Chapter 6

Production of Recombinant Proteins by *Agrobacterium*-Mediated Transient Expression

Holger Spiegel, Stefan Schillberg, and Greta Nölke

Abstract

The agroinfiltration of plant tissue is a robust method that allows the rapid and transient expression of recombinant proteins. Using wild-type plants as biomass, agroinfiltration exploits the ability of plants to synthesize even complex multimeric proteins that require oxidative folding and/or post-translational modifications, while avoiding the expensive and time-consuming creation of stably transformed plant lines. Here we describe a generic method for the transient expression of recombinant proteins in *Nicotiana benthamiana* at the small to medium laboratory scale, including appropriate binary vectors, the design and cloning of expression constructs, the transformation, selection, and cultivation of recombinant *Agrobacterium tumefaciens*, the infiltration of plants using a syringe or vacuum device, and finally the extraction of recombinant proteins from plant tissues.

Key words Extraction, GV3101, pAIX, *Nicotiana benthamiana*, Syringe method, Vacuum infiltration

1 Introduction

The transient expression of recombinant proteins is one of the most interesting concepts in the field of molecular farming [1]. In contrast to stably transformed plant lines [2], production processes based on transient expression can be established rapidly (within a few months, or even weeks depending on the availability of plant biomass) and offer a high degree of flexibility. Because wild-type plants are used as biomass for production, the host system is generic and the recombinant *Agrobacterium tumefaciens* strains used in such processes can easily be switched when modifications are required in the target protein. This is especially valuable in the midst of rapidly spreading epidemic or pandemic diseases, when the prompt manufacture of recombinant proteins (especially diagnostic reagents, therapeutic antibodies, and vaccine candidates, many of them complex glycoproteins [3]) is essential, as

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demonstrated during the 2014 outbreak of Ebola hemorrhagic fever and, more recently, in the case of COVID-19 [4–7].

In plant biotechnology, engineered strains of *A. tumefaciens* are used to deliver genetic material to plants. The technology utilizes the natural mechanism of T-DNA transfer and leads to the genomic integration of genetic elements originating from the *A. tumefaciens* tumor-inducing (Ti) plasmid [8–10]. Several *A. tumefaciens* strains have been modified to carry a so-called disarmed Ti plasmid that lacks the tumor-inducing ability of wild-type plasmids but retains the functions required for T-DNA transfer to infected host cells [11]. In binary vector systems, the disarmed Ti plasmid also lacks a functional T-DNA, but is paired with a second plasmid carrying the T-DNA left and right border sequences flanking a selectable marker that functions in plants and an expression cassette for the recombinant protein of interest [12]. The Ti plasmid, which in its natural form is very large (up to 235 kb) and difficult to manipulate in the laboratory, therefore provides the T-DNA transfer functions in *trans* to the binary vector, which is small and suitable for cloning. For this reason, the smaller vector carries origins of replication for both *Escherichia coli* and *A. tumefaciens*, and markers for antibiotic resistance to allow selection in bacterial culture.

The expression cassette between the T-DNA borders can be tailored to control expression levels and subcellular targeting in plant cells. Examples include the inclusion of scaffold attachment regions (SARs) [13], appropriate regulatory sequences (promoter, 5′ and 3′ untranslated regions, and a transcriptional terminator/polyadenylation site), and targeting signals (leader peptides for secretion, plastid or vacuolar targeting signals, or retrieval sequences that cause recombinant proteins to accumulate in the endoplasmic reticulum) [14]. To facilitate generic purification strategies, the cassette may also include peptide tag sequences such as His_6 or fusion partners such as the IgG Fc domain. When the recombinant protein consists of more than one distinct subunit (e.g., an antibody consisting of two identical heavy chains and two identical light chains), there are several available strategies including the placement of multiple tandem expression cassettes within the same T-DNA and the preparation of separate binary vectors (and separate *A. tumefaciens* strains) for each subunit delivered by co-infiltration [15, 16]. The tandem expression cassette strategy ensures that all transgenes co-integrate at the same locus and is therefore preferred for stable transformation, because it avoids the risk of meiotic segregation. However, the efficiency of cloning and T-DNA transfer begins to decline as the size of the T-DNA increases, with a practical limit of 15–20 kb. The use of identical promoters for two or more expression cassettes may also promote recombination events that lead to the loss of genes or gene fragments. For transient expression, co-infiltration with different *A. tumefaciens* strains has many advantages, including simpler

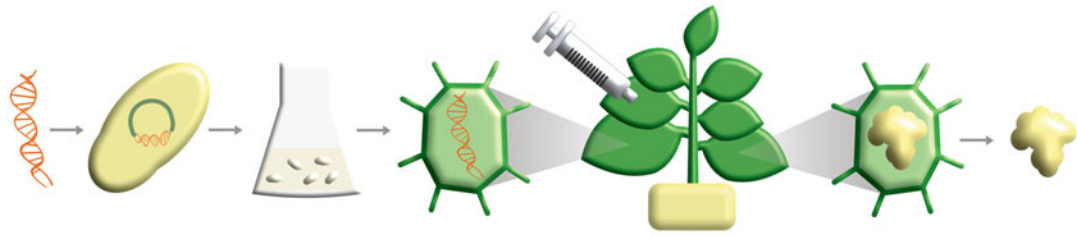

Fig. 1 Schematic overview of the workflow for transient expression in plants mediated by *A. tumefaciens*

cloning, more efficient bacterial transformation, the ability to optimize the expression of multiple proteins or protein subunits by adjusting the ratio of different strains in the *Agrobacterium* suspension [17, 18], and the ability to add further *Agrobacterium* strains, for example carrying a vector encoding a silencing supressor to further improve recombinant protein yields [19].

Having prepared the binary expression vector(s), a simple workflow can be used to produce and purify recombinant proteins by transient expression in *N. benthamiana* (Fig. 1). All the materials and methods needed to perform these steps are described below.

2 Materials

Prepare all solutions using deionized water and use them at room temperature (22 °C) unless otherwise noted. Strictly adhere to all safety regulations that apply to the chemicals described herein. For example, appropriate personal protective equipment should be worn, including gloves and safety glasses, when handling concentrated acids or bases. Also, follow local waste disposal guidelines and any regulations that apply to the handling of genetically modified organisms (GMOs).

2.1 Construct Cloning and Sequencing

1. Eppendorf multiporator.
2. 2-mm electroporation cuvette.
3. Vector pAIX-c or pAIXpress (derivatives of pTra [20])—Fraunhofer IME, Germany (Fig. 2).
4. Oligonucteotides for colony PCR and sequencing. pAIX-backbone-specific forward primer PS5′ and pAIX-backbone-specific reverse primer PS3′ for colony PCR and sequencing (5′-GAC CCC TCC TCT ATA TAA GG-3′).
5. *Agrobacterium tumefaciens* strain GV3101::pMP90RK [GmR, KmR, RifR] [21].
6. *Escherichia coli* cloning strain (e.g., DH5 alpha).
7. Restriction and DNA modifying enzymes and *Taq* polymerase: NcoI, NotI, XbaI, T4 DNA ligase, and *Taq* polymerase.

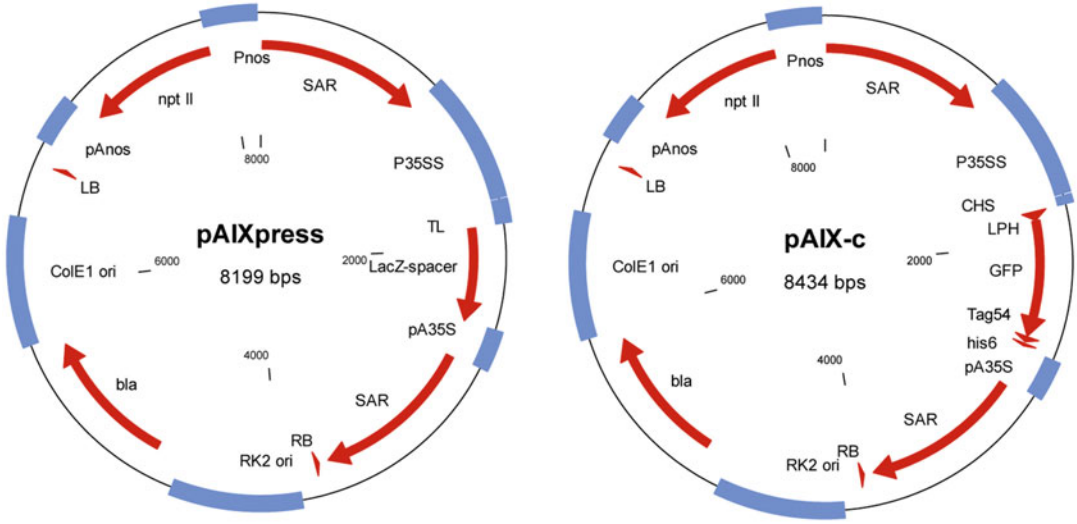

Fig. 2 Maps of the binary vectors pAIX-c and pAIXpress. The plasmids are derivatives of pTRA [20] and likewise contain two origins of replication (ColE1 *ori* for *E. coli* and RK2 *ori* for *A. tumefaciens*) and an ampicillin-resistance cassette (*bla*) as a bacterial selection marker. Recombinant protein expression is driven by the Cauliflower mosaic virus 35S promoter with a duplicated enhancer region (P35SS), the 5′ untranslated region of the chalcone synthase (CHS) gene from *Petrosinella* (pAIX-c) or the 5′ untranslated region of Tobacco etch virus [21] (pAIXpress) and the Cauliflower mosaic virus 35S polyadenylation signal (pA35SS). Scaffold attachment regions (SAR) were introduced next to the right and left borders (RB and LB) of the T-DNA to improve gene expression. For the generation of stable transgenic lines, the T-DNA contains the kanamycin-resistance gene (*nptII*) under the control of the nopaline synthase promoter (pNos) as a plant selection marker

8. Lysogeny broth (LB)—*E. coli* growth medium: 5 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl.
9. Yeast extract broth (YEB)—*A. tumefaciens* growth medium: 5 g/L tryptone, 5 g/L beef extract, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L MgSO4, adjust to pH 7.

2.2 Agrobacterium Cultivation

1. Yeast extract broth (YEB)—*A. tumefaciens* growth medium: 5 g/L tryptone, 5 g/L beef extract, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L $MgSO_4$, adjust to pH 7.0.

2.3 Plant Cultivation and Infiltration

1. *Nicotiana benthamiana* plants (Rdr1 insertion genotype) [22] (*see* **Note 1**).
2. Desiccator with connections and tubing.
3. Rotary vane vacuum pump.
4. 1-mL Ominifx F syringe.
5. 2× infiltration medium: 100 g/L sucrose, 3.6 g/L glucose, 1 g/L Ferty-II-Mega Fertilizer (Planta, Regenstauf, Germany), adjust to pH 5.4–5.8.
6. Acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone), 200 mM stock in dimethylsulfoxide (DMSO).

2.4 Protein Extraction

1. Default extraction buffer (PBS, pH 7.4): 8 g/L, NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, adjust to pH 7.4 with 100 mM HCl.
2. Blender.
3. Laboratory scale.
4. Miracloth.

3 Methods

3.1 Preparation of Expression Constructs

Synthetic genes or PCR products can be introduced into the pAIX vector of choice (pAIX-c or pAIXpress). Both vectors (Fig. 2) are derivatives of pTRA [20] and share the same backbone genetic elements (T-DNA, selection marker cassettes, SARs) as well as the promoter, 5′/3′ untranslated regions, and terminator of the expression cassette. The preferred vector selection and cloning strategy depends on the recombinant protein of interest, and specific requirements for product purification and detection. Cloning procedures such as restriction enzyme digestion, ligation, and isolation of plasmid DNA should be carried out according to standard methods [23]. Vector pAIX-c should be chosen if the preferred strategy is to direct the recombinant protein through the secretory pathway, including the endoplasmic reticulum and Golgi body. This will support oxidative folding (the formation of disulfide bridges), subunit assembly, and *N*-linked glycosylation. The expression cassette (Fig. 3) can be tailored by using different pairs of restriction sites for cloning. If the transgene is inserted at the NcoI/NotI sites, the recombinant protein will carry C-terminal His_6 and 54 k tags [27] to facilitate product detection and purification. However, using the XbaI site instead of NotI will remove the C-terminal tags. In this case, the inserted gene sequence may contain custom tags (if required) and must feature a stop codon. Vector pAIXpress should be chosen if the recombinant protein is intended to accumulate in the cytosol (default pathway) or any compartment other than the secretory pathway (in which case, targeting signals must be provided on the inserted gene). The gene of interest should be inserted at the NcoI/XbaI sites (Fig. 3). This will remove a *lacZ* fragment that can be used diagnostically to exclude transformants carrying undigested or religated pAIXpress vectors using classic blue-white selection [26].

Enzymes should be used according to the manufacturers' instructions in terms of amounts, buffers and incubation conditions.

1. Digest 2–4 μg of pAIX and the cDNA insert (synthetic gene or PCR product) with NcoI and NotI or NcoI and XbaI, according to the chosen cloning strategy.

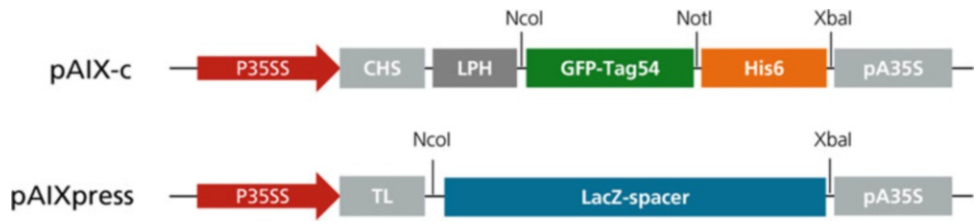

Fig. 3 Maps of the expression cassettes of the vectors pAIX-c (upper panel) and pAIXpress (lower panel). Recombinant protein expression is under the control of the cauliflower mosaic virus 35S promoter with duplicated enhancer region (P35SS) and its corresponding polyadenylation signal (pA35SS). Vector pAIX-c features the 5′ untranslated region of the chalcone synthase (CHS) gene, whereas pAIXpress features the 5′ untranslated region of tobacco etch virus (TL) [21]. In pAIX-c, a gene encoding green fluorescent protein (GFP) from *Aequorea victoria* [24] serves as a spacer that can be also used as a visual marker during test transformations. In pAIXpress, a gene encoding the β-galactosidase (LacZ) α-subunit from *Escherichia coli* [25] is used as a spacer that facilitates the blue-white selection of positive clones [26]. Restriction sites that can be used for subcloning are indicated

2. Isolate the digested pAIX backbone and cDNA by preparative gel electrophoresis, and purify the fragments using a commercial gel isolation kit (*see* **Note 2**).
3. Ligate 50–100 ng of the pAIX backbone with a 5–10-fold molar excess of cDNA.
4. Use the completed ligation reactions for the direct transformation of chemically competent *E. coli* cells.
5. Allow the cells to recover for 20–60 min in LB medium at 37 °C.
6. Streak the cells onto plates of lysogeny broth (LB) agar supplemented with 100 μg/mL ampicillin and incubate overnight at 37 °C (*see* **Note 3**).

3.2 Identification of Recombinant E. coli Cells

1. Check for recombinant *E. coli* cells by colony PCR [28] using the PS5′ and PS3′ or custom gene-specific primers (*see* **Note 4**) or by restriction digestion of plasmid DNA prepared from LB liquid cultures using standard methods [23].
2. Confirm successful cloning by sequencing [29] the recovered plasmids (*see* **Note 5**).

3.3 Preparation of Electrocompetent A. tumefaciens Cells

1. Inoculate 100 mL of YEB containing 25 μg/mL rifampicin and 25 μg/mL kanamycin with an aliquot of cryo-preserved *A. tumefaciens* cells (GV3101::pMP90RK).
2. Grow the culture at 28 °C shaking at 160 rpm until the OD_{600} reaches ~2.5, which should take 24–48 h (*see* **Note 6**).
3. Transfer the culture to ice and store for at least 5 min.
4. Transfer cultures to pre-chilled 50-mL Falcon tubes and centrifuge at 3000 × *g* for 10 min.
5. Discard the supernatant and resuspend cells in 50 mL ice-cold sterile water.

6. Centrifuge the suspension as described in **step 4** and resuspend the cells in 25 mL ice-cold sterile water.
7. Centrifuge the suspension as described in **step 4** and resuspend the pellet in 10 mL ice-cold sterile water containing 10% (v/v) glycerol.
8. Repeat **step 7** but resuspend the cells in 3 mL ice-cold sterile water containing 10% (v/v) glycerol and prepare 50-μL aliquots in sterile 1.5-mL reaction tubes.
9. Store the reaction tubes containing electrocompetent *A. tumefaciens* cells immediately at −80 °C (*see* **Note 7**).

3.4 Electroporation of A. tumefaciens

1. Thaw an aliquot of electrocompetent *A. tumefaciens* cells (*see* Subheading 3.3) on ice.
2. Add 200–500 ng of the pAIX plasmid DNA containing the gene of interest and mix gently with the thawed cells.
3. Transfer cells to a pre-chilled 2-mm electroporation cuvette and apply a pulse of 2.5 kV for 5 ms using an Eppendorf multiporator.
4. Immediately add 950 μL YEB and transfer the cells to sterile 1.5-mL tubes.
5. Incubate the cells at 28 °C for 2–3 h, shaking 160 rpm (*see* **Note 8**).
6. Use a spatula to plate in a descending order 5, 1, and 0.5 μL of the culture on YEB plates containing 25 μg/mL rifampicin, 25 μg/mL kanamycin, and 50 μg/mL carbenicillin for selection (*see* **Note 9**).
7. Incubate the plates for 3–4 days at 28 °C (*see* **Note 10**).

3.5 Identification of Recombinant A. tumefaciens Cells

1. Check at least five *A. tumefaciens* clones growing on selection plates by colony PCR as described below (*see* **Note 11**).
2. Pick colonies using plastic tips (*see* **Note 12**) and transfer each colony to a YEB master plate containing 25 μg/mL rifampicin, 25 μg/mL kanamycin, and 50 μg/mL carbenicillin for selection, afterwards use the to transfer the remaining cell into 19 μL sterile water in a reaction tube.
3. Incubate the master plate at 28 °C for 1–2 days, then seal and store the plate at 4–8 °C for up to 4 weeks.
4. Prepare a PCR master mix with 0.2 μM of each primer (PS5′ and PS3′) and standard PCR ingredients (10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, and *Taq* DNA polymerase).
5. Add 6 μL of the PCR master mix to the 19 μL bacterial suspension.
6. Include a positive control (pAIX vector DNA) and a negative control (water).

Table 1
PCR program

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	95	300	1×
Denaturation Annealing Elongation	95 55 72	30 30 60/kb	25×
Final elongation	72	10	1×
Storage	20	∞	1×

7. Run the PCR program shown in Table 1 below.
8. Analyze PCR products by agarose gel electrophoresis [30] to identify positive recombinant *A. tumefaciens* clones.

3.6 Cultivation of Recombinant A. tumefaciens Cells

1. Inoculate 3 mL of YEB containing 25 μg/mL rifampicin, 25 μg/mL kanamycin, and 50 μg/mL carbenicillin with a recombinant *A. tumefaciens* colony (*see* **Note 12**).
2. Incubate at 28 °C for 48 h, shaking at 160 rpm.
3. Enlarge the culture to an appropriate volume (depending on the amount of plant material to be infiltrated) by adding YEB containing 25 μg/mL rifampicin, 25 μg/mL kanamycin, and 50 μg/mL carbenicillin for selection, and incubate at 28 °C for 24–48 h, shaking at 160 rpm, until the OD_{600} reaches 3–6 (*see* **Note 13**).
4. Prepare glycerol stocks by carefully mixing 500 μL of the culture with 500 μL 100% glycerol and store at −80 °C.

3.7 Preparation of the Infiltration Solution

1. Determine the OD_{600} of the *A. tumefaciens* culture.
2. Adjust the culture to $OD_{600} = 1$ with 2× infiltration medium and an appropriate volume of sterile water (*see* **Note 14**).
3. Add acetosyringone from a 200 mM stock solution in DMSO to a final concentration of 200 μM and incubate the infiltration solution for 30 min at room temperature.

3.8 Cultivation of N. benthamiana Plants

1. Germinate *N. benthamiana* seeds preferentially on Rockwool blocks using a hydroponic cultivation system, or in standard soil and pots.
2. Irrigate plants with a 0.1% (w/v) Ferty-2-Mega in a greenhouse or phytochamber (25/22 °C day/night temperature, 16-h photoperiod, 70% relative humidity).
3. Plants are ready for infiltration 6–8 weeks after seeding (*see* **Note 15**).

3.9 Plant Infiltration and Incubation

Two different infiltration and incubation procedures can be applied according to the number of expression constructs to be tested and the scale: (A) syringe-based infiltration of single or multiple leaves on intact plants, and (B) vacuum-based infiltration of entire intact plants. Syringe-based infiltration requires much smaller culture volumes than vacuum infiltration (*see* **Note 16**) and is more suitable for testing different variants in parallel, whereas vacuum infiltration is typically used for the larger-scale production of antigens for detailed characterization (e.g., structural analysis). Note that all work involving GMOs (*A. tumefaciens*) must be carried out in a designated S1 environment, and all used material should be properly decontaminated according to local regulations.

3.9.1 Syringe-Based Infiltration (A)

1. A laboratory coat and safety glasses must be worn for this procedure.
2. Select suitable plants (*see* **Note 14**) and prepare them for infiltration by misting and incubation under light for at least 60 min.
3. Place a single plant on an autoclavable or disposable tray.
4. Aspirate 1 mL infiltration solution into a 1-mL syringe without a needle and position the syringe by gently pressing the tip against the lower surface of a suitable leaf (*see* **Note 17**) close to a main leaf vein. Start infiltrating the solution into the leaf tissue by slowly depressing the plunger. Infiltrated tissue appears darker and slightly translucent compared to non-infiltrated areas (Fig. 4a). Depending on the operator's skill and experience (and the condition of the leaf), 50–500 µL of solution can be infiltrated into leaf tissue at one contact point. If the infiltration does not proceed or if the syringe needs to be refilled, change the contact point.

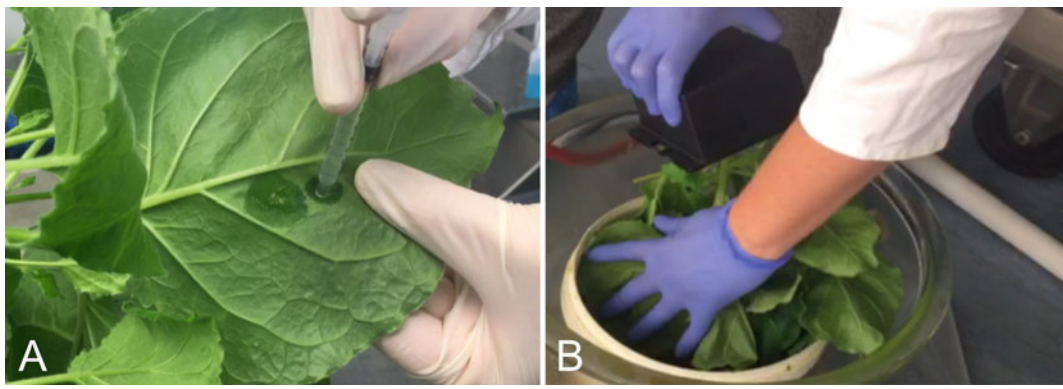

Fig. 4 Agroinfiltration procedures. Panel A shows the syringe-based infiltration of *A. tumefaciens* into *N. benthamiana* leaf tissue. Panel B shows the placement of *N. benthamiana* in a beaker (within a desiccator vessel) filled with the suspension of *A. tumefaciens* ready for vacuum infiltration

5. Repeat the procedure until the desired number of leaves and/or contact points has been infiltrated.
6. It is possible to use different leaves of the same plant to infiltrate different constructs. In this case it is important to label the leaves and/or contact points carefully and to avoid cross contamination from dripping infiltration solution.
7. Transfer plants to a fresh tray and incubate them for 3–10 days (typically 5 days) in a contained growth chamber (16-h photoperiod, 10,000 lux, 22 °C, 60% humidity). Check the plants every 2 days for sufficient watering. If the plants appear dry, they should be misted daily by spraying with water.

3.9.2 Vacuum Infiltration (B)

1. Select suitable plants (*see* **Note 14**) and prepare them for infiltration by misting and incubation under light for at least 60 min.
2. Make sure the plants fit within the beaker that matches the available infiltration vessel (desiccator).
3. Fill the beaker (typically a 5-L plastic beaker) to 80% capacity with infiltration solution.
4. Carefully invert the plants and position them above the infiltration solution (Fig. 4b), making sure that all leaves are completely submerged (*see* **Note 18**). Prevent the root block from slipping into the solution using wooden sticks and/or adhesive tape.
5. Place the beaker with the submerged plant into an appropriate desiccator (typically a 20-L vessel), close the lid and reduce the pressure to <20 mbar using a vacuum pump.
6. Carefully release the vacuum after 5–10 min (*see* **Note 19**).
7. Incubate the plants hanging upside down for 3–10 days in a contained growth chamber (16-h photoperiod, 10,000 lux, 22 °C, 60% humidity). Check the plants every 2 days for sufficient watering. If the plants appear dry, they should be misted daily by spraying with water.

3.10 Extraction of Total Soluble Protein (TSP)

1. Harvest infiltrated leaf material 3–10 days post-infiltration (dpi). Typically 5 dpi is sufficient to obtain high yields of recombinant protein.
2. Determine the weight of the infiltrated leaf material (*see* **Note 20**).
3. For small-scale extraction (1–20 g leaf material), grind leaf material to a fine powder in liquid nitrogen using a mortar and pestle and add 2–3 mL of extraction buffer per gram of leaf material.
4. For large-scale extraction, a blender or juicer can be used to obtain the TSP. Add 2–3 mL of extraction buffer per gram of

leaf material before blending, or after the extraction when using a juicer (*see* **Note 21**).

5. After extraction, clarify the crude plant extract by filtering through a double layer of Miracloth.
6. Check and, when necessary, adjust the pH of the extract to pH 7.5 using 1 M Tris pH 8.8 (*see* **Note 21**).
7. Centrifuge the extract at 40,000 × *g* for 15 min at 4 °C to remove remaining fibers and other insoluble compounds.
8. The clarified extract can be used for the analysis of recombinant protein expression and recovery during downstream processing, using methods such as SDS-PAGE [31], immunoblotting [32], or surface plasmon resonance spectroscopy [33].

4 Notes

1. Take care when sourcing *A. tumefaciens*. There are several distinct strains named GV3101, which carry different modified helper plasmids. The recommended GV3101::pMP90RK strain carries a helper plasmid with a kanamycin-resistance selection marker. Other GV3101 strains carry helper plasmids that confer gentamycin resistance.
2. To avoid the recovery of incompletely digested and religated plasmids, restriction digestion of the plasmid can be followed by treatment with calf intestinal alkaline phosphatase to remove terminal phosphate groups before setting up the ligation reaction.
3. Cells can also be plated without prior incubation if longer growth times are acceptable.
4. Always include a positive control (pAIX vector) when performing check PCRs to confirm that the reagents and method are working.
5. The oligonucleotides PS3′ and PS5′ can be used as sequencing primers. If the insert is larger than 1 kb, the design and use of additional internal primers is recommended to improve sequence coverage.
6. Growth time can vary, and is dependent on inoculation density. Expect delays when small colonies or small volumes of lower OD_{600} cultures are used for inoculation. Always include kanamycin to apply selection pressure on the Ti helper plasmid and do not include carbenicillin when growing wild-type GV31011:: pMP90RK.
7. Shock-freeze the cells in liquid nitrogen before storage at −80 °C.

8. Make sure that cells are kept at 28 °C during regeneration and growth. Higher incubation temperatures (e.g., 37 °C) may encourage the loss of the Ti helper plasmid and reduce yields during transient expression.
9. The transformation of electrocompetent *A. tumefaciens* is normally very efficient, so use low volumes for plating to avoid crowded colonies on the selection plates, which may limit selection pressure and prevent the picking of single clones.
10. Cells may grow very slowly during the initial phase after transfer from the selection plate to liquid medium. Growth performance may also depend on the transgene. However, incubation times exceeding 3 days are problematic because the antibiotics lose their activity.
11. Sometimes two populations of colonies can arise after transformation, comprising a few large colonies and many smaller ones. Pick from both populations when identifying positive clones. In our experience, large colonies are less likely to carry the recombinant expression vector.
12. It is absolutely essential to use plastic rather than wooden tips when picking *A. tumefaciens* colonies for the inoculation of cultures. The use of wooden tips usually results in the absence of growth and rapid browning of the culture medium.
13. To ensure fast growth when scaling up to higher culturevolumes, use sufficient amounts of inoculation material. If possible use a 1/100 (v/v) ratio of a culture with an $OD_{600} > 3$.
14. Generally an OD_{600} of 0.5–1 is optimal for transient expression based on infiltration with *A. tumefaciens*. When different cultures are mixed, the OD_{600} of the mixture should also stay within this range. The expression of monoclonal antibodies is usually achieved by mixing two cultures of *A. tumefaciens* carrying the plasmids encoding the heavy and light chains, respectively. Another *A. tumefaciens* strain expressing the p19 silencing suppressor [34] is typically used at a final OD_{600} of 0.1–0.2 within such a mixture.
15. Avoid using older plants that are flowering or have recently flowered because expression levels are generally lower than those in young plants.
16. The manual infiltration of leaves using a syringe requires care and practice to adjust the position and pressure. Avoid larger injuries in the leaf tissue during this procedure.
17. Older leaves are larger and usually easier to infiltrate than young leaves but tend to produce lower yields of recombinant proteins. The third youngest leaf is a good default choice.
18. It is important to avoid the nicking of leaves and veins when submerging the plant in the infiltration solution.

19. Carefully release the vacuum when no more gas bubbles are seen emerging from the leaf surface.
20. After infiltration and incubation, leaves can be stored frozen at −20 °C for short periods or −80 °C for longer periods.
21. Refer to Chapters 9 and 10 for more details regarding protein extraction and purification.

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