Infiltration of *Nicotiana benthamiana* Protocol for Transient Expression via *Agrobacterium*

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[Abstract] Transient expression in tobacco plant (*Nicotiana benthamiana*) is used to determine the subcellular location of a protein of interest when tagged with a reporter such as green fluorescent protein (GFP), or to mass produce proteins without making transgenic plants. The root tumor bacteria, *Agrobacteria*, are used to introduce the target gene expression cassette into benthamiana mesophyll cells.

Materials and Reagents

1. *Agrobacterium* strain hosting a plant expression construct (usually driven by *Cauliflower mosaic virus* 35S promoter)
2. Healthy *Nicotiana benthamiana* (*N. benthamiana*) plants 2-4 weeks
3. MES
4. $MgCl_2$ stock
5. Antibiotics
6. Acetosyringone
7. LB media with appropriate antibiotics (see Recipes)
8. Acetosyringone stock (see Recipes)
9. MES-K (see Recipes)
10. Resuspension solution (see Recipes)
11. Acetosyringone datasheet (Sigma-Aldrich) (see Recipes)

Equipment

1. Centrifuge for 50 ml tubes
2. Spectrometer
3. Syringe
4. UV lamp (optional)
5. Fluorescence microscope (optional)
6. Confocal laser scanning microscope (optional)

Procedure

1. Inoculate one single colony of *Agrobacterium* in 5 ml LB with appropriate antibiotics. Grow overnight at 28-30 °C.
   Note: I usually use 100 µg/ml gentamicin (maintain the virulence of Agrobacterium strain GV3101) and 50 µg/ml spectinomycin (selective marker for shuttle vector) for most of the shuttle vectors.
2. Use 1 ml of the overnight culture to inoculate 25 ml LB (with same antibiotics, plus 20 µM acetosyringone added after autoclaving and immediately before use) and grow overnight.
3. Measure the A_{600} of overnight culture.
4. Precipitate the bacteria (5,000 *x g*, 15 min), resuspend the pellet in Resuspension Solution. The final A_{600} should be adjusted to 0.4.
5. Leave on the bench (room temperature) for 2-3 h (or overnight) before infiltration.
6. Perform the infiltration with 5 ml syringe. Simple press the syringe (no needle) on the underside of the leaf (*Note: Avoid cotyledons*), and exert a counter-pressure with finger on the other side. Successful infiltration is often observed as a spreading “wetting” area in the leaf.
7. (Optional) Check the GFP fluorescence by a portable long-wavelength UV lamp 2-5 days after infiltration. This only applies to strong expression of GFP signal (as green from red background).
8. Observe the fluorescence labeled protein under a fluorescent microscope or confocal laser scanning microscope. Or harvest leaves for protein purification.

Recipes

1. LB media with appropriate antibiotics
   Usually two antibiotics used: one to maintain *Agrobacteria* virulence, one for the shuttle vector
2. Acetosyringone stock
   100 mM in ethanol, stored at -20 °C
3. MES-K (0.5 M) (pH 5.6)
   First make 0.5 M MES, adjust pH with KOH to 5.6
4. Resuspension solution
   10 mM $MgCl_2$
   10 mM MES-K (pH 5.6)
   Autocloave 15 min
   100 µM acetosyringone (*note: Added after autoclaving and immediately before using*)

5. Acetosyringone datasheet

Synonyms	3', 5'-Dimethoxy-4'-hydroxyacetophenone
Synonyms	Acetosyringone
4'-Hydroxy-3', 5'-dimethoxyacetophenone	
Molecular Formula	$C_{10}H1_2O_4$
Molecular Weight	196.20
CAS Number	2478-38-8
Beilstein Registry Number	1966119
EG/EC Number	2196105
MDL number	MFCD00008748

References

1. Li, X., Chanroj, S., Wu, Z., Romanowsky, S. M., Harper, J. F. and Sze, H. (2008). A distinct endosomal Ca^{2+}/Mn^{2+} pump affects root growth through the secretory process. *Plant Physiol* 147(4): 1675-1689.

