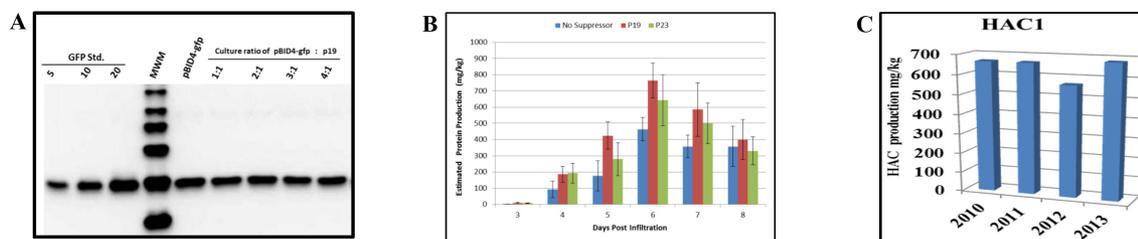
Figure 5. Effects of silencing suppressors on transient protein production in *N. benthamiana* leaves. (A) Western blot analysis of GFP protein following co-infiltration of pBID4-GFP and p19 at different ratios. Samples collected at 7 dpi (25 µg of fresh leaf weight equivalent was loaded per lane). **(B)** 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. Please click here to view a larger version of this figure.

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 promoter[41], or *Lactuca sativa*, *Solanum lycopersicum* and *Arabidopsis thaliana* infiltrated with the C58C1 strain of *A. tumefaciens* carrying the beta-glucuronidase reporter gene[57]. *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 movement[18], 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 systemically[18]; 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 fusion[18]. 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 flagellin[58-60]. 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 plasmid[18].

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 hr using the same Milli-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 hr and pBID4 (the launch vector) is very stable during the 8 hr-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 expression[40]. 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 µM were shown to slightly increase transient gene expression[40]. 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 hr, and found no difference in GFP expression. Interestingly, GV3101 cultures harboring pBID4-GFP and diluted in Milli-Q water to an A_{600} 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 production[49]. 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* infiltration[2]. 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* × *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 PTGS[26], which can be overcome by co-expression of gene silencing suppressors of plant virus origin[62]. 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 tissues[34]. 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 vector[63]. 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 kilograms 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.fhcmb.org/.

Disclosures

We have nothing to disclose.

Acknowledgements

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