Video Article

Efficient Agroinfiltration of Plants for High-level Transient Expression of Recombinant Proteins

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Abstract

Mammalian cell culture is the major platform for commercial production of human vaccines and therapeutic proteins. However, it cannot meet the increasing worldwide demand for pharmaceuticals due to its limited scalability and high cost. Plants have shown to be one of the most promising alternative pharmaceutical production platforms that are robust, scalable, low-cost and safe. The recent development of virus-based vectors has allowed rapid and high-level transient expression of recombinant proteins in plants. To further optimize the utility of the transient expression system, we demonstrate a simple, efficient and scalable methodology to introduce target-gene containing *Agrobacterium* into plant tissue in this study. Our results indicate that agroinfiltration with both syringe and vacuum methods have resulted in the efficient introduction of *Agrobacterium* into leaves and robust production of two fluorescent proteins; GFP and DsRed. Furthermore, we demonstrate the unique advantages offered by both methods. Syringe infiltration is simple and does not need expensive equipment. It also allows the flexibility to either infiltrate the entire leave with one target gene, or to introduce genes of multiple targets on one leaf. Thus, it can be used for laboratory scale expression of recombinant proteins as well as for comparing different proteins or vectors for yield or expression kinetics. The simplicity of syringe infiltration also suggests its utility in high school and college education for the subject of biotechnology. In contrast, vacuum infiltration is more robust and can be scaled-up for commercial manufacture of pharmaceutical proteins. It also offers the advantage of being able to agroinfiltrate plant species that are not amenable for syringe infiltration such as lettuce and *Arabidopsis*. Overall, the combination of syringe and vacuum agroinfiltration provides researchers and educators a simple, efficient, and robust methodology for transient protein expression. It will greatly facilitate the development of pharmaceutical proteins and promote science education.

Video Link

The video component of this article can be found at https://www.jove.com/video/50521/

Introduction

Since the 1970s, plants have been explored as alternatives to mammalian, insect, and bacterial cell cultures for the commercial production of recombinant proteins and protein therapeutics [1]. Plant-based systems for the expression of biopharmaceuticals have shown promise in recent years as several novel treatments for diseases, like Gaucher's Disease [2], and avian H5N1 influenza [3], have shown success in clinical trials. The development of competent mechanisms for recombinant protein expression in plants in the decades since those initial experiments has created the potential for plant-based systems to alter the current paradigm of protein production for three primary reasons. Firstly, there is a notable decrease in cost as mammalian, insect, and bacterial bioreactors require considerable startup costs, expensive growth media, and complicated processes for downstream purification [4]. The creation of stable transgenic plant lines also allows them to outpace the scalability of other expression systems as protein expressing plants could be grown and harvested on an agricultural scale [5]. Secondly, plant-based expression systems significantly reduce the risk of transmitting a human or animal pathogen from the protein-expressing host to humans, demonstrating superiority in public safety [6]. Lastly, plants utilize a eukaryotic endomembrane system that is similar to mammalian cells, allowing for proper post-translational modification of proteins including glycosylation and the assembly of multiple-subunit proteins [7]. This ability puts plant-based systems ahead of those based on prokaryotic systems, such as bacteria, since a wider number of pharmaceutical recombinant proteins, including monoclonal antibodies (mAbs), have a more complicated structure and require extensive posttranslational modifications or assembly [8].

There are two major approaches to expressing recombinant proteins in plants. The first is the development of a stably transgenic line, where DNA coding for the target protein is cloned into an expression cassette and introduced to either the nuclear or chloroplast genomes. In doing so, the foreign DNA becomes heritable through succeeding generations and allows for tremendously improved scalability, far beyond that of other expression systems [1]. Introduction of exogenous DNA to the nuclear genome is usually achieved by *Agrobacterium tumefaciens* infection of plant tissue or, less often, by microprojectile bombardment of the tissue [9]. Plant hormones are then used to induce differentiation and growth

of transgenic plant tissue such as roots and leaves. Transformation of the chloroplast genome cannot be achieved with *A. tumefaciens*, but relies entirely on gold or tungsten particles coated with DNA fired ballistically into plant cells. The second method of expressing recombinant protein in plants is through transient expression [10]. In this scenario, virus-derived vectors harboring the gene of interest are delivered *via A. tumefaciens* to fully developed plants through a process called agroinfiltration. Instead of integrating into the plant genome, the delivered gene construct will then begin to direct the transient production of the desired protein, which can be harvested and isolated after a short incubation period. Transient gene expression offers the advantage of greater overall protein accumulation as well as an improved time of protein production, as plants will be ready to harvest approximately 1-2 weeks after agroinfiltration [11]. This is significantly faster than the processes of generation, selection, and confirmation of stable transgenic plant lines, which can take several months to a year. This however, is also the limitation of the transient expression system, as it will not yield genetically stable plant lines that can be used to generate a seed bank for large scale commercial production. Despite this, approaches have been developed to improve large scale transient expression. Here we demonstrate one method of generation of transient protein-expressing *Nicotiana benthamiana* plants using deconstructed viral vectors delivered by *A. tumefaciens*.

Two major methods are being developed for the delivery of *A. tumefaciens* into plant tissue: bench scale infiltration *via* syringe and large scale infiltration *via* vacuum chamber. Both protocols are described here using *N. benthamiana,* which is closely related to the common tobacco plant, as the host plant for transient expression of two fluorescent proteins: the green fluorescent protein (GFP) from jellyfish *Aequorea victoria* and the red fluorescent protein from Discosoma coral (DsRed) [12,13]. *N. benthamiana* is the most common host plant for recombinant protein because it is amenable to genetic transformation, can yield high amounts of biomass rapidly, and is a prolific seed producer for scale-up production [14]. Another advantage of using *N. benthamiana* as hosts for protein expression is the availability of a variety of expression vectors [2,5]. In this study, two deconstructed viral vectors, one based on a tobacco mosaic virus (TMV) RNA replicon system (MagnICON vectors) and the other derived from the bean yellow dwarf virus (BeYDV) DNA replicon system (geminiviral vectors) [4,11,15-18] , are used to carry the GFP and DsRed gene and deliver them into *N. benthamiana* cells *via A. tumefaciens.* Three DNA constructs will be used for GFP or DsRed expression with MagnICON vectors. They include the 5' module (pICH15879) containing the promoter and other genetic elements for driving the expression of the target gene, the 3' module containing the gene of interest (pICH-GFP or pICH-DsRed), and the integrase module (pICH14011) coding for an enzyme that integrates the 5' and 3' modules together upon expression [8,15]. Three DNA constructs are also needed for expression with geminiviral vectors. In addition to vectors containing the replicon of the target gene (pBYGFP or pBYDsRed), a vector coding for the replication protein (pREP110) is required for the amplification of the target replicon [11,14,16]. Furthermore, the inclusion of a vector encoding the silencing suppressor p19 from tomato bushy stunt virus is desired for high level target gene expression [11,16].

There are generally three major steps for the introduction of genes of recombinant proteins into plant cells by agroinfiltration including plant growth, *A. tumefaciens* culture preparation, and infiltration. As every step is critical for the ultimate success of this procedure, therefore, a detailed description for each is provided for both syringe infiltration and vacuum infiltration below.

Protocol

1. Plant Growth

1. Place 60 peat pellets into a propagation tray. Add 4 L of tap water and let the peat pellets absorb the water for 2 hr.
2. Add 2 *N. benthamiana* seeds into each peat pellet using a seeder, cover the tray with a transparent plastic dome, and allow them to germinate in a 25 °C, 84% humidity environment with a 16/8 hr day/night cycle (**Figure 1A**).
3. Remove the dome two weeks after seeding, drain water from the tray, and add 2 L of Jack's fertilizer at a concentration of 1.48 g/L (**Figure 1B**). Continue to grow plants in a 25 °C, 50% humidity environment with a 16/8 hr day/night cycle. Supply 2 L of Jack's fertilizer every 2 days per tray.
4. At week 4, transfer the plants with peat pellets to a new tray that hosts six plants to provide adequate space for further growth (**Figure 1C**) until they are ready to be infiltrated at 6 weeks of age (**Figure 1D**).

2. *A. tumefaciens* Culture Preparation

2.1 Preparation for Syringe Infiltration

1. Streak *A. tumefaciens* GV3101 strains containing the geminiviral vectors p19, pREP110, pBYGFP, and pBYDsRed on LB Agar plates containing kanamycin (100 µg/ml). Streak one strain per plate and grow at 30 °C for 48 hr.
2. Similarly, streak and grow GV3101 strains harboring the MagnICON vectors of the 5' module (pICH15879), the 3' module containing the target gene of GFP or DsRed (pICH-GFP or pICH-DsRed), and Integrase (pCH14011) on LB Agar plates containing carbenicillin (100 µg/ml).
3. From a single colony on the LB Agar plate, inoculate GV3101 strains harboring geminiviral vectors into 3 ml of YENB media (0.75% Bacto yeast extract, 0.8% Nutrient Broth, adjust pH to 7.5 with NaOH) + kanamycin (100 µg/ml) in a 15 ml round-bottom culture tube, grow the liquid culture at 30 °C in a shaker overnight with a 300 rpm rotation rate.
4. Similarly, inoculate and grow GV3101 strains harboring MagnICON vectors in YENB media + carbenicillin (100 µg/ml) in a shaker overnight at 30 °C.
5. Measure and record OD_{600} values for each liquid culture with a spectrophotometer and use the formula below to calculate the necessary volume (V_{sub}) to be subcultured for a new culture with a starting OD_{600} of 0.025 in 10 ml of YENB media with appropriate antibiotics.
   V_{sub} (ml) = (10 ml) x (0.025) / OD_{600}
6. Transfer V_{sub} (ml) of the overnight culture to (10 - V_{sub}) ml of YENB media with appropriate antibiotics for each strain. Grow the new culture overnight at 30 °C in a shaker with a 250 rpm rotation rate until the OD_{600} value is in the range of 1.7-2.0.
7. Measure and record OD_{600} values for each liquid culture and use the formula below to calculate the necessary volume (V_{inf}) to be diluted in 50 ml of infiltration buffer to give a final OD_{600} of 0.12 for each strain.
   V_{inf} (ml) = (50 ml) x (0.12) / OD_{600}
8. Transfer V_{inf} (ml) of each culture to a microcentrifuge tube and spin down the cells by centrifugation at 12,000 x g for 2 min, remove supernatant and then resuspend the cells in 10 ml infiltration buffer (10 mM MES, pH 5.5; 10 mM $MgSO_4$) by vortexing briefly.

9. Mix resuspended cells of pBYGFP + pREP110 + p19, spin the cells down at 12,000 x g for 2 min, and resuspend in total of 50 ml of infiltration buffer. This *Agrobacteria* combination is for geminiviral expression of GFP.
10. Similarly, mix the following combinations of *Agrobacteria* cells, spin down and resuspend them in 50 ml of infiltration buffer. They include (1) pBYDsRed + pREP110 + p19 for geminiviral expression of DsRed, (2) pBYGFP + pBYDsRed + pREP110 + p19 for geminiviral co-expression of GFP and DsRed, (3) pREp110 + p19 as negative control of geminiviral expression, (4) pICH-GFP + pICH15879 + pCH14011 for MagnICON expression of GFP, (5) pICH-DsRed + pICH15879 + pCH14011 for MagnICON expression of DsRed, and (6) pICH15879 + pCH14011 as negative control for MagnICON expression.

2.2 Preparation for Vacuum Infiltration

1. Inoculate and culture each GV3101 strain containing geminiviral vectors or MagnICON vectors into 15 ml of YENB media with appropriate antibiotics in a 50 ml autoclaved flask and grow overnight as described for syringe infiltration above.
2. Measure and record OD_{600} values for each liquid culture with a spectrophotometer and use the formula below to calculate the necessary volume (V_{sub}) to be subcultured for a new culture with a starting OD_{600} of 0.025 in 250 ml of YENB media with appropriate antibiotics.
   V_{sub} (ml) = (250 ml) x (0.025) / OD_{600}
3. Transfer V_{sub} (ml) of the overnight culture to (250 - V_{sub}) ml of YENB media in a 1 L autoclaved flask with appropriate antibiotics for each strain and grow the new culture overnight as described for syringe infiltration above.
4. Measure and record OD_{600} values for each liquid culture and use the formula below to calculate the necessary volume (V_{inf}) to be diluted in 3 L of infiltration buffer to give a final OD_{600} of 0.12 for each strain.
   V_{inf} (ml) = (3000 ml) x (0.12) / OD_{600}
5. Pellet and resuspend V_{inf} (ml) of each culture in infiltration buffer as described for syringe infiltration and mix the resuspended cultures according to the combinations described for syringe infiltration. Repellet the mixed *Agrobacteria* cells and resuspend them in 3 L of infiltration buffer.

3. Infiltration

3.1 Syringe Infiltration

1. Choose four 6-week old *N. benthamiana* plants with 5 leaves each. The first three leaves counting from the top of each plant will be infiltrated entirely with one of the combinations of *A. tumefaciens* strains in infiltration buffer. The fourth leaf will be spot-infiltrated with all four strain combinations for geminiviral expression or all three combinations for MagnICON expression. The last leaf on each plant will be infiltrated with combinations of negative controls.
2. Create a small nick with a needle in the epidermis on the back side of the leaf (**Figure 2A**). Attention: make sure not to scratch so hard as to pierce the leaf through both sides, as the *Agrobacteria* cells in the infiltration mixture will pass through the puncture to the other side of the leaf.
3. Take a firm hold of the front side of the leaf and while applying gentle counter pressure to the nick with the thumb of one hand, inject the *Agrobacterium* mixtures in infiltration buffer into the nick with a syringe without a needle (**Figure 2B**). Note: As the *Agrobacterium* mixture enters the intercellular space of the leaf, the infiltrated area will turn visibly darker green (**Figure 2C**).
4. Continue to inject the *Agrobacterium* mixtures into the nick until the darker green circle stops to expand. Create another nick and repeat steps 3.1.2-3.1.4 until the entire leaf is infiltrated and the whole leaf turns darker green for the first three leaves and the last leaf.
5. For the fourth leaf of each plant, all four (geminiviral vector) or three (MagnICON vector) combinations will be infiltrated into it. Make one nick for each combination of *Agrobacterium* strains, and infiltrate each nick with one combination.
6. After infiltration, move plants back to the growth room and monitor protein expression between 2-15 days post infiltration (dpi).

3.2 Vacuum Infiltration

1. Place a tub into a vacuum desiccator and transfer 3 L infiltration buffer containing the *Agrobacterium* strains to the tub. Connect the desiccator to a Vacuubrand diaphragm vacuum pump (**Figure 3A**).
2. Place a plant upside down on the desiccator plate (**Figure 3B**) and lower the plate with the plant until the entire leaf and stem system is submerged into the infiltration buffer with the plate resting atop of the tub. Place the desiccator O ring along the rim and put the desiccator lid on the chamber (**Figure 3C**).
3. Turn on the vacuum pump and start timing when the vacuum reaches 100 mbar. Slowly open the release valve on the desiccator after 1 min at 100 mbar to allow entrance of *Agrobacteria* into the interstitial spaces of submerged plant tissue. Repeat this step one additional time to ensure good infiltration.
4. After infiltration, take the plant out of the desiccator and put it back to its upright position. Move plants back to the growth room and monitor protein expression between 2-15 dpi.

4. Fluorescent Protein Detection and Photography

1. Starting from 2 dpi, move plants into dark room and shine UV light on the back side of infiltrated leaves with a hand-held UV lamp.
2. Observe the green fluorescence of GFP or the red fluorescence of DsRed. GFP gives off a stronger signal with long wave UV light, while DsRed is stronger under short wave UV light.
3. Take photographs of fluorescent leaves with a regular digital camera with no flash.
4. Move plants back to the growth room after observation or photography.

Representative Results

1. Expression of Fluorescent Proteins by Syringe Infiltration

To demonstrate the effectiveness of syringe infiltration of *Agrobacterium* into plant tissue, we tested the expression of two fluorescent proteins - GFP and DsRed - by two different deconstructed plant viral vectors - geminiviral and MagnICON - in *N. benthamiana*. For *N. benthamiana* leaves that were entirely infiltrated with *Agrobacteria* containing geminiviral vectors, GFP expression was observed over the entire leaf area under UV light starting from as early as 2 dpi and reached peak accumulation at 4 dpi (**Figure 4C**). This early GFP expression by geminiviral vector is consistent with previous results of other recombinant proteins [14,16,18,19]. In contrast, leaves infiltrated with MagnICON vector-containing *Agrobacterium* combinations showed GFP fluorescence only after 5 dpi and reached its maximum accumulation at 7 dpi (**Figure 4D**). No green fluorescence was observed from leaves infiltrated with negative control *Agrobacterium* mixtures of pREP110 + p19 (**Figure 4B**) or pICH15879 + pCH14011 (data not shown), indicating that the fluorescence was specific to the GFP gene and was not the result of background fluorescence from the leaves. At its peak accumulation, the fluorescence of MagnICON vector-expressed GFP is more intense than that of geminiviral vector (**Figure 4C** and **4D**), similar to results previously obtained for other recombinant proteins [8,18]. The temporal expression pattern and the relative fluorescent intensity of DsRed are similar to that of GFP (data not shown). No major necrosis was observed on leaves infiltrated with GFP constructs (**Figure 4A**). Similar results were also observed for DsRed-expressing plants, indicating that neither fluorescent protein is highly toxic to plant cells. However, local necrosis at the nick site was observed and they appeared as "holes" in the photographs (**Figure 4**). When both florescent proteins were expressed on the same leaf *via* geminiviral vectors with syringe spot-infiltration, they were detected with their expected fluorescent color in the spot where they were infiltrated (**Figure 5**). Interestingly, co-infiltration of GFP and DsRed resulted in yellowish fluorescence (**Figure 5**). The intensity of DsRed fluorescence appeared to be weaker than that of GFP on the same leaf. However, this may not reflect the weaker expression of DsRed, but rather due to the less-than-optimal excitation of DsRed by UV light. Overall, our results demonstrate that syringe infiltration efficiently introduces recombinant gene-carrying *Agrobacteria* into plant leaves and resulted in robust expression of our target proteins. In addition, we demonstrated that this method allows the flexibility to either infiltrate entire leaves for maximal accumulation of one target protein or to spot-infiltrate multiple protein targets with multiple vectors for comparing their expression and expression pattern.

2. Expression of Fluorescent Proteins by Vacuum Infiltration

Vacuum infiltration was also examined to develop a scalable agroinfiltration method that can be used for large-scale production of recombinant proteins by plant transient expression systems. Our results indicate that the temporal expression pattern of GFP or DsRed by geminiviral and MagnICON vectors was not altered by the change of infiltration method and remained similar to that of syringe infiltration. As the entire shoot of a plant is submerged in the infiltration mixture during vacuum infiltration, fluorescence was observed for GFP (**Figure 6**) for all leaves of the infiltrated plants. Compared with syringe infiltration, it is more robust and can achieve infiltration of each plant with a much shorter time frame. For example, it takes a skilled student 15 min to syringe-infiltrate one entire 6-week old *N. benthamiana* plant. In contrast, the same plant can be infiltrated in 3 min by vacuum from start to finish and multiple plants can be infiltrated at the same time.

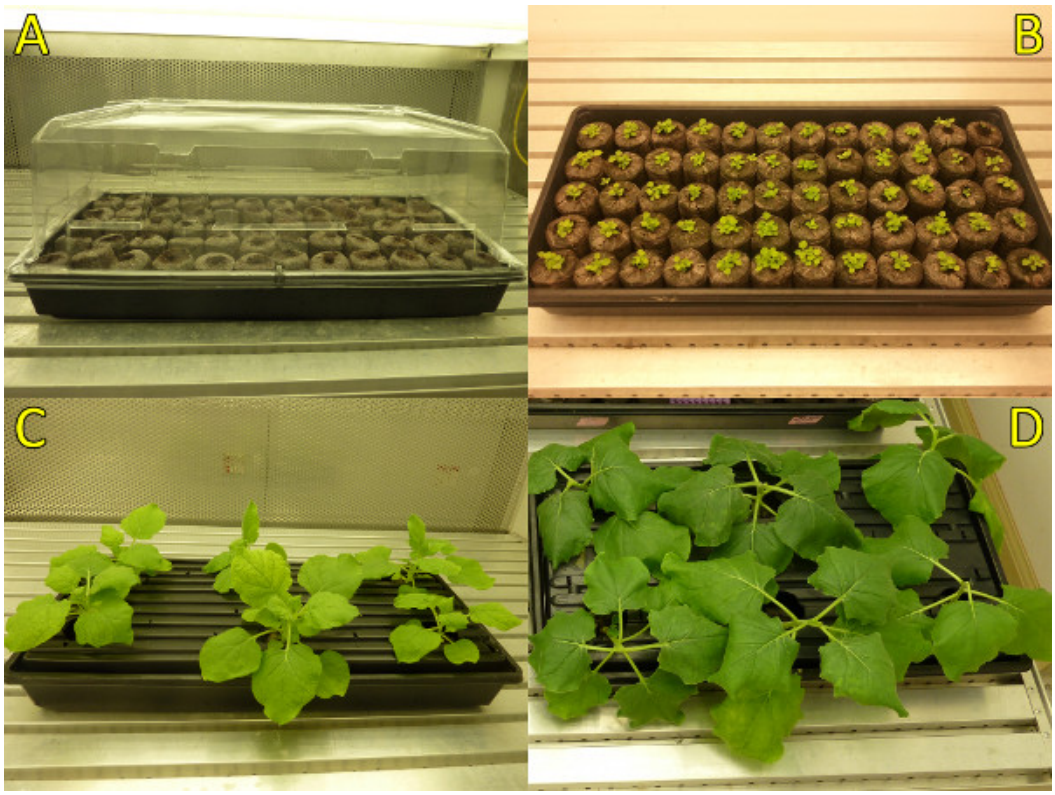

Figure 1. *N. benthamiana* plant growth with peat pellets and growth trays. Wild-type plants at 0 **(A)**, 2 **(B)**, 4 **(C)** and 6 **(D)** weeks after seeding.

Figure 2. Syringe infiltration of *N. benthamiana* leaves with *Agrobacterium tumefaciens*. Strain GV3101 of *A. tumefaciens* harboring GFP-expressing MagnICON vectors were resuspended in infiltration buffer and loaded into a syringe without a needle. A nick was created with a needle on the back side of a 6-week old plant leaf **(A)**. The opening of the syringe was positioned against the nick **(B)** and *Agrobacteria* in infiltration buffer were injected into the intercellular space of the leaf *via* the nick **(C)**.

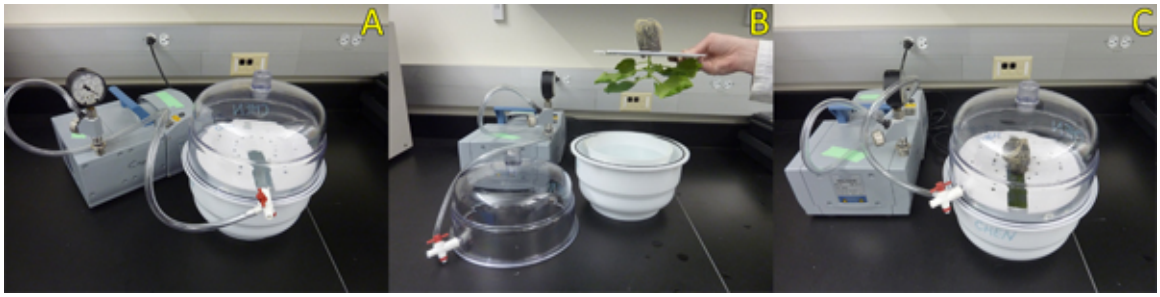

Figure 3. Vacuum infiltration of *N. benthamiana* leaves with *Agrobacterium tumefaciens*. Strain GV3101 of *A. tumefaciens* containing target protein-expressing vectors were resuspended in infiltration buffer and loaded into a 3 L tub. The tub was then placed into a vacuum desiccator that was connected to a vacuum pump **(A)**. A 6-week old plant was placed upside down on the desiccator plate **(B)**. The entire leaf and stem system was then submerged into the tub as the plate was placed atop the chamber **(C)**. Agroinfiltration was achieved by applying and releasing a vacuum at 100 mbar twice for 1 min. Click here to view larger figure.

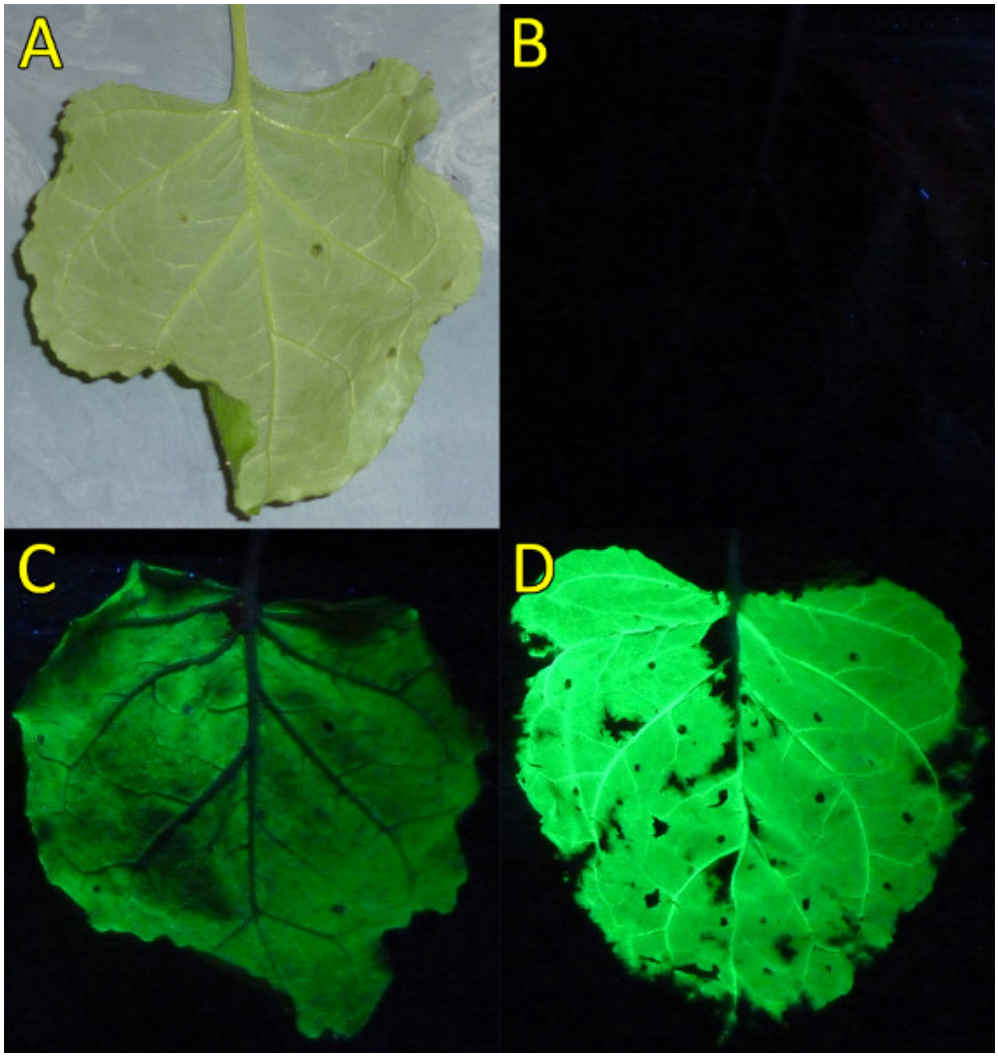

Figure 4. Expression of GFP in syringe agroinfiltrated leaves. Entire *N. benthamiana* leaves were infiltrated with strain GV3101 of *A. tumefaciens* harboring the GFP gene in a geminiviral **(A** and **C)** or MagnICON vector **(D)**. Negative control leaves were infiltrated with *Agrobacteria* strains that do not contain that GFP gene **(B)**. Leaves were photographed under white light **(A)** or UV light **(B-D)** at 4 dpi **(A-C)** or 7 dpi **(D)**.

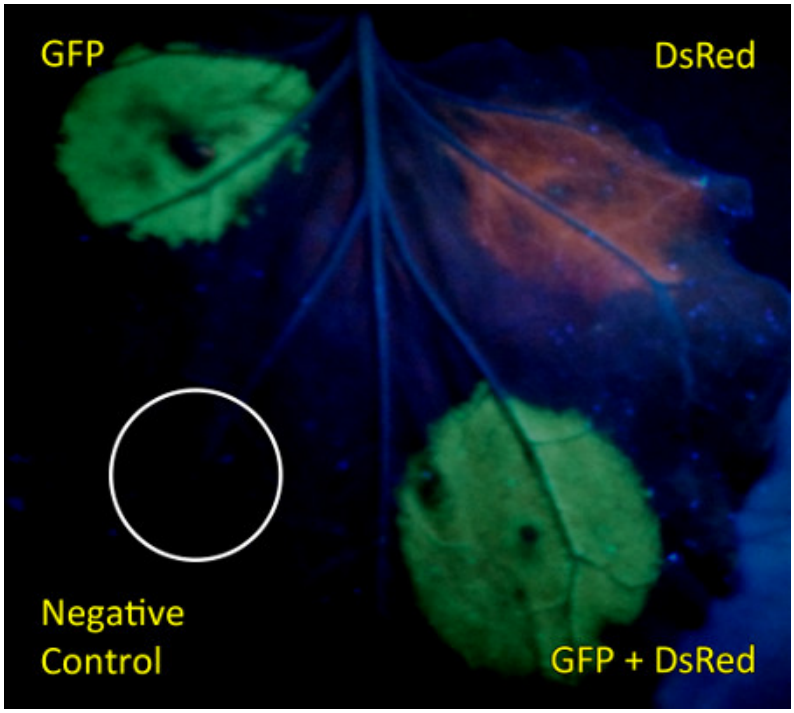

Figure 5. Expression of GFP and DsRed in syringe spot-agroinfiltrated leaves. *N. benthamiana* leaves were spot-infiltrated with GV3101 harboring the GFP, DsRed, or both GFP and DsRed genes in geminiviral vectors. A negative control spot was infiltrated with pREP110+p19. Leaves were photographed under UV light at 4 dpi.

Figure 6. Expression of GFP in vacuum agroinfiltrated leaves. *N. benthamiana* leaves were infiltrated with GV3101 harboring the GFP gene in MagnICON vectors. Leaves were photographed under UV light at 7 dpi.

Discussion

The increasing demands for protein-based pharmaceuticals worldwide require new production platforms that are robust, scalable, low-cost and safe. Plants have shown to be one of the most promising alternative production systems for pharmaceutical protein production. In recent years, the development of deconstructed virus-based vectors has enabled transient expression of proteins in plants, which greatly enhances the speed and yield of plant expression systems [2,10]. To further optimize the utility of the transient expression system, we demonstrate a simple yet efficient and scalable approach to introduce target-gene containing *Agrobacterium* into plant tissue. Our results indicate that agroinfiltration with either the syringe or vacuum method has resulted in the efficient introduction of *Agrobacterium* into the plant leaves and robust production of two fluorescent proteins, GFP and DsRed.

To ensure the efficient agroinfiltration and protein production, the following critical parameters must be carefully controlled. Deviations from these parameters may result in low agroinfiltration efficiency and in turn, low target protein expression.

1. Plant developmental stage and health. The most likely variable in this method between laboratories is the plant material for infiltration. While plants may appear phenotypically similar, their developmental stage and physiological state affect their competency in expressing recombinant proteins significantly. Specific parameters which have impacts on plant growth and protein expression levels include temperature, humidity, light intensity, supply of fertilizer, plant inoculation age, and time required for the maximum accumulation of target protein after leaf infiltration. Plants must consistently receive equal amounts of water and fertilizer daily. Minor changes in the plant growth conditions may drastically change the final size of plants and their ability to express recombinant proteins. Our previous studies indicate that plants grown under natural light yielded more leaf biomass, but protein yield is much less than that grown under artificial light [4]. Therefore, using artificial light is the method of choice for plant growth. Our results also show that a 16 hr light/8 hr dark cycle at 25 ± 0.5 °C is the optimal condition to grow *N. benthamiana* plants under such artificial lighting [4]. We demonstrated that under these conditions, 6-week plants are the optimal age for GFP and DsRed expression as they produce high-levels of fluorescent proteins while the biomass yield is adequate. Plants older than 6-weeks produce more biomass but are too tall to fit into the infiltration chamber [4]. In addition, flowers start to develop after 6 weeks of growth, negatively affecting recombinant protein expression [4]. Consequently, 6-week plants contain the optimal leaf material that balances the combined need of biomass yield, protein accumulation, and the ease of agroinfiltration.

2. Growth and infiltration concentration of *Agrobacterium*. Another key point in this methodology is the control of growth and infiltration concentration of *A. tumefaciens*, as measured by the OD_{600}. In each culture and subculture step, strains of *A. tumefaciens* must be grown to, but not over the designated OD_{600}. We have examined multiple concentrations of *A. tumefaciens* for the final infiltration of *N. benthamiana* leaves [4]. Low *Agrobacterium* concentration will result in the insufficient delivery of target genes into plants, leading to low protein expression. On the other hand, if the infiltration concentration of *Agrobacterium* is too high, it will trigger a hypersensitive response in the infiltrated tissue and lead to necrosis [20]. Our results demonstrated that OD_{600} = 0.12 *Agrobacterium* strain balances the need for maximum delivery of gene construct without causing tissue necrosis and cell death. The desired OD_{600} density of *Agrobacterium* can be obtained by using consistent culture media, temperature and culture time.

3. For vacuum infiltration, it is important to follow the designated vacuum pressure and infiltration duration. Our results indicate that one 3 L tub of *Agrobacterium* infiltration mixture can be used to infiltrate approximately 30 plants. If more than 30 plants need to be infiltrated, a new batch of *Agrobacterium* infiltration mixture needs to be supplied.

4. The specific parameters presented in this paper are optimized for expression of proteins using *N. benthamiana* plants grown under specific conditions described above with deconstructed virus-based vectors. As discussed earlier, the most difficult parameter to control in this methodology is the plant material. We have expressed a variety of vaccines and therapeutic proteins using plants and the infiltration procedure we described here and obtained excellent results in all cases [4,8,14,18,21-23]. These results demonstrated that the conditions we have developed are optimal for protein expression. However, if different host plant species, expression vectors, or different *N. Benthamiana* growing conditions were used for infiltration, each parameter in this methodology would need to be re-optimized by experiments.

Overall, we have demonstrated that agroinfiltration with syringe and vacuum is a simple yet efficient methodology to introduce target gene carrying *Agrobacterium* into plants for transient expression of recombinant proteins. Both methods have their unique advantages depending on the goal of research and production. Syringe infiltration is simple, requires only small volumes of *Agrobacterium* culture and does not need expensive pumps and vacuum chambers. As demonstrated in this report, it has the flexibility to either infiltrate the whole leaf with one target gene or use spot infiltration to introduce genes of multiple targets on one leaf. The entire leaf infiltration method can be used for small laboratory scale expression of recombinant protein for their biochemical characterization, purification, and preclinical functional studies [1]. In contrast, spot infiltration can be used to express multiple protein targets on one leaf to compare their yield, expression kinetics and toxicity to plants. It can also be used to compare the expression of one reporter protein such as GFP or DsRed driven by different expression vectors on the same leaf. As a result, different vector's ability in driving protein accumulation and their expression kinetics can be characterized. The simplicity of syringe infiltration also enables it a feasible tool to teach and train high school and undergraduate students in the subject of biotechnology and genetic engineering.

In comparison with syringe infiltration, vacuum infiltration requires the investment of vacuum pumps and chambers and larger volumes of *Agrobacterium* cultures. Therefore, it is not the method of choice when just a few plants need to be infiltrated. However, it provides the scalability that cannot be matched by syringe infiltration. It is more robust and can infiltrate large numbers of plants in a short period of time. With the scale presented in this report, we are able to produce gram level of purified pharmaceutical proteins sufficient for a Phase I human clinical trial [4]. Furthermore, this process can be further scaled-up for commercial manufacture of pharmaceutical proteins from plants. For example, processes are being designed to vacuum infiltrate three metric tons of *N. benthamiana* plants per hour in a scale-up operation [24]. Another advantage of vacuum infiltration is that it can be used to agroinfiltrate plant species that are not amenable for syringe infiltration.

In summary, the combination of syringe and vacuum infiltration provides researchers, biotechnologists and educators a simple, efficient, robust and scalable methodology for transient expression of recombinant proteins in plants. It will greatly facilitate the development and production of pharmaceutical proteins and promote science education.

Disclosures

The authors have no competing financial interests.

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