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VIGS Assays

Haili Zhang[1] and Yule Liu[2*]

[1]ChengDu Institute of Biology, Chinese Academy of Sciences, Cheng Du, China; [2]School of Life Sciences, Tsinghua University, Beijing, China

*For correspondence: yuleliu@mail.tsinghua.edu.cn

[Abstract] Virus-induced gene silencing (VIGS) is a powerful method to study gene function in plants. Tobacco rattle virus (TRV)-based VIGS vector is the most efficient VIGS vector so far. This method was originally developed by the Dinesh-Kumar's group(Liu *et al*., 2002). Here, we describe a rapid and high efficient TRV-based VIGS method for knocking down genes in *Nicotiana benthamiana*. For TRV-based VIGS, *Agrobacterium* culture containing pTRV1 and *Agrobacterium* culture containing pTRV2 with plant target gene fragment are mixed and infiltrated into the lower leaves of plant. After 2-3 weeks post infiltration, plant target gene will be silenced.

Materials and Reagents

1. 6-leaf-stage *Nicotiana benthamiana* plants
   Note: Nicotiana benthamiana can be obtained from our lab (Figure 1).

Figure 1. 6-leaf-stage *Nicotiana benthamiana* plant

2. Bacterial strains
   a. *Escherichia coli* strains, such as DH5α
   b. *Agrobacterium* strains, such as GV3101
   Note: All strains were obtained from our lab.
3. pTRV1 and pTRV2-LIC based expression vectors (Dong *et al*., 2007)
   a. pTRV1: a T-DNA vector containing duplicated CaMV 35S promoter, NOS terminator and cDNA clone of TRV RNA1 of Ppk20 strain.

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b. pTRV2-LIC: a T-DNA vector containing duplicated CaMV 35S promoter, NOS terminator and cDNA clone of TRV RNA2, of which non-structural genes were replaced by a multiple cloning site (MCS).
c. pTRV2-NbPDS: gene fragement of NbPDS was inserted at MCS into pTRV2-LIC. This construct was usually used as a control to show the successful gene silencing.
d. pTRV1 and pTRV2-LIC could be ordered at http://www.arabidopsis.org/abrc/catalog/vector_3.html. Stock numbers are CD3-1039 and CD3-1042, respectively.

4. Media for *Agrobacteria*
   a. Liquid Luria-Bertani (LB) medium
   b. Solid LB plates with 0.12% agar
   Note: LB medium is autoclaved under 120 °C for 20 min.
5. Antibiotics
   a. Kanamycin
   b. Rifampicin
   c. Gentamicin
6. Easy Taq DNA polymerase (Beijing TransGen Biotech)
7. dNTP (Roche)
8. TIANprep Mini Plasmid Kit (Beijing TransGen Biotech)
9. $MgCl_2$ (Sigma-Aldrich)
10. MES (AMRESCO)
11. Acetosyringone (Sigma-Aldrich)
12. DMSO (AMRESCO)
13. Infiltration buffer (see Recipes)

Equipment

1. Centrifuge tubes
2. Plant growth chamber (24 °C, 16 h photoperiod conditions, 50% huminity)
3. Sterile 1 ml syringe without needle
4. Sterile bacterial culture tubes
5. Centrifuge
6. PCR instrument
7. 37 °C and 28 °C incubators with shaking

Procedure

1. Clone plant target gene fragment into pTRV2-LIC as described previously (Liu *et al.*, 2002), and transform it into *Escherichia coli* DH5α. Positive clones were picked up

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and the plasmid DNA was amplified by PCR, using specific primer pairs, and then confirmed for correct insertion by DNA sequencing. Grow a positive clone in 5 ml LB liquid medium (containing 50 µg/ml Kanamycin) in 37 °C incubator at 200 rpm shaking overnight. Collect the bacteria by 14,000 *x g* centrifuging for 1 minute at room temperature and then extract plasmids using mini plasmid kit.

2. Transform pTRV1, pTRV2 or its derivatives into Agrobacterium strain GV3101 respectively. Transformed *Agrobacteria* were grown for 2 days on LB plates containing 50 µg/ml Kanamycin, 30 µg/ml Rifampicin and 50 µg/ml gentamicin.
3. Pick several clones and confirm that the grown *Agrobacteria* contain correct plasmid using PCR with specific primers.
4. Grow one positive clone from each transformant containing pTRV1, pTRV2 or pTRV2 derivatives in 5 ml liquid LB media (containing 50 µg/ml Kanamycin, 50 µg/ml Rifampicin and 50 µg/ml Gentamicin) in 28 °C incubator shaking at 200 rpm overnight.
   Note: Inoculating Agrobacteria into media for culturing should be done on super-clean bench, all equipment used needs to be sterile.
5. Take the culture tubes out of the incubator. Adjust all the *Agrobacterium* culture to OD_{600}=1.0. Taking equal volume of *Agrobacterium* culture (OD_{600} = 1.0) with pTRV1 and that with pTRV2 or pTRV2 derivatives. Mix them together and pellet by centrifuging at 3,000 *x g* for 5 min, at room temperature.
6. Pour off the supernatant, re-suspend the *Agrobacterium* pellet in infiltration buffer of equal volume to that of *Agrobacterium* culture (as to keep OD_{600} at around 1.0). Keep the re-suspended culture at room temperature for 2-4 h.
7. Select 6-leaf-stage plants and inflitrate the re-suspended *Agrobacterium* culture into abaxial side of expanded leaves, using 1 ml syringe (without needle). 2 or 3 leaves of each plant need to be injected. Plants were grown in a growth room with a 16-h/8-h photoperiod at a light intensity of 10,000 lux at 24 °C. Figure 2 shows the schematic diagram of infiltration. Figure 3 showed the leaf state right afer inoculation.
   Note: Each leaf for inoculation is often injected 2 circles with 1 cm diameters.

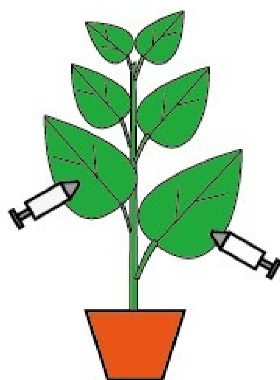

Figure 2. Schematic diagram of infiltration

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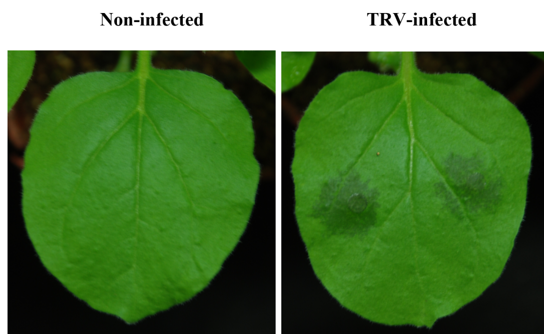

Figure 3. The right picture showed the state right after inoculation and the left one showed a non-infected leaf

8. 2 weeks post inoculation, target gene will be silenced at whole plant level. The new leaves always show the strong silencing phenotype, and are appropriate for following study. Figure 4 shows the upper leaves of plants that PDS gene was silenced by VIGS.

Figure 4. Successful silencing of Nb*PDS*. Photograph was taken 2 weeks post inoculation.

Recipes

1. Infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone) (100 ml)
   1 M $MgCl_2$: 20.33 g $MgCl_2$ dissolved in 100 ml dH_2O, autoclaved by 120 °C, 20 min. 1 M $MgCl_2$ stock was stored at 4 °C.
   1 M MES: 21.325 g MES dissolved in 100 ml dH_2O, filter sterilized with 0.22 μm filter membrane
   200 mM acetosyringone: 0.3924 g acetosyringone dissolved in 10 ml DMSO. 1 M MES stock was stored at room temperature. 200 mM acetosyringone was stored at -20 °C.
   100 ml infiltration buffer
   1 ml 1 M $MgCl_2$
   1 ml 1 M MES

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100 µl 200 mM acetosyringone

Add dH_2O to 100 ml

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This protocol was adapted from the research article: Wang *et al.* (2013).

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