

Transient Expression Assay in *NahG* Arabidopsis Plants Using *Agrobacterium tumefaciens*

Pepe Cana-Quijada¹, Eduardo R. Bejarano¹, Rosa Lozano-Durán² and Tábata Rosas-Díaz², *

¹Instituto de Hortofruticultura Subtropical y Mediterránea “La Mayora”, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Dept. Biología Celular, Genética y Fisiología, Universidad de Málaga, Campus Teatinos, Málaga, Spain; ²Shanghai Center for Plant Stress Biology/CAS Center for Excellence in Molecular Plant Science, Chinese Academy of Sciences, Shanghai, China

*For correspondence: tabatarosas@sibs.ac.cn

[Abstract] *Agrobacterium*-mediated transient expression has greatly contributed to research in molecular plant biology but has low efficiency and inconsistency in *Arabidopsis thaliana* (*Arabidopsis*). Here, we describe a simple, efficient and fast protocol to make transient gene expression in *NahG* Arabidopsis plants using *Agrobacterium tumefaciens*. This protocol has been successfully used to assess protein sub-cellular localization and accumulation, enzyme activity, and protein-protein interaction. In addition, this assay overcomes the use of *Nicotiana benthamiana* plants as a surrogate system for transient gene expression assays. Finally, the use of this protocol does not require complex inoculation methods or specific growth conditions, and can be used with different *Agrobacterium* strains with similar results.

Keywords: *NahG* Arabidopsis, *Agrobacterium*, Transient expression, Transient transformation, Protein accumulation

[Background] *Agrobacterium tumefaciens* (hereafter referred to as *Agrobacterium*)-mediated transient transformation assays have greatly contributed to research in molecular plant biology. These methods have many advantages over the laborious and time-consuming stable transformation approaches including, among others, a higher efficiency, simplicity, and fast, consistent results when the transient transformation assays are carried out in *Nicotiana benthamiana*. On the other hand, these assays are inefficient and lack robustness when carried out in the model plant *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), forcing *Arabidopsis* researchers to use *N. benthamiana* as a heterologous system, which entails obvious limitations and might generate misleading results.

Many efforts have been made in the past to increase the efficacy of *Agrobacterium*-mediated transient transformation in *Arabidopsis* (reviewed in Krenek *et al.*, 2015). Recently, we described a protocol to perform transient gene expression using *NahG* Arabidopsis plants, overcoming previous limitations (Rosas-Díaz *et al.*, 2017). Using Arabidopsis *NahG* plants, which contain low levels of salicylic acid (SA) due to the expression of an SA hydroxylase from the bacterium *Pseudomonas putida* (Lawton *et al.*, 1995), we have been able to obtain high accumulation of marker proteins such as GUS and GFP, and carry out sub-cellular localization and protein-protein interaction experiments. Remarkably, this protocol for transient expression can be used with, at least, three widely used *Agrobacterium* strains, LBA4404,

GV3101 and C58C1. In summary, this protocol shows that expression of the *NahG* transgene greatly enhances the efficiency of *Agrobacterium*-mediated transformation in rosette leaves in *Arabidopsis*, enabling the routine use of this technique in the model plant.

Materials and Reagents

A. Materials

1. Soil mix or substrate such as Compo Sana® Universal Ligera (COMPO, TSUSTPROF25L)
2. Plant pots such as Desch vol 11 (Desch Plantpak, catalog number: 1055278)
3. Seed tray—40 cavities
4. Plant trays
5. Cling film
6. Syringes 1 ml or 2 ml
7. Tissue paper
8. Petri dishes
9. Falcon tubes
10. Eppendorf tubes
11. Pipette tips
12. Sterile toothpicks
13. Syringe filter 0.22 µm

B. Biological materials

1. *Arabidopsis thaliana NahG* seeds (Lawton *et al.*, 1995)
2. *Agrobacterium tumefaciens* strain (LBA4404, GV3101 or C58C1) carrying a binary vector with the gene of interest

C. Reagents

1. Sterile deionized water
2. Glycerol (CARLO ERBA Reagents, catalog number: 453752)
3. NaCl (AppliChem, catalog number: 121659.1210)
4. Tryptone (Biolife, catalog number: 412290)
5. Yeast Extract (AppliChem, catalog number: 403687.1210)
6. Bacteriological agar (MICROKIT, catalog number: BCB006+)
7. MES (2-(N-morpholino) ethanesulfonic acid) (Sigma-Aldrich, catalog number: M2933)
8. MgCl₂ (AppliChem, catalog number: 131396.1210)
9. DMSO (Sigma-Aldrich, catalog number: M81802)
10. Acetosyringone (Sigma-Aldrich, catalog number: D134406)
11. Rifampicin (Duchefa Biochemie, catalog number: R0146.0005)
12. Tetracycline (Sigma-Aldrich, catalog number: T3383)

13. Gentamycin (Sigma-Aldrich, catalog number: G3632)
14. Spectinomycin (Duchefa Biochemie, catalog number: S0188.0005)
15. Kanamycin (Sigma-Aldrich, catalog number: K4378)
16. LB medium (see Recipes)
17. 1 M MES (see Recipes)
18. 1 M MgCl₂ (see Recipes)
19. 0.1 M Acetosyringone (see Recipes)
20. Infiltration solution (see Recipes)
21. Antibiotics solution (depending on construct and *Agrobacterium* strain, see Recipes)

Equipment

1. Sterile Erlenmeyer flasks
2. Plant growth chamber capable of sustaining 21 °C under short-day conditions (8 h light/16 h dark) with 140-150 μmol m⁻² sec⁻¹ light intensity (Radiber SA, catalog number: AGP-1400)
3. Spectrophotometer capable of OD₆₀₀ measurements such as Shimadzu UV-1601 (Shimadzu, catalog number: 206-67001-34)
4. Automatic P1000, P200 and P20 micropipettes
5. Incubator set at 28 °C such as Incubator D-6450 Hanau (Heraeus Instruments, model: D-6450)
6. Incubator shaker capable of sustaining 28 °C and 180 rpm such as New Brunswick™ I26 (Eppendorf, New Brunswick scientific, model: I26, catalog number: M1324-0000)
7. Centrifuge for 50 ml tubes such as Rotofix 32A (Hettich, catalog number: 1206-01)
8. Autoclave

Procedure

A. Plant growth conditions

Note: The preparation of Arabidopsis NahG plants is a key step in obtaining a satisfactory transient gene expression/protein expression. In this protocol, ~4 weeks old Arabidopsis NahG plants are used for Agrobacterium infiltration.

1. Sow seeds in water-soaked soil mix in a plant pot. Cover the pot with cling film and place it in a growth chamber with 8 h light/16 h dark cycle at 21 °C.
2. Grow until the seedlings have two true leaves (around 7-10 days).
3. Carefully transplant seedlings to the final destination in seed trays with 40 cavities.
4. Grow plants for approximately 3 more weeks inside the growth chamber with 8 h light/16 h dark cycle at 21 °C.
5. Plants are ready for infiltration when they have a robust rosette of 10-12 leaves and have not started flowering.

B. *Agrobacterium* cultures

Note: This protocol can be used with, at least, three different commonly used strains of Agrobacterium: LBA4404, GV3101 and C58C1.

1. Using a glycerol stock and a sterile toothpick, streak the *Agrobacterium* clone(s) to be used in LB solid plates supplemented with the appropriate antibiotics. Place the plates inside a 28 °C incubator for 48 h to obtain fresh and single colonies.
2. The day before starting the infiltration, start liquid *Agrobacterium* cultures in LB liquid medium using the fresh colonies on the plates. Pick *Agrobacterium* biomass from a single colony, using a sterile toothpick, place it inside a sterile Erlenmeyer flask with 20 ml LB liquid media supplemented with the appropriate antibiotics, and culture them at 28 °C and 180 rpm overnight.

C. Infiltration

1. *Agrobacterium* preparation

- a. Pour saturated cultures into 50 ml Falcon tubes. Spin down cells at 4,000 x g for 10 min.
- b. Discard LB medium supernatant by decanting. Eliminate as much supernatant as possible.
- c. Resuspend with vortex the cell pellets using 1 volume of freshly prepared infiltration buffer.
- d. After resuspension, leave cultures for 2-4 h in darkness at room temperature.
- e. Prepare a 1/20 dilution of the saturated culture, measure OD₆₀₀ and calculate necessary volume to have a final OD₆₀₀ of 0.05. Dilute using infiltration buffer.

2. Infiltration procedure

- a. Fill a 1 or 2 ml needleless syringe with the resuspended culture at a final OD₆₀₀ of 0.05. Perform the infiltration by pressing the syringe (without needle) on the abaxial side of the leaf while exerting counter-pressure with a fingertip on the adaxial side. Observe how the liquid spreads within the leaf if the infiltration is successful. Infiltrate whole leaves (ca. 100 µl of bacterial suspension/leave).
- b. Dry the excess of culture from the leaf surface using tissue paper (Figure 1, Video 1).
- c. Two to four days after infiltration, observe fluorescence of infiltrated proteins or harvest infiltrated leaves to do a protein extraction.

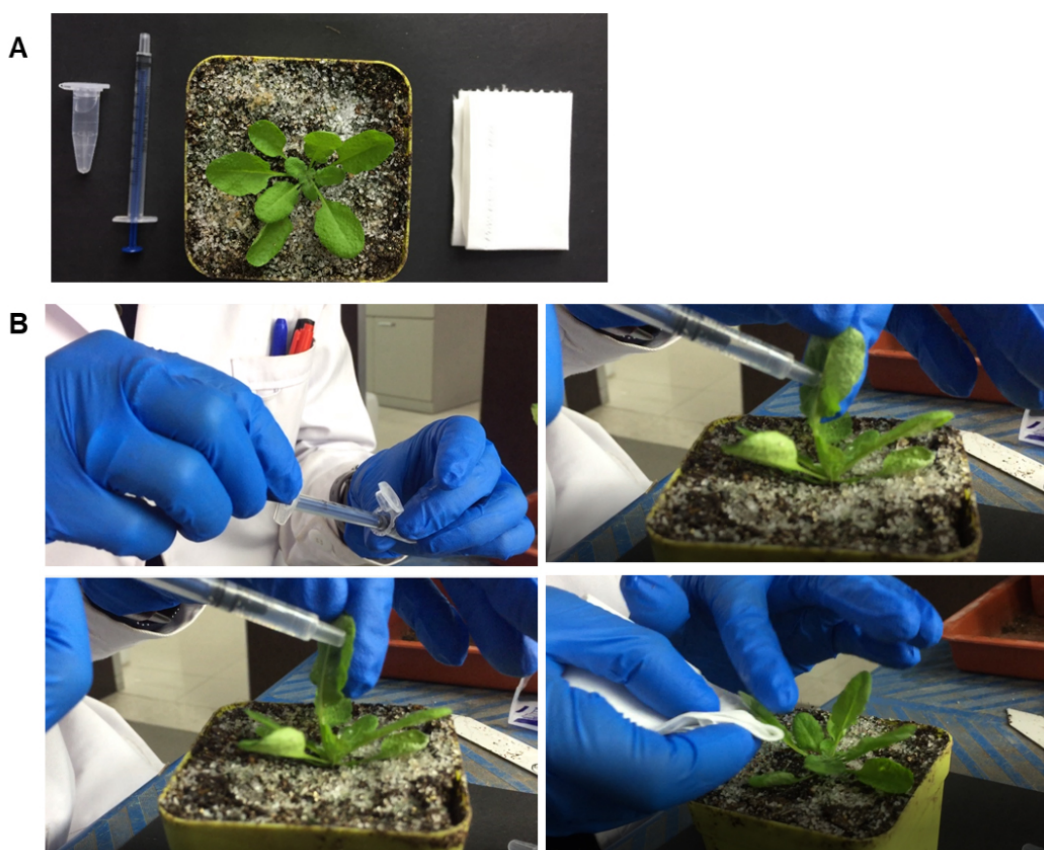
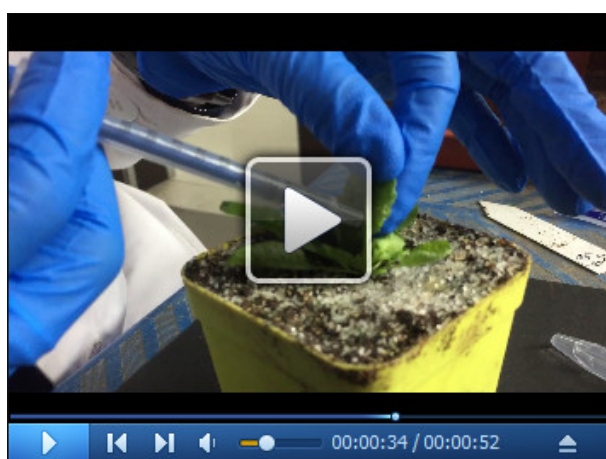


Figure 1. Experimental procedure of transient expression in *NahG Arabidopsis thaliana* plants using *Agrobacterium tumefaciens*. A. Eppendorf tube with *Agrobacterium* carrying the desired construct, 1 ml syringe, 4-week-old *NahG Arabidopsis* plants, and piece of paper. B. Plant infiltration (see details in the procedure section).



Video 1. Experimental procedure of transient expression in *NahG Arabidopsis thaliana* plants using *Agrobacterium tumefaciens*

Notes

1. Some leaves might suffer necrosis after infiltration with the bacterial suspension.
2. Growing *NahG* Arabidopsis under long day condition (16 h light/8 h dark cycle), might not affect the transient expression if the plants have not started flowering. However, plant leaves are bigger and easier to infiltrate when plants are grown in short day conditions.
3. It is also possible to adjust the OD₆₀₀ first and then incubate the cultures for 2-4 h in darkness.
4. Infiltrate no more than 4-5 leaves per plant. Survival rate of the plants diminishes considerably when more leaves are infiltrated.
5. Using this protocol the level of protein accumulation is optimal at 3 days after infiltration.
6. Practically any leaf of the rosette can be infiltrated with positive results but the eldest leaves tend to show a lower signal and the youngest leaves tend to show a stronger response to the bacterial inoculation, with the concomitant necrosis.
7. Drying the excess of culture is not determinant for the success of the infiltration but highly recommendable due to leaves sticking together when wet, causing higher levels of necrosis.

Recipes

1. LB medium (1 L)
NaCl 5 g
Tryptone 10 g
Yeast extract 5 g
(Only for solid medium) Bacteriological agar 16 g
2. 1 M MES (100 ml)
17.5 g MES
Complete with sterile deionized water
Adjust the pH to 5.6
Sterilize by filtration
Store at room temperature
3. 1 M MgCl₂ (100 ml)
20.3 g MgCl₂
Complete with sterile deionized water
Sterilize by autoclaving
Store at room temperature
4. 0.1 M Acetosyringone (10 ml)
0.196 g acetosyringone
10 ml DMSO
Prepare 1 ml aliquots and store them at -20 °C

5. Infiltration solution (100 ml)

Reagent	Volume	Final concentration
1 M MES	1 ml	10 mM
1 M MgCl ₂	1 ml	10 mM
0.1 M acetosyringone	100 µl	0.1 mM

Top up with sterile deionized water

Note: Prepare infiltration solution just before use.

6. Antibiotics solution

Antibiotic	Final concentration	Solvent
Rifampicin	25 µg/ml	DMSO
Tetracycline	5 µg/ml	Ethanol
Gentamycin	50 µg/ml	ddH ₂ O
Spectinomycin	50 µg/ml	ddH ₂ O
Kanamycin	50 µg/ml	ddH ₂ O

Acknowledgments

The authors thank Huang Tan for his technical help during the imaging. This work was supported by the Spanish Ministerio de Ciencia y Tecnología (AGL2016-75819-C2) and the Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences. This protocol is derived from a previous publication (Rosas-Díaz *et al.*, 2017).

Competing interests

The authors declare that they have no conflict of interest.

References

1. Krenek, P., Samajova, O., Luptovciak, I., Daskocilova, A., Komis, G. and Samaj, J. (2015). [Transient plant transformation mediated by *Agrobacterium tumefaciens*: Principles, methods and applications](#). *Biotechnol Adv* 33(6 Pt 2): 1024-1042.
2. Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995). [Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene](#). *Mol Plant Microbe Interact* 8(6): 863-870.
3. Rosas-Díaz, T., Cana-Quijada, P., Amorim-Silva, V., Botella, M. A., Lozano-Duran, R. and Bejarano, E. R. (2017). [Arabidopsis NahG plants as a suitable and efficient system for transient expression using *Agrobacterium tumefaciens*](#). *Mol Plant* 10(2): 353-356.