Development and Expression of Subunit Vaccines Against Viruses in Plants

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Abstract

Various systems exist for the robust production of recombinant proteins. However, only a few systems are optimal for human vaccine protein production. Plant-based transient protein expression systems offer an advantageous alternative to costly mammalian cell culture-based systems and can perform posttranslational modifications due to the presence of an endomembrane system that is largely similar to that of the animal cell. Technological advances in expression vectors for transient expression in the last two decades have produced new plant expression systems with the flexibility and speed that cannot be matched by those based on mammalian or insect cell culture. The rapid and high-level protein production capability of transient expression systems makes them the optimal system to quickly and versatility develop and produce vaccines against viruses such as 2019-nCoV that have sudden and unpredictable outbreaks. Here, expression of antiviral subunit vaccines in \textit{Nicotiana benthamiana} plants via transient expression is demonstrated.

Key words Plant-produced vaccines, Agroinfiltration, Transient expression, Vaccines, \textit{Nicotiana benthamiana}

1 Introduction

Flaviviruses such as the yellow fever and dengue viruses are responsible for causing illness in a large portion of the global population [1–3]. The 2015 outbreak of the flavivirus Zika (ZIKV) in the Americas and Caribbean was linked to devastating congenital conditions in newborns such as microcephaly and Guillain-Barre syndrome in adults [1–3]. Unfortunately, neither a vaccine nor a specific treatment is available, calling for the urgent development of ZIKV vaccines. Like all flaviviruses, the virally encoded envelope (E) protein covers the entire virion surface, consequently making it the main antigenic target of the immune system and neutralizing antibodies [4]. Thus, the ability to quickly express and evaluate the ZIKV E protein and its various domains becomes an important issue, highlighting the need for flexible and fast systems for vaccine protein production [5].
A wide range of recombinant protein production systems exist and include systems based on mammalian, insect, bacterial, and yeast cell culture [6]. However, these cell-based systems command a large investment in capital required to establish the necessary infrastructure for protein production. Furthermore, the output of proteins can reach a cap very quickly, hindering large-scale production of biologic proteins. Plant-based protein expression systems offer numerous advantages over traditional cell culture-based systems, such as greater scalability, faster protein production times, lower startup costs, and potential of great biomass production by employing commercial agriculture practice [6]. Furthermore, plants possess the eukaryotic endomembrane system which enables plants to perform posttranslational modifications that are important in the protein folding, structure, stability, and function [7]. In addition, plants also offer the advantage of a lower risk of harboring animal pathogens, thereby reducing the risk of inadvertent contamination of the final drug products [8, 9].

Recombinant protein expression in plants can be achieved from two sources: the nuclear genome and the plastid genome. Because posttranslational modifications are important in the function of therapeutic proteins, expression of proteins from the nucleus is preferred [10]. Nuclear or plastid stable expression of transgenes can be heritable through succeeding generations in the form of seeds, which allows transgenic plants to be grown on agricultural scales [10]. Despite the advantages of stable transformation, production of a plant line that stably expresses a transgene is laborious and extremely time consuming, and biosafety regulations are in place to restrict growing transgenic plants in open fields [11].

Transient expression is another option for the expression of recombinant proteins in plants. Here, the gene of interest (GOI) is expressed during its transient presence in the host cell [12]. Decades of research have led to the development of systems that use the ability of *Agrobacterium tumefaciens* (*A. tumefaciens*), a Gram-negative soil bacterium, in transferring its plasmid DNA to plant cells. In agroinfiltration, *A. tumefaciens* bacteria carrying the GOI are infiltrated into the interstitial space of the mesophyll of leaves to deliver the GOI into plant cells to drive target protein production [13, 14]. When compared to other methods, transient protein production via agroinfiltration reaches greater protein accumulation levels in much less production lead times. Furthermore, the risk of inadvertent environmental contamination is virtually eliminated since agroinfiltration is done indoors and infiltrated plants are grown in enclosed areas [13]. Due to the underlying genetic mechanisms of transient expression, infiltration must be repeated for each batch of recombinant protein desired. However, transient expression offers a significantly improved yield, biosafety, and production speed of recombinant proteins, which makes it the best
selection for multiple applications including basic research and industrial-scale production of plant-made vaccine recombinant proteins.

The last two decades have seen advances in the development of numerous vectors for transient expression of recombinant proteins in plants. The more recently developed “deconstructed viral” vectors have gained favor due to their superior performance in the production of high levels of recombinant protein production with unprecedented speed [15, 16]. Deconstructed viral vectors also offer advantages such as improved biosafety and reduced environmental risks due to the elimination of infectious viral functions [17, 18]. In particular, the MagnICON and geminiviral deconstructed viral vectors are described here. The MagnICON vectors are RNA replicons based on the genomes of the tobacco mosaic virus and the noncompeting potato virus X [19]. On the other hand, the geminiviral vectors are a DNA replicon system based on the bean yellow dwarf virus and it is comprised of three parts that can be combined into a single vector: the pBY-GOI segment containing the replicon of the target gene, the pREP110 construct coding for the Rep/RepA protein necessary for replication, and the p19 that codes for a posttranscription silencing suppressor [20, 21]. Noteworthy, one or more replicons can be encoded in a single geminiviral vector to produce several different proteins or a protein with multiple hetero-subunits [21]. Additionally and in contrast to MagnICON vectors, geminiviral vectors have a broader range of plant hosts. Both systems have been successfully used to produce plant-made pharmaceuticals [22, 23]. Results from our laboratory and others have shown that transient expression with “deconstructed” plant viral vectors allows for the production of up to 5 mg of antivirus vaccines per gram of leaf fresh weight within 4–10 days of vector inoculation [16, 24–33]. Therefore, the rapid and high-level protein production capability of these new transient expression systems makes them the optimal system to produce and evaluate vaccine candidates for viral diseases with unpredictable and sudden outbreaks. To transiently express vaccine proteins in plants, the GOI has to be cloned into an appropriate transient expression vector and this GOI-carrying “deconstructed” plant viral vector is then introduced into A. tumefaciens.

The introduction of GOI into plant cells can be done by agroinfiltration during which the transient expression vector is transferred into plant cells by A. tumefaciens. Syringe infiltration is the most common method of agroinfiltration in the laboratory for small-scale GOI delivery as it allows multi-GOI infiltration on different parts of the plant or combinations thereof.

Nicotiana benthamiana (N. benthamiana) has been the plant of choice for transient protein production since it offers advantages such as a thoroughly characterized biology, high biomass yield, abundant availability of compatible expression vectors, and
amenability to agroinfiltration [8]. Agroinfiltrated plants will return to greenhouses to allow target protein production. With MagnICON or geminiviral vectors, transiently expressed target proteins generally reach their peak accumulation between 5 and 10 days post-agroinfiltration. Leaves will be harvested and total soluble proteins will be extracted from the plant material [34–38]. The target protein will then be purified by a target-protein-specific process [39, 40]. Purified target proteins will be further characterized by biochemical and immunological assays and ultimately tested in animal models. Therefore, before expressing a target protein in plants, a thorough purification strategy should be in place to remove unwanted compounds so that the final recombinant protein preparation is conducive to downstream applications and assays. Here, a protocol designed to produce a subunit vaccine based on ZIKV E protein by transient expression via syringe agroinfiltration of *N. benthamiana* plants is presented.

### 2 Materials

Prepare all media using double-distilled water and sterilize via autoclave or a filtration method that is capable of excluding bacterial contamination. Please observe biological and chemical waste disposal regulations when disposing waste material.

#### 2.1 Plant Growth Materials

1. T.O. Plastics (Clearwater, MN) SFT-1020-OPEN-NH standard flat carry tray.
2. T.O. Plastics STI-804 standard insert.
3. Humidity dome.
4. Peat pellets (Jiffy-7727W/Hole, Canada).
5. Handheld seeder (e.g., Vibro HandSeeder VHS-1).
6. Water-soluble fertilizer: 15–16–17.
7. *N. benthamiana* seeds.
8. Linear fluorescent bulbs Sylvania FO32/25 W/835/XP/SS/ECO3 (Wilmington, MA).

#### 2.2 Bacterial Culture

1. *A. tumefaciens* strain GV3101 carrying the GOI.
2. 100 mg/mL (1000×) Carbenicillin disodium (*see Note 1*).
3. 16.65 mg/mL (333×) Rifampicin.
4. Yeast extract nutrient broth (YenB) medium: 1% Nutrient broth, 0.5% yeast extract. When preparing agar plates, add 1.5% agar to the YenB medium.
5. Shaker incubator.
6. Spectrophotometer capable of reading absorbance at 600 nm.
2.3 Infiltration

1. 15 mL Syringe.
2. Sterile thumbtack.
3. Rubber bands.
4. Tray.
5. Absorbing pad.
6. 2 × 100 mL Plastic beakers.
7. Goggles.
8. Gloves.
9. 2-(N-morpholino) ethanesulfonic acid (MES) buffer (1 ×: 10 mM MES hydrate (FW 195.24), 10 mM MgSO₄ hydrate (FW 246.47)): Mix in deionized water and adjust pH to 5.5 with sodium hydroxide (NaOH). Sterilize by passing the MES buffer through a 0.2 μM filter.
10. Phenylmethylsulfonyl fluoride (PMSF) 1 M stock.

2.4 Harvesting

1. Razor blade(s).
2. Backing board.
3. Blender.
4. Centrifuge bottles/caps.
5. Funnel.
6. PBS buffer, pH 5.2.
7. PMSF.
8. Ice container.
9. Ice.
10. Cheese cloth.
11. 0.5–0.2 μM Vacuum filtration device.

2.5 Immobilized Metal Affinity Chromatography

1. Ring stand.
2. Chromatography column.
3. Nickel-nitrilotriacetic acid (Ni-NTA) agarose beads.
4. Imidazole buffers (50, 100, 300, 500 mM).
5. 50 and 15 mL conical tubes.

3 Methods

Plants are grown inside a climate-controlled room at 25 °C, 60–80% humidity, and with a 16/8-h day/night cycle under artificial fluorescent lighting (85–100 Lux).
3.1 Plant Growth

1. Place 8 standard inserts and 32 peat pellets inside the flat carry tray. Add 1–2 L water and allow the pellets to expand to their full size.

2. Using a handheld seeder, add 2–4 *N. benthamiana* seeds to the center of each pellet. Add enough deionized water to cover the bottom quarter of the peat pellet (see Note 2).

3. Cover the tray with a humidity dome (Fig. 1) and allow the seeds to germinate for 1 week in the growth chamber. Beginning with week 2, shift the dome so that a small opening is created (see Note 3).

4. After 2 weeks, remove the dome and add 2 L of 1.48 g/L fertilizer to the tray. Continue to grow plants under the same environmental conditions, adding fertilizer every 2 days or as necessary (see Notes 4 and 5).

5. Starting with week 5, remove all but five of the largest leaves on the plant. They should ideally be evenly spaced.

6. Continue to fertilize the plants and remove any new sprouting leaves, leaving a total of just five leaves to fully grow, up until the end of week 6, at which point the plants are ready for infiltration (Fig. 2).

3.2 *A. tumefaciens* Culturing and Preparation for Syringe Infiltration

1. Streak 50–100 μL (5.0 × 10^7–1.0 × 10^8 CFU) *A. tumefaciens* GV3101 strains containing ICON vector modules carrying the gene of interest (e.g., ZIKV E protein) on LB-agar plates with 100 μg/mL carbenicillin and 333 μg/mL of rifampicin. Grow for 48 h at 30 °C [5].

Fig. 1 Peat pellets and growth trays with a humidity-promoting dome. After sowing with *N. benthamiana* seeds the tray should be covered to promote germination.
2. Inoculate a 10 mL liquid YenB starter culture from a single colony for each strain. Grow at 30 °C in a shaker (200 RPM) for 16–20 h, until the OD600 reaches at least 1.0 (measure OD600 with a spectrophotometer).

3. Use the culture to inoculate a new 100 mL YenB subculture containing the appropriate antibiotics for each strain and incubate at 30 °C and 200 rpm for 16–20 h to reach an approximate OD600 of 1.5–1.8.

4. Centrifuge the cultures at room temperature and 6000 × g for 15 min. Remove the supernatant to obtain a bacterial pellet.

5. Resuspend the pellet in 10 mL MES buffer and measure the OD600 (see Note 6).

6. If multiple vector modules are used, mix equal amounts of each vector-carrying *Agrobacterium* strain together in 200 mL of MES buffer. Use the following formula to calculate the volume of each of the strains to transfer. The final OD600 of each strain should be equally divided to obtain a total OD600 of 0.21 (see Note 7):

   \[ V_{\text{subculture}} = \frac{(\text{OD600} \times 0.21)(200 \text{ mL})}{\text{OD600}} \]

3.3 **Syringe Agroinfiltration**

1. Gather a tray, paper towels, adsorbent pad, syringe, thumbtack, and rubber band.

2. Assemble the syringe/thumbtack infiltration setup using a rubber band (see Fig. 3 and Note 8).

3. Pour approximately 200 mL of infiltration media into a plastic 300 mL beaker (see Note 9).

4. With the syringe, siphon approximately 10 mL of the infiltration media.
5. Pick a leaf and target infiltration area. With the hand not holding the syringe, place a tip of a finger against the back side of the leaf to act as a support for infiltration.

6. With the hand holding the syringe, lightly pierce a small hole with the thumbtack and place the nozzle of the syringe over the hole.

7. Slowly push on the syringe plunger and observe infiltration media spread throughout the leaf (Fig. 4) (see Note 10).

8. Pick a different spot on the leaf and infiltrate again. Once the entire leaf takes on a darker color due to the infiltration media, the leaf is done.

9. Infiltrate all leaves in plants and place back on tray.

10. Once all plants on tray have been infiltrated, fill the tray with tap water up to 1/3 of the peat pellet, return tray to growth chamber, and monitor expression (see note: leaves should be harvested in approximately 1 week).
3.4 Harvesting, Isolation, and Characterization of Recombinant Zika Envelope Protein

1. Harvest the plant leaves on the sixth day post-infiltration by cutting the leaf stem from the plant.
2. Remove the central vein from the leaf using a razor blade leaving behind just the two halves of the leaf. Discard the central vein.
3. Prepare the homogenization buffer using the following calculation:

\[
\text{Homogenization buffer} = (\times \text{g of leaves}) \times \left( \frac{1.5 \text{ mL}}{\text{g of leaves}} \right)
\]

4. The homogenization buffer should contain phenylmethylsulfonyl fluoride at a final concentration of 2 mM.
5. Place bottles on ice and funnel on top of them with the cheese-cloth over the funnel.

**Fig. 4** Syringe agroinfiltration of *N. benthamiana* leaves. Slowly depress the plunger and allow the leaf to take up the infiltration media while noting pressure and relative rate of flow/spreading of the infiltrate. Once the rate of flow slows down, pick a different part of the leaf and begin the infiltration process again. Infiltration is complete once the entire leaf has changed to a slightly darker shade of green.
6. Add the homogenization buffer to the blender and add the leaves.
7. Blend at low speed until the leaves have largely been broken down. Thereafter, increase the blender speed to high for about 1 min.
8. Pour the homogenate on the cloth on top of the funnel. Allow the homogenate to flow through the cloth for about a minute.
9. Carefully wrap the homogenate with the cloth and gently squeeze to allow filtration of the homogenate to flow through the funnel and into the bottle(s) (see Note 11).
10. Spin the sample at 14.8K × g for 30 min at 4 °C, twice.
11. Pour the supernatant onto a large plastic container (on ice) and add a stir bar.
12. Adjust the pH to 5.2 and let sit overnight at 4 °C or a minimum of 4 h. The isoelectric point of the ribulose-1,5-bisphosphate, an enzyme involved in the first major step of carbon fixation, is reached at a pH of 5.2, thus promoting the removal of this most abundant plant leaf protein via isoelectric point precipitation.
13. Transfer the sample to centrifuge bottles and spin again at 14.8K × g for 30 min at 4 °C, twice to remove the precipitated protein.
14. Transfer the sample to a plastic container and adjust the pH to 7.0.
15. Transfer the sample to centrifuge bottles and spin at 14.8K × g at 4 °C.
16. Transfer the sample to a vacuum-filtering device to remove fine particles that can potentially and quickly foul the column.
17. Using immobilized metal affinity chromatography, flow the sample through Ni²⁺ His-Bind column in accordance with the manufacturer’s instructions (MilliporeSigma, Billerica, MA).
18. Analyze purified ZIKA envelop protein by SDS-PAGE and Western blot under reducing conditions [5]. The expected molecular weight of plant-produced Zika E protein is approximately 52.7 kDa (see Fig. 5).

4 Notes

1. All antibiotics must be filter sterilized.
2. Use 2–4 seeds to maximize the possibility of germination.
3. The use of a dome significantly aids in maintaining a high level of humidity and minimal airflow. Beginning with week 2, the
dome is shifted to unseat it, thereby allowing airflow and a subsequent decrease in humidity.

4. The condition and overall quality of the host plant are the utmost important factors in the final yield and quality of the target recombinant protein. Thus, it is imperative to monitor the color of the plants and avoid the use of any leaves that are yellowing, necrotic, beginning to wilt, or thin to the touch (may be dehydrated). These changes or abnormalities can translate to significant changes in recombinant protein yield and quality.

5. Avoid overwatering the plants since it will lead to the overgrowth of roots and cover the trays. Ideally, it is best to re-fertilize when the tray has become close to dry and the peat pellets are still visibly moist.

Fig. 5 SDS-PAGE and Western blot analysis of plant-produced Zika E expressed in *N. benthamiana* plants and purified via Ni$^{2+}$-immobilized metal affinity chromatography. (a) Plant-produced Zika envelope recombinant protein was analyzed on a 12% SDS-PAGE Coomassie-stained gel. The far-right lane shows the plant-produced Zika envelope recombinant protein slightly above the 50 kDa marker (arrow), as expected for the calculated MW of 52.7 kDa. The far-left lane contains total leaf-soluble protein and the second lane from the right contains Ni$^{2+}$-immobilized metal affinity chromatography flow-through. (b) Western blot analysis of plant-produced ZIKV E protein. The left lane contains extract from uninfiltrated leaves, whereas the right lane contains extract from the leaves agroinfiltrated with the plant-produced Zika envelope recombinant protein construct. The plant-produced Zika envelope recombinant protein is noted slightly above the 50 kDa mark, as expected.
6. The resuspension of the bacterial cells in a 1/10 volume of MES effectively concentrates the cells and allows for the manipulation of smaller volumes.

7. Good results with GFP have indicated an OD600 value of 0.07. However, optimum yields and quality of the target recombinant protein may require a slightly higher OD600. Additionally, when expressing multiple recombinant proteins or multiple subunit recombinant proteins, it is possible to modulate the level of expression and ratios of the recombinant proteins by simply adjusting their respective OD600s.

8. The use of a needle to puncture the leaf is not necessary since the plant tissue is soft and easily punctured with a thumbtack. It is also helpful to trim off the edges on one side of the plastic component of the thumbtack. This will aid in the stability of the assembled apparatus. The use of a thumbtack attached to the syringe significantly reduces any accidental deep punctures to fingers. Furthermore, the attachment of the piercing thumbtack to the syringe eliminates the constant lifting and releasing of a thumbtack needed to initiate infiltration on different parts of the leaves.

9. Prior to starting the infiltration process, it is helpful to place a few paper towels under the front part of the tray so as to elevate it and allow infiltrate overflow to run to the back of the tray. This significantly helps keep the process tidy.

10. Avoid initiating infiltration on any leaf vein, especially the central vein. It is extremely difficult to infiltrate from the central vein and counterproductive. It is important to start the infiltration process slowly and gauge the pressure and rate of spread of the infiltration media. If too much pressure is applied a hole may be blown on the leaf tissue. Furthermore, blistering of the leaf may occur at which point one should back off and reinitiate the infiltration process at a different part of the leaf.

11. When squeezing the homogenate in the cheesecloth, it is best if all sides and angles are squeezed in alternating fashion. This will ensure that all areas of the homogenate are pressed and will also minimize the possibility of bursting the cheesecloth and spreading the homogenate all over the work area.

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