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Simplified Protocol to Demonstrate Gene Expression in *Nicotiana benthamiana* Using an *Agrobacterium*-Mediated Transient Assay

Satyam Vergish[1], Ryan Wolf[2], and Wen-Yuan Song[1,*]

[1]Department of Plant Pathology, University of Florida, Gainesville, FL, USA
[2]Santa Fe High School, Alachua, FL, USA
*For correspondence: wsong@ifas.ufl.edu

Abstract

Agrobacterium-mediated transient gene expression in *Nicotiana benthamiana* is widely used to study gene function in plants. One dramatic phenotype that is frequently screened for is cell death. Here, we present a simplified protocol for *Agrobacterium*-mediated transient gene expression by infiltration. Compared with current methods, the novel protocol can be done without a centrifuge or spectrometer, thereby suitable for K-12 outreach programs as well as rapidly identifying genes that induce cell death.

Key features

- The protocol simplifies the widely used *Agrobacterium*-mediated transient gene expression assay [1] and can be completed within one week when plants are available.
- Rice *XB3* gene can induce a dramatic and easily identifiable cell death phenotype in *Nicotiana benthamiana.*
- Allows identification of cell death–inducing genes and is suitable for teaching.
- Compared to the currently used methods, our protocol omits the use of agroinfiltration buffer, pH meter, temperature-controlled growth chamber, centrifuge, and spectrophotometer.

Keywords: *Agrobacterium*, *Nicotiana benthamiana*, Agroinfiltration, Cell death, Gene expression, Ion leakage

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Graphical overview

***Agrobacterium* infiltration (agroinfiltration) of *Nicotiana benthamiana*.** The photo demonstrates the method of agroinfiltration into the abaxial side of leaves using a needleless syringe.

Background

Agrobacterium-mediated transient transformation of the plant *Nicotiana benthamiana* is a powerful tool widely used in molecular biology studies [2,3]. This assay is ideal for rapidly demonstrating gene expression, especially when used for the visible cell death phenotype induced by specific plant genes from various species. While holding summer professional development workshops for pre-college educators, we found that the current protocols for *Agrobacterium*-mediated transient gene expression are difficult to use in pre-college classrooms due to lack of the necessary equipment and reagents.

The rice (*Oryza sativa*) cell surface innate immune receptor XA21 specifies resistance to bacterial strains of *Xanthomonas oryzae* pv. *oryzae* [4]. XA21 interacts with a number of proteins in vitro and in vivo. One protein that XA21 interacts with is called XB3 for XA21 binding protein 3 [5]. XB3 induces highly visible cell death in *N. benthamiana* when over-expressed [1]. To support public education and quickly identify cell death–inducing genes, we developed a novel protocol to simplify the current *Agrobacterium*-mediated transient gene expression assay.

Materials and reagents

1. *N. benthamiana* seeds (available from numerous plant science research labs)
2. pC1300S-XB3: *Agrobacterium tumefaciens* strain EHA105 (Intact Genomics, catalog number: 1084-06) harboring the construct pC1300S-XB3 [1]
3. EV: *A. tumefaciens* strain EHA105 harboring pC1300S (empty vector)
4. P19: *A. tumefaciens* carrying the p19 construct. The P19 protein from tomato bushy stunt virus is an efficient suppressor of posttranscriptional gene silencing, which enables higher expression of the gene of interest [6]
5. Luria-Bertani broth (LB Broth) (Fisher Scientific, catalog number: 10855001)
6. Kanamycin sulfate (Fisher Scientific, catalog number: BP906-5)
7. Rifampicin (PhytoTechnology Lab, catalog number: R501)

8. 100 mM acetosyringone (PhytoTechnology Lab, catalog number: A1104)
9. Filtered water
10. Soilless potting mix (Growing Mix, PRO-LINE HFC/B HydraFiber, from Jolly Gardener)

Solutions

1. Kanamycin sulfate stock solution (50 mg/mL) (see Recipes)
2. Rifampicin stock solution (50 mg/mL) (see Recipes)
3. Infiltration buffer [1] (see Recipes)

Recipes

1. **Kanamycin sulfate stock solution (50 mg/mL)**
   Dissolve 0.5 g of kanamycin sulfate (powder) in 10 mL of distilled water at room temperature. Filter through a 0.2 µm filter. Aliquot in 1 mL fractions and store in a -20 °C freezer.

2. **Rifampicin stock solution (50 mg/mL)**
   Dissolve 0.5 g of rifampicin (powder) in 10 mL of 100% methanol at room temperature. Aliquot in 1 mL fractions and store in a -20 °C freezer.

3. **Infiltration buffer [1]**
   Dissolve 1.95 g of MES [2-(N-Morpholino)ethanesulfonic acid] and 1.96 g of $MgCl_2$ in 1,000 mL of sterile water at room temperature. Adjust pH to 5.6. Aliquot in 1 mL fractions and store in a -20 °C freezer.

Laboratory supplies

1. 9-cm square disposable pots with drainage holes (any manufacturer)
2. 15 mL culture tubes (Carolina Biological Supply, catalog number: 215090)
3. 50 mL conical screw cap tubes (Carolina Biological Supply, catalog number: 215095)
4. Syringes (1 cc) (Carolina Biological Supply, catalog number: 697765)
5. 10 mm cork borer (Carolina Biological Supply, catalog number: 712202)

Equipment

1. Forceps for transplanting seedlings (Carolina Biological Supply, catalog number: 624790)
2. Incubator shaker (New Brunswick Scientific, model: C24KC)
3. Conductivity meter with electrode (Eutech, model: Cond 6+ ECCON603PLUS)
4. Deep freezer with -20 °C (any manufacturer)
5. Bunsen burner (Carolina Biological Supply, catalog number: 706706)
6. Laminar flow hood (any manufacturer)

Procedure

A. Growth conditions for *N. benthamiana* plants

1. Fill a 9 cm disposable square pot with moist potting soil and sprinkle 20–50 *N. benthamiana* seeds on the surface of the soil. Cover the pot with plastic wrap or a plastic dome to create a humid environment. Place the pot for two weeks in a warm location with a day length of 16 h (24 °C) and a period of darkness of 8

h (21 °C) each day. Once the seeds have germinated and the seedlings are 1–1.5 cm tall, remove the plastic wrap. Water the plants twice a week (~20 mL water/pot).

2. After two weeks, transplant the germinated seedlings to new pots containing moist potting soil (one seedling per pot) and grow them in the above-mentioned conditions.
3. Water the plants regularly (~20 mL water/pot, every 48 h), keeping the soil consistently moist but not waterlogged and growing the plants under the same conditions as described above for another five weeks until they reach the 4–6 true-leaf stage.

Note: Keep an eye on the plants for pests and diseases. Fertilize plants with a balanced liquid fertilizer every 2–3 weeks during the growing season, if required. The optimal stage for Agrobacterium *infiltration (agroinfiltration) for the experiment occurs when the plants reach a young vegetative stage, characterized by actively dividing and expanding leaf area.*

B. Preparation of *A. tumefaciens* culture

1. Inoculate individual *A. tumefaciens* EHA105 strains carrying p19, pC1300S-XB3, or pC1300S (EV) (Figure 1) into three separate 15 mL culture tubes each containing 3 mL of LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (30 µg/mL) in a laminar flow hood. Place the culture tubes in a shaker set at 28 °C and a rotation rate of 220 rpm and incubate for 36–40 h in the dark.

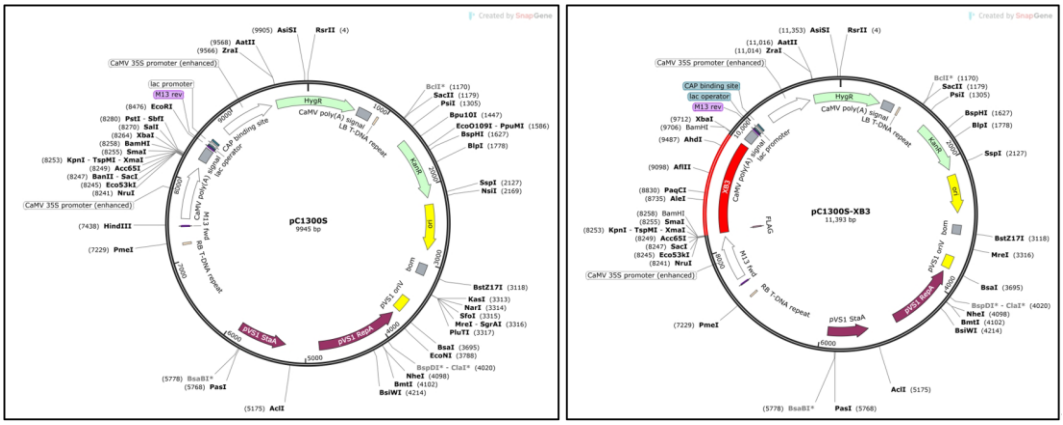

Figure 1. Maps of the pC1300S (EV, kindly provided by Dr. Yinong Yang) and pC1300S-XB3 [1] constructs used in this study. P19 is described in Voinnet et al. [6].

2. Establish secondary cultures by taking 25 µL from the primary culture and inoculating into 50 mL conical screw cap tubes filled with 15 mL of LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (30 µg/mL). Grow the new culture at 28 °C overnight in the dark reaching the logarithmic growth phase of the bacteria.
3. Dilute the overnight bacterial culture into LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (30 µg/mL) based on the visual guidance provided in Figure 2, which covers a range of dilutions of the control LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (30 µg/mL) to translucent and turbid cultures containing bacterial cells. Add 30 µL of 100 mM acetosyringone into 15 mL of bacterial culture.

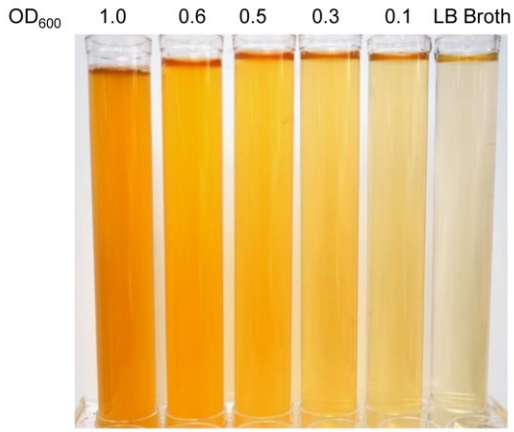

Figure 2. Reference for bacterial dilution for agroinfiltration experiments. An overnight *Agrobacterium* culture containing pC1300S-XB3 in LB broth supplemented with kanamycin (100 µg/mL) and rifampicin (30 µg/mL) is diluted to different bacterial densities (within the range of OD_{600} 0.1 to 1). The visual representation of *Agrobacterium* at varying densities offers a visual target for dilution without a spectrometer.

4. Incubate the diluted bacterial suspensions for an additional 3 h at room temperature to activate the virulence machinery and prime the cells for successful genetic transformation.

Note: If a laminar flow hood is not available, bacterial inoculation can be performed using a Bunsen burner. A temperature range of 28–30 °C is optimal for the growth and replication of A. tumefaciens. *Overnight growth of* Agrobacterium *culture often leads to an* OD_{600} *value within the range of 0.8–1.0.*

C. Agroinfiltration using pC1300S-XB3 and controls

1. Two *Agrobacterium* cultures will be combined: either pC1300 (EV) and p19 or pC1300S-XB3 and p19 in a proportion of 3:2 to prepare samples for infiltration.
2. Inject the abaxial side (lower surface) of *N. benthamiana* leaves with the two prepared *Agrobacterium* mixtures using needleless syringes under controlled pressure. Separate the injection sites of each test mixture.
3. Incubate the infiltrated plants at room temperature with humidity for a defined period of 12–72 h. This incubation is essential for the plants to acclimatize to the introduced genetic material and to enable the expression of the transferred genes.

Notes:

a. The volume of bacterial cultures used for infiltration can be scaled accordingly. Always include the empty vector control (pC1300S) as one of the infiltration spots in your experiments to validate the success of the infiltration and to identify any potential issues with the transformation process. We have noticed that healthy plant conditions are crucial for the success of the infiltration experiments. Sometimes, this is not easy to visualize from plant appearance. The empty vector control serves as the "baseline" to phenotype the effect of XB3.

b. As shown in Figure 3, similar results were obtained when LB broth was used to replace infiltration buffer for diluting bacterial cultures.

D. Monitor cell death response

The infiltrated leaves are tested for the expression of the *XB3* gene by monitoring cell death detected as visible grey patches 12–48 h after agroinfiltration (Figures 3A and 3B).

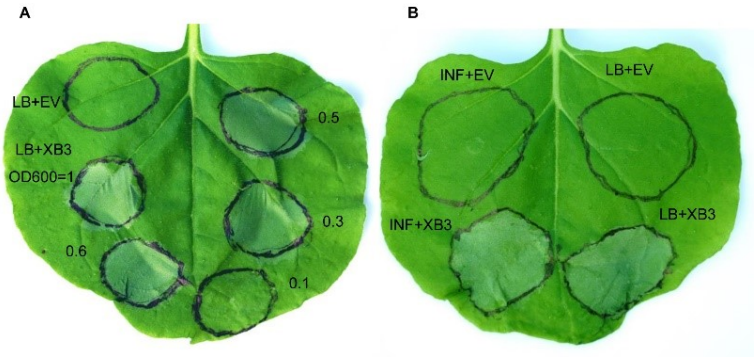

Figure 3. Leaf collapse induced by expression of XB3. (A) Cell death phenotypes induced by various densities of the agrobacterial culture harboring pC1300S-XB3. Photo taken 48 h after infiltration. EV, empty control. Visible cell death phenotypes were observed when the leaves were infiltrated with the *Agrobacterium* at densities ranging from OD_{600} = 0.3 to 1. (B) Cell death phenotypes induced by the *Agrobacterium* harboring pC1300S-XB3 in LB medium and infiltration buffer at OD_{600} of 0.5. The figure shows an *N. benthamiana* leaf 48 h after infiltration by four different solutions. Top-left quadrant of the leaf was infiltrated by *Agrobacterium* containing the empty vector diluted in infiltration buffer and the bottom-left quadrant was infiltrated with *Agrobacterium* containing XB3 expression plasmid (pC1300S-XB3) diluted in infiltration (INF) buffer at OD_{600} of 0.5. Similar cell death was observed when LB was used to dilute the *Agrobacterium* cultures for infiltration (Right).

E. Ion leakage assay

1. Electrolyte leakage of infiltrated leaves can be quantified to assess cellular mortality. Punch out three leaf discs, each 10 mm in diameter, from each region infiltrated by *Agrobacterium* using a 10-mm cork borer.
2. Submerge these leaf discs in 10 mL of water. Incubate for 2 h at room temperature with gentle agitation of 160 rpm.
3. Measure the conductivity of the resulting solution using the COND 6+ conductivity meter according to the manufacturer's instructions (Figure 4). In order to calibrate the conductivity meter, rinse the electrode with sterile water and calibrate it with water, as in our experiment, water served as standard.

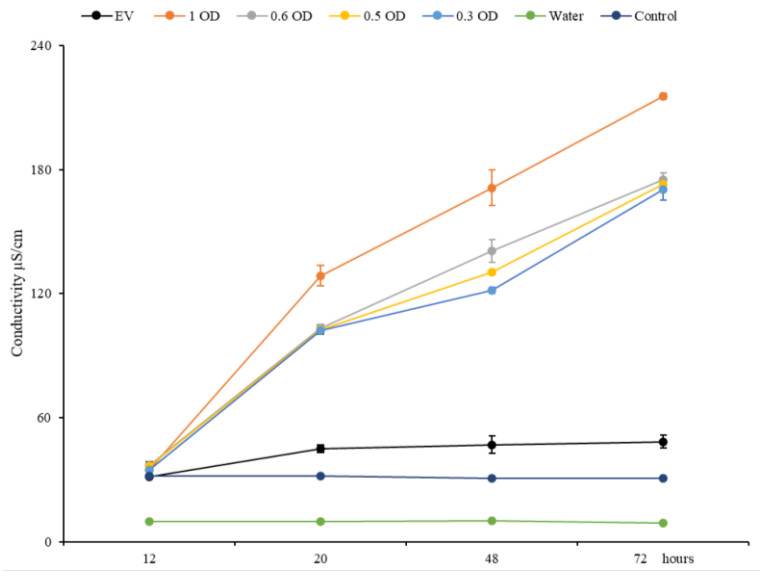

Figure 4. Ion leakage induced by the expression of XB3. The graph represents the ion leakage from *N.*

benthamiana leaf tissues isolated at different time points (12, 20, 48, and 72 h) after infiltration with *Agrobacterium* cultures containing pC1300-XB3 at OD600 values of 1.0, 0.6, 0.5, and 0.3 along with *Agrobacterium* culture containing the empty vector (EV) pC1300S. Water, conductivity of water. Control, ion leakage from healthy, non-infiltrated leaves.

Validation of protocol

This protocol was modified from Huang et al. [1] and reproducible (Figures 3 and 4).

General notes and troubleshooting

General notes

1. Our protocol presents advantages over conventional agroinfiltration methods [1,7–9]. We eliminate the centrifugation step, as it is cost-prohibitive for many school programs, and bypass the use of a spectrophotometer to measure OD at a specific wavelength. Additionally, we replace the agroinfiltration buffer with LB to reduce chemical costs. Target bacterial density is instead assessed using Figure 2. The figures demonstrate the efficiency of LB-based agroinfiltration, and the cell death response induced by XB3 expression.
2. EHA105 possesses a wide host range and is suitable for delivering genetic material to various plant species. Alternatively, researchers may opt for other *Agrobacterium* strains with similar capabilities, ensuring compatibility with the specific plant species under investigation and the intended genetic modifications.
3. A wide range of the concentrations of agrobacterial cultures (OD_{600} from 0.3 to 1) harboring the *XB3* gene can induce cell death response. If an incubator shaker is not available, a flask with a magnetic stir bar can be used to culture agrobacterial cells on a magnetic stir plate.
4. For the ion leakage assay, if a COND 6+ conductivity meter is not available, a less expensive TDS sensor might be used [10].

Troubleshooting

Reduced transformation efficiency. Overgrown *Agrobacterium* cultures may lead to a reduction in plant transformation efficiency. Avoid plant leaves with visible signs of disease, stress, or damage. We have noticed that certain types of soil affect the growth of *N. benthamiana*. If plants grow poorly, we recommend using Growing Mix, PRO-LINE HFC/B HydraFiber.

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Competing interests

The authors declare no competing interests.

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