

Virus-based MicroRNA Silencing

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[Abstract] Virus-based microRNA silencing (VbMS) is a viable and prompt method to screen and characterize the function of microRNAs (miRNAs) in plants. The *Tobacco rattle virus* (TRV)-based VbMS method was originally developed by the Yule Liu's group (Sha *et al.*, 2014) using miRNA target mimic (TM) methodology. Here, we describe the TRV-based VbMS method for silencing endogenous miRNA in *Nicotiana benthamiana* and tomato via *Agrobacterium* infiltrations. For each assay, *Agrobacterium* cultures containing pTRV1 and specific pTRV2e derivative harboring TM fragments are mixed and infiltrated into plant tissues. Generally within 3 weeks, the target miRNAs gene will be silenced and the newly developed tissues will exhibit corresponding phenotypes.

Materials and Reagents

1. Centrifuge tubes
2. Sterile 1 ml syringe (needle removed)
3. Sterile bacterial culture tubes
4. Plant materials
Nicotiana benthamiana, tomato (cultivar MoneyMaker)
Note: seeds can be obtained from Yule Liu's lab.
5. Bacteria strains
 - a. *Escherichia coli*: DH5 α , *ccdb* survival (Thermo Fisher Scientific, Invitrogen™, catalog number: A10460)
 - b. *Agrobacterium tumefaciens*: GV3101, GV2260 (alternative to GV3101)
Note: All strains can be obtained from Yule Liu's lab.
6. Plasmids
 - a. pTRV1 (Dong *et al.*, 2007): a T-DNA vector containing 2 x 35 s promoter, Nos terminator and full cDNA of TRV RNA1 (from Ppk20 strain).
 - b. pTRV2e (Sha *et al.*, 2014): a T-DNA vector containing 2 x 35 s promoter, Nos terminator and cDNA clone of TRV RNA2, of which the sub-genomic promoter of coat protein from *Pea early brown virus* (PEBV) (Wang *et al.*, 1997) and a ligation

independent cloning (LIC) cassette are inserted immediately downstream of the TRV CP gene.

- c. pTRV2e-GFP: *GFP* gene was inserted at LIC cassette into pTRV2e. This construct can be used in a control assay to show successful exogenous expression.
 - d. The pTRV1 (Arabidopsis, ABRC, catalog number: CD3-1039) and pTRV2e (Arabidopsis, ABRC, catalog number: CD3-1866) vectors can be ordered at the Arabidopsis Biological Resource Center (ABRC, <http://www.arabidopsis.org/>).
7. Culture Media
 - a. Liquid Luria-Bertani (LB) medium
 - b. Solid LB medium plate with 1.5% agar
Note: LB medium is autoclaved at 120 °C for 20 min before appropriate antibiotics are added.
 8. Antibiotics
 - a. Kanamycin (Sangon Biotech, USP Grade)
 - b. Rifampicin (Sangon Biotech, USP Grade)
 - c. Gentamicin (Sangon Biotech, USP Grade)
 9. PCR reagents
 - a. EasyTaq DNA polymerase (Beijing TransGen Biotech, catalog number: AP112)
 - b. EasyPfu DNA polymerase (Beijing TransGen Biotech, catalog number: AP211)
 - c. dNTP Mix (Roche Diagnostics, catalog number: 04729706103)
 10. Infiltration reagents
 - a. Dimethyl sulfoxide, DMSO (AMRESCO, ACS grade)
 - b. MgCl₂ (Beijing Chemical Works, Analytical pure) (see Recipes)
 - c. 2-(N-Morpholino) ethanesulfonic acid, MES (AMRESCO, Regent Grade) (see Recipes)
 - d. Acetosyringone (3, 5-Dimethoxy-4-hydroxyacetophenone) (AS) [Sigma-Aldrich, Purity (HPLC)] (see Recipes)
 - e. Infiltration buffer (see Recipes)

Equipment

1. Plant growth chamber (24 °C, 16 h/8 h light/dark photoperiod, 40-80% humidity)
2. Centrifuge
3. PCR instrument
4. 37 °C and 28 °C incubators with shaking

Procedure

1. TMs designing.

- a. The TM molecules were designed empirically by adding 3-4 nucleotides into the complementary sequences between sites opposite to the 10th and 11th nt of the targeted miRNA.
 - b. Keep the other position with base-pairing to the miRNA.
- Note: Figure 1 shows an example for TM design.*

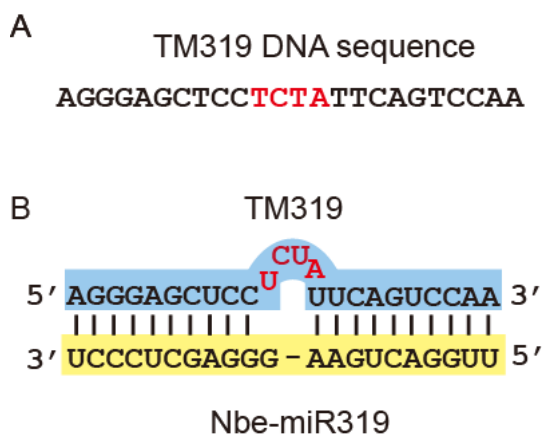


Figure 1. Example for TM design. A. DNA sequence of TM for miRNA319 (TM319). B. Base pairing of TM319 and Nbe-miR319. Red letters are nucleotides inserted in to the complementary sequences between sites opposite to the 10th and 11th nt (denoted by “-”).

2. Cloning.
 - a. The miRNA TM fragment was inserted into pTRV2e at LIC cassette as described in the previous study (Sha *et al.*, 2014).
 - b. Correct constructs were screened by PCR and must be confirmed by DNA sequencing.
3. Plasmid extraction.
 - a. Correct clones were grown in 2~5 ml liquid LB medium (containing 50 µg/ml Kanamycin) at 37 °C, with shaking at 200 rpm for 16 h.
 - b. Collect the bacteria and extract plasmids using Alkaline Lysis Method (Sambrook, 2001).
4. *Agrobacterium* transformation.
 - a. Transform pTRV1, pTRV2e or its derivatives into *Agrobacterium* strain GV3101 (or GV2260) respectively.
 - b. Grown for 2 days on solid LB media (containing 50 µg/ml Kanamycin, 50 µg/ml Rifampicin) at 28 °C.
 - c. Confirm that the *Agrobacteria* contain desired plasmid by PCR using specific primers and streak the correct transformants on LB plate.
5. Preparation of Agro-infiltrates.

- a. Grow correct transformants containing pTRV1, pTRV2e or pTRV2e derivatives (Sha *et al.*, 2014, Figure 1) in 5 ml liquid LB media respectively (containing 50 µg/ml Kanamycin, 50 µg/ml Rifampicin) in 28 °C incubator shaking at 200 rpm overnight.
 - b. Collect the culture media and adjust each agrobacterium culture to OD₆₀₀=1.0. Mix equal volume of agrobacterium culture (OD₆₀₀=1.0) of pTRV1 and that of pTRV2e or pTRV2e derivatives together.
 - c. Pellet the mixed agrobacteria by centrifuging at 3,000 x g for 5 min at room temperature.
 - d. Discard the supernatant and re-suspend the pellet with equal volume of infiltration buffer (to keep OD₆₀₀ ≈1.0).
 - e. Incubate the re-suspended agrobacteria at room temperature for 2.5-6 h.
Note: Inoculating agrobacteria into media for culturing should be done on clean bench and all equipment used needs to be sterile.
6. Plant infiltration.
- a. Select 6-leaf-stage plants for VbMS assay. Infiltrate the re-suspended agrobacteria into the abaxial side of 3-4 expanded leaves (avoid the mid vein) with 1 ml needless syringe.

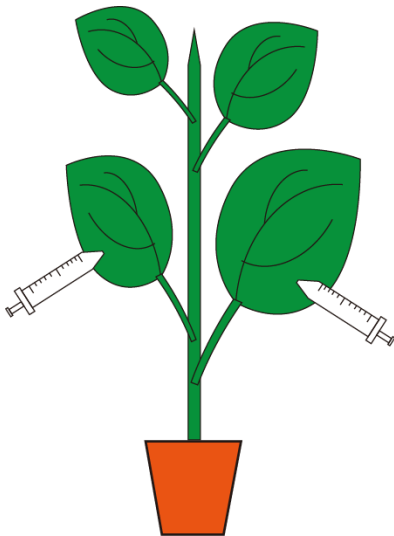


Figure 2. Schematic diagram of *Agrobacterium* infiltration

7. Plant growth and evaluation of miRNA silencing effects.
 - a. Grow the infiltrated plants at 24 °C with a 16 h/8 h light/dark photoperiod and the light intensity is 200 µmol m⁻² s⁻¹.
In 2-3 weeks post inoculation the target miRNA will be silenced at the whole plant level.
 - b. The newly developed organs will show phenotypes of strong silencing of the corresponding miRNA. These tissues can be used for appropriate experiments.

Note: Figure 2 shows a schematic overview of infiltration procedure. Each leaf is often injected at 2-4 sites throughout the leaf lamina, each injection site has a diameter >1 cm.

Recipes

1. 1 M MgCl₂
20.33 g MgCl₂·6H₂O dissolved in 100 ml ddH₂O, autoclaved by 120 °C, 20 min, stored at 4 °C.
2. 1 M MES
21.325 g MES dissolved in 100 ml ddH₂O, sterilized via filtration through 0.22 µm membrane, stored at room temperature.
3. 200 mM acetosyringone (AS)
0.3924 g AS dissolved in 10 ml DMSO, stored at -20 °C as 1 ml aliquots.
4. Infiltration buffer (10 mM MgCl₂, 10 mM MES, 200 µM AS)
Add 1 ml MgCl₂ (1 M), 1 ml MES (1 M), 100 µl AS (200 mM), add ddH₂O to 100 ml.

References

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