Virus-Induced Gene Silencing as a Tool for Delivery of dsRNA into Plants

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Protocol

Virus-Induced Gene Silencing as a Tool for Delivery of dsRNA into Plants

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INTRODUCTION

The inherent RNA silencing mechanism in plants has been effectively manipulated as a tool for the targeted down-regulation of genes. Numerous methods have been employed to initiate this homology-based RNA degradation process, but all rely on the activity of double-stranded RNAs (dsRNAs) corresponding to the gene of interest. Virus-induced gene silencing (VIGS) has gained acceptance as the tool of choice for transient induction of silencing. It involves creation of engineered viruses carrying sequences corresponding to the host gene to be silenced. Infection leads to synthesis of viral dsRNA, an intermediate step in viral replication. This activates the anti-viral RNA silencing pathway, resulting in down-regulation of the host gene transcript. While several VIGS vectors have been developed, the Tobacco Rattle Virus (TRV) provides the most robust results in terms of efficiency, ease of application, and absence of disease symptoms. Engineered TRV vectors carrying host-derived segments are transformed into *Agrobacterium tumefaciens*, which is then introduced into the plant. This protocol outlines a simple procedure for introducing the TRV-based binary vectors pTRV1 and pTRV2 into solanaceous plants such as *Nicotiana benthamiana* and *Solanum lycopersicon* (tomato), as well as *Arabidopsis*.

RELATED INFORMATION

This protocol is an updated version of **Delivery of dsRNA into Plants by VIGS Methodology** (Burch-Smith et al. 2006a).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Agrobacterium tumefaciens, strain GV2260 or GV3101, containing pTRV1

GV2260 is ideal for silencing in N. benthamiana, *whereas GV3101 is used for tomato and* Arabidopsis.

Agrobacterium tumefaciens, strain GV2260 or GV3101, containing pTRV2 alone (empty plasmid; negative control), pTRV2 with a fragment of the *phytoene desaturase* gene (*PDS*; positive control), or pTRV2 with a fragment of the target gene

To ensure efficient VIGS, use of both positive and negative controls is advised. PDS *silencing leads to a visible bleached phenotype that can be easily scored (*Fig. 1*).*

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<R>Infiltration medium
Luria Broth (LB) liquid medium containing appropriate antibiotics
Plant of interest, e.g., *N. benthamiana* (four-leaf stage), *S. lycopersicon* (two-leaf stage), or *Arabidopsis* (three- to four-leaf stage)

Equipment

Air compressor, portable (e.g., Campbell Hausfeld) (for spray inoculation; see Step 6.v)
Airbrush, artist's (e.g., Paasche V-180 or equivalent) (for spray inoculation; see Step 6.v)
Beaker, plastic, 500 mL (for vacuum infiltration; see Step 6.vii)
Centrifuge
Desiccator, 240-mm diameter (Bel-Art Products) (for vacuum infiltration; see Step 6.ix)
Incubator preset to 26°C
Gloves, latex (for syringe infiltration; see Step 6.iii)
Growth carts
Razor blade (for syringe infiltration; see Step 6.ii)
Shaker
Spectrophotometer
Syringe, needleless, 1 mL (for syringe infiltration; see Step 6.i)
Vacuum pump (Fisher-Biotech FB-DVP-0352 or equivalent) (for vacuum infiltration; see Step 6.ix)

METHOD

Preparing Agrobacterium

1. Inoculate *Agrobacterium* GV2260 or GV3101 containing the TRV1 and TRV2 vectors in LB containing appropriate antibiotics. Grow the culture overnight at 26°C with shaking.

   Five milliliters of Agrobacterium *culture is sufficient to inoculate three to four plants. The culture volume can be scaled up based on the number of plants to be used for silencing.*

2. Collect the cells by centrifugation at 3000 rpm for 15-30 min. Resuspend the *Agrobacterium* pellet in infiltration medium.

3. Adjust the OD_{600} to 1.0 for *N. benthamiana* infiltration, 1.5 for *Arabidopsis* infiltration, or 1.5-2.0 for tomato infiltration. If the vacuum infiltration method (see Steps 6.vii-6.x) is used for introducing *Agrobacterium*, adjust the OD_{600} to 0.4 for pTRV1 and 0.2 for pTRV2.

4. Incubate the cultures at room temperature for 3-6 h.

   A minimum of 3 h ensures sufficient activation of the Agrobacterium Vir *genes that enhance transformation efficiency.*

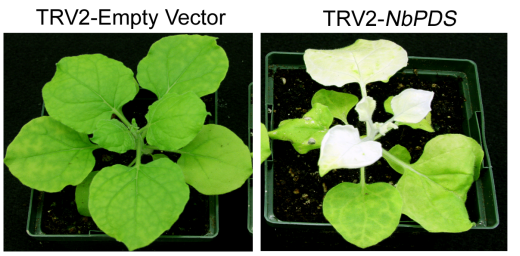

FIGURE 1. Silencing of the *phytoene desaturase* gene (*PDS*) using VIGS. *N. benthamiana* plants were infiltrated with *Agrobacterium* containing either the empty vector TRV2 (TRV2-Empty Vector) or TRV2 with a *PDS* fragment (TRV2-*NbPDS*) and photographed 12 d post-infiltration. Plants with the empty TRV vector appear similar to healthy uninoculated plants, whereas TRV2-*NbPDS*-inoculated plants have newly emerged leaves with the photobleached phenotype characteristic of *PDS* silencing.

Infiltration of *Agrobacterium*

5. Mix the *Agrobacterium* cultures containing TRV1 and TRV2 1:1 just before infiltration.
6. Introduce *Agrobacterium* into the leaves of the plants of interest using one of the following methods:

   Use the spray inoculation or vacuum infiltration methods for VIGS in tomato plants.

   Syringe infiltration:

   i. Load a 1-mL needleless syringe with the *Agrobacterium* mixture.

   ii. Using the corner of a razor blade, gently nick the underside of the lower two leaves of the plant.

   iii. Place the mouth of the syringe on the nick. Create a seal on the other side of the leaf by placing a finger from the other gloved hand just beneath the nick.

   iv. Using minimal pressure, inject the *Agrobacterium* culture into the leaf. Repeat this step until the two lower leaves has been completely infiltrated.

   When performing multiple silencing experiments, take care to avoid cross-contamination. Use fresh gloves and syringes for each infiltration.

   Spray inoculation:

   v. Load the *Agrobacterium* solution into an artist's airbrush that is attached to a pressure compressor set to 80 psi.

   vi. Spray the ventral side of the two lower leaves from a distance of ~8 in. for 1-5 sec.

   Vacuum infiltration:

   vii. Transfer the culture into a 500-mL beaker.

   viii. Turn the plant upside down. Submerge the plant completely inside the *Agrobacterium* solution.

   ix. Place the beaker containing the submerged plant inside a vacuum desiccator attached to a vacuum pump.

   x. Evacuate the chamber using a maximum vacuum of 29.5 cm Hg for 90 sec.
7. Grow the plants in growth carts. Maintain at an ambient temperature of 25°C (for *N. benthamiana*) and ~21°C (for tomato and *Arabidopsis*).

   For N. benthamiana*, VIGS efficiency is high under continuous light, whereas a 16-h light/8-h dark cycle is ideal for tomato and* Arabidopsis.
8. Visualize the VIGS phenotype 6-10 d post-infiltration.

   *The upper (i.e., uninoculated) leaves of plants infiltrated with the positive control (TRV2-*PDS*) should develop a bleached phenotype indicating efficient silencing (*Fig. 1*). Plants infiltrated with the TRV2 empty plasmid should resemble healthy uninoculated plants.*
9. Quantify the RNA levels of the silenced genes using northern blot hybridization or quantitative polymerase chain reaction (qPCR) methods.

TROUBLESHOOTING

Problem: Uniform silencing is not observed.
[Step 8]
Solution: Multiple factors can affect the efficiency of VIGS:

1. Growth conditions: *Agrobacterium* transformation efficiency is optimal at 16°C-21°C, whereas VIGS efficiency for *N. benthamiana* is good at ~23°C-25°C. Tomato plants show drastically reduced VIGS efficiency at temperatures above 21°C. For *Arabidopsis*, short day conditions (8 h light/16 h dark) are suboptimal for VIGS induction.

2. Age of the plant: If the plants are older than the stages indicated above, this can lead to a decrease in efficiency.

3. Gene fragment chosen for silencing: Fragments of 300-1500 bp have been used successfully to induce VIGS. Silencing efficiency is reduced below 300 bp, whereas with longer fragments there is a strong possibility of the insert being lost from the recombinant virus. Different gene fragments can show variability in VIGS efficiency. Try to insert fragments from different regions within the same gene.

DISCUSSION

Elucidation of plant gene function has conventionally employed the use of stable insertions in the gene of interest. Homology-based silencing techniques have proven to be an attractive alternative that can overcome some of the limitations seen with gene knockouts. dsRNA can be introduced into plants either as stable hairpin-RNA-synthesizing transgenes or by infection with recombinant viruses that carry segments of plant genes (Baulcombe 1999; Wang and Waterhouse 2002). The advantage of VIGS is that it precludes the need for laborious and time-consuming plant transformation. It is possible to silence multiple classes of closely related genes, as well as genes where true knockouts are embryo-lethal. It is also more amenable to high-throughput studies and has been used successfully in model and non-model host systems (Lu et al. 2003; Burch-Smith et al. 2004; 2006b; Constantin et al. 2004; Fofana et al. 2004; Ding et al. 2006). TRV has been perfected as a VIGS vector: It provides robust silencing, has a broad host range, can infect meristematic tissue, and produces only mild disease symptoms (Ratcliff et al. 2001; Liu et al. 2002; Burch-Smith et al. 2006b). Using *Agrobacterium* to introduce TRV to the host also overcomes the need for cumbersome in vitro transcription of viral RNA or biolistic delivery. Recently, the Turnip Yellow Mosaic Virus has been adapted for VIGS in *Arabidopsis* and provides robust silencing following simple mechanical inoculation with a plasmid-carrying engineered virus (Pflieger et al. 2008). Despite these advantages, it must be noted that VIGS provides only transient silencing and can only lead to reduced transcript accumulation and not a complete loss of transcript.

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