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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*: DH5α, *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. $MgCl_2$ (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.

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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-paring to the miRNA.
Note: Figure 1 shows an example for TM design.

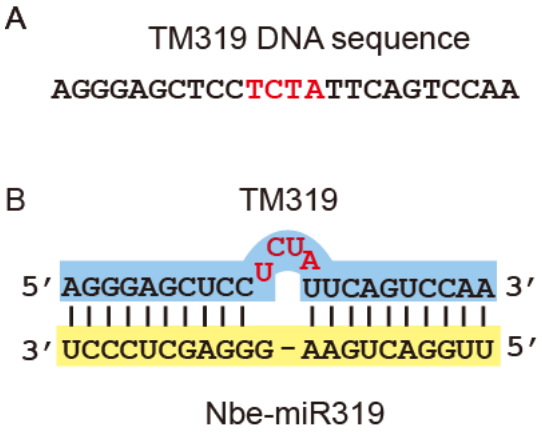

Figure 1. Example for TM design. A. DNA sequence of TM for miRNA319 (TM319). B. Base paring of TM319 and Nbe-miR319.Red letters are nucleotides inserted in to the complementary sequences between sites opposite to the 10th and 11th nt (denoted by "-").

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 OD_{600}=1.0. Mix equal volume of Agrobacterium culture (OD_{600}=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 OD_{600} ≈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 midvein) with 1 ml needless syringe.

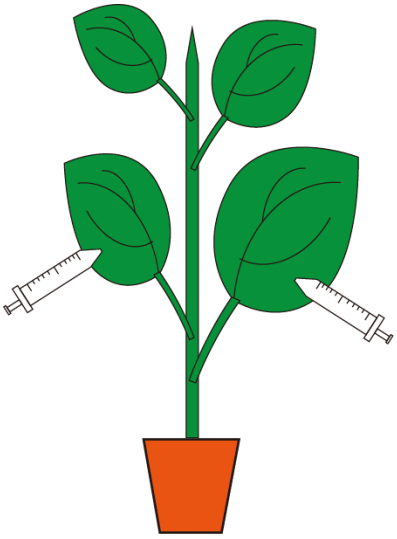

Figure 2. Schematic diagram of *Agrobacterium* infiltration

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 $MgCl_2$
   20.33 g $MgCl_2 \cdot 6H_2O$ dissolved in 100 ml ddH_2O, autoclaved by 120 °C, 20 min, stored at 4 °C.
2. 1 M MES
   21.325 g MES dissolved in 100 ml ddH_2O, 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 $MgCl_2$, 10 mM MES, 200 μM AS)
   Add 1 ml $MgCl_2$ (1 M), 1 ml MES (1 M), 100 μl AS (200 mM), add ddH_2O to 100 ml.

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