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Article *in* Methods in molecular biology (Clifton, N.J.) · June 2014

DOI: 10.1007/978-1-4939-0922-3_7 · Source: PubMed

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Virus-induced Gene Silencing in Plant MAPK Research

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Key words: mitogen-activated protein kinase, *Nicotiana attenuata*, virus-induced gene silencing, transient transformation

Summary

Virus-induced gene silencing (VIGS) technology has become more and more widely used in various plant species for rapid screening of gene functions. VIGS does not require time-consuming tissue culture steps that are needed for stable transformation in most plant species and it can be used for studying gene function even in plants that are very difficult to stably transform. Furthermore, VIGS technology provides high gene silencing efficiency (up to 95%) and specificity. Here, we describe a VIGS protocol that can be used for studying the functions of MAPKs and other genes in a wild tobacco species, *Nicotiana attenuata*. This method is also suitable for other *Nicotiana* species and tomato with minor modifications.

1. Introduction

Gene silencing is probably the most important means of studying gene function. Mostly, this is done by transforming plants with vectors containing a fragment of the target gene in an anti-sense or inverted repeat fashion. *Agrobacterium*- or biolistics-mediated plant stable transformation is available for a small number of plant species. However, except Arabidopsis (*Arabidopsis thaliana*), whose transformation can be easily done by floral dipping [1], transformation generally requires lengthy tissue culture procedures. This has greatly limited functional analyses of genes in these plants, especially in the age of functional genomics.

VIGS is a technique that utilizes recombinant viruses to specifically silence genes of interest in plants. In plants, post-transcriptional gene silencing (PTGS) is an innate resistance to limit viral proliferation. The underlying mechanism of this form of resistance is very similar to the RNA interference (RNAi). To initiate VIGS, a viral vector carrying a fragment of the target gene is introduced into plant cells by *Agrobacterium*- or biolistics-based transient transformation, and consequently, plants start to accumulate recombinant viruses, which in turn induce silencing of the plant target genes. Compared with gene silencing mediated by stable transformation, VIGS procedures have several advantages: 1) Viral vectors are simple to construct; 2) VIGS does not require tissue culture and thus can be used to study functions of many genes in short time; 3) VIGS can be used in many plant species, such as tomato, tobacco, Arabidopsis, and even in plants that are hard to stably transform, such as dicots, grape vine and soybean, and monocots, barley and maize [2]. Given the relatively high throughput property of VIGS technology, it is an excellent tool to study functional genomics. Plant mitogen-activated protein kinases (MAPKs) belong to a large gene family and have important signaling functions [3] and VIGS has been successfully used to obtain plants silenced in target MAPK genes in short time [4-8].

A tobacco rattle virus (TRV)-based VIGS system has often been used in a few solanaceous plants, namely, tobacco (*Nicotiana tabacum*), *N. attenuata*, *N. benthamiana*, and tomato (*Solanum lycopersicum*). Compared with other viruses, such as potato virus X (PVX), TRV more effectively infects solanaceous plants and can uniformly spread to most parts of a plant including newly emerging leaves, and this results better silencing efficiency and can be used for studying many plant organs, including leaves, flowers, and roots [9]. TRV has a positive-strand bipartite viral genome. RNA1 was cloned into pBINTRA6, and RNA2 was cloned into a construct named pTV00 [9]. A fragment of the gene of interest can be cloned into pTV00 to silence this gene in the host plants. Here we describe a detailed protocol, in which the TRV-based VIGS system [9,10] is used to silence target MAPK genes for studying their functions in *N. attenuata* [4]. This method is also suitable for studying other genes and in other *Nicotiana* species and tomato with minor modifications [6,11].

2. Materials

Prepare all solutions using pure or ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Waste should be disposed following regulations, including the genetically modified bacteria and plants.

2.1. Preparation of *Agrobacterium* strains

2.1.1. Preparation of *A. tumefaciens* GV3101 Electro-Competent Cells

1. Sterile YEP medium (10 g/L yeast extract, 10 g/L peptone, 5 g/L sodium chloride; adjusted to pH 7.0) (0.5 L are enough to prepare about 60 aliquots of competent cells).
2. Antibiotics (25 mg/ml rifampicin and 5 mg/ml tetracycline stock solutions, store at -20ºC)
3. Sterile H_2O (1 L). Sterile 10% (v/v) glycerol (0.5 L).
4. Sterile conical flasks/Erlenmeyer flasks (1.0 L).

5. Sterile 1.5 mL and 50 mL centrifuge tubes.
6. Spectrophotometer for determination of bacterial density (OD_{600}).
7. Refrigerated centrifuge.
8. Shaker for cultivation of bacterial cultures.
9. Freezer (−80°C).

2.1.2. Preparation of Gene-Specific VIGS Constructs

1. Gene-specific PCR primers.
2. Restriction enzymes and buffers.
3. DNA ligase kit; PCR polymerase kit.
4. *E. coli* competent cells.
5. Sterile liquid LB medium and LB agar with antibiotics (50 mg/L kanamycin).
6. Gel and plasmid DNA extraction kits (e.g., MN NucleoSpinExtract II; MN NucleoSpin Plasmid; http://www.mn-net.com).
7. Agarose gel electrophoresis equipment (chamber, power supply, gel trays, etc.).
8. PCR machine, centrifuge, and electroporation instrument.
9. Shaker for cultivation of bacterial cultures.

2.1.3. Transformation of *A. tumefaciens* GV3101

1. Sterile electroporation cuvette, 1 mm gap (use UV or 70% ethanol to sterilize); electroporation instrument.
2. Sterile S.O.C. media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose)
3. Sterile LB agar and liquid LB medium with antibiotics.
4. Shaker for cultivation of bacterial cultures at 28°C.
5. Sterile 50% glycerol.

2.2. Materials for plant growth

1. *N. attenuata* Torr. ex S. Watson seeds.
2. Seed sterilization solution, freshly prepared: 2% (w/v) dichloroisocyanuric acid sodium salt with 0.005% Tween-20 in H_2O (1 mL is enough for 50-100 seeds).
3. Stock solution of 0.1 M GA_3 (gibberellic acid) in ethanol. Keep at 4ºC.
4. 50 × diluted liquid smoke (House of Herbs, Passaic, NJ), in H_2O, autoclaved.
5. Sterile H_2O (100 mL).
6. Petri dishes with 0.6% plant agar- or phytagel-supported Gamborg's B5 cultivation media (1× strength).
7. Small Teku plastic pots and 1 L pots; soil.
8. Growth chamber for plates with seedlings (16 h light/8 h dark, 26°C, 150-200 µmol $s^{-1}m^{-1}$ PAR); air-conditioned (22-24ºC) growth chamber for plant cultivation.

2.3. Materials for Bacteria Cultivation and Plant Inoculation

1. *A. tumefaciens* GV3101 strains carrying constructs: pBINTRA6, pTV00 (empty vector control), pTV-(MAPK Gene-of-Interest; hereafter GOI), and pTV-PDS (fragment of phytoene desaturase gene).
2. *N. attenuata* plants in young rosette stage, growing in a climate chamber at a constant temperature program of 22-24°C, 16 h day/8 h night, ca. 65% relative humidity.
3. YEP medium: 10 g/L yeast extract, 10 g/L peptone (from soy), and 5 g/L sodium chloride, autoclaved; LB medium can also be used as a substitute for YEP, but *A. tumefaciens* grows more slowly in LB.
4. 1,000× kanamycin stock solution in H_2O (50 mg/mL), filter-sterilized.
5. Erlenmeyer flasks for growing Agrobacterium cultures, autoclaved.
6. Shaker for cultivation of bacterial cultures in flasks at 28°C.
7. Spectrophotometer for determination of bacterial density.

8. Centrifuge.
9. Resuspension buffer: 5 mM $MgCl_2$/5 mM 2-*N*-morpholino ethanesulfonic acid (MES) (adjust pH of MES to 6.0 with KOH).
10. 1 mL syringes without needle.

3. Methods

3.1. Construction of VIGS Vectors

3.1.1. Preparation of *A. tumefaciens* GV3101 Electro-Competent Cells

1. Inoculate 20 mL YEP-medium containing 25 mg/L rifampicin and 5 mg/L tetracycline with *A. tumefaciens* GV3101 and incubate in a shaker overnight at 200 rpm and 28°C.
2. Inoculate 0.5 L YEP medium (no antibiotics) in a sterile 1 L conical flask with 10 mL of the overnight-pre-culture. Let the bacteria grow at 200 rpm and 28°C for about 8 h until they reach an OD_{600} of 0.5–0.7 (logarithmic growth phase).
3. Fill the bacterial cultures into sterile 50 mL centrifuge tubes (maximum 40 mL volume) and spin them in a fixed angle rotor at 3800 × g for 10 min at 4°C.
4. Pour off the supernatants, thereby preserving the pellets. Refill the tubes with the same volume of the remaining bacterial culture. Repeat the centrifugation step to pellet all bacteria. Keep cell suspensions or pellets on ice between each centrifugation step!
5. Carefully remove all supernatants. If necessary, briefly spin the tubes again after emptying and remove the last drops with a pipette tip. It is important to remove as much salt as possible to obtain cells with lowest possible conductivity for highest possible competence.

6. Resuspend the cells in 10 mL pre-cooled sterile H_2O (resuspend the pellet gently without vortexing). Combine the bacterial suspensions from the individual tubes.
7. Spin for 10 min and remove the supernatant as before. Continue with two more cycles of washing and until all bacteria are pooled into one 50 mL centrifuge tube.
8. Resuspend the cells in 40 mL 10% glycerol. Centrifuge and remove supernatant as before. Repeat at least 3 times.
9. Resuspend the final pellet in 1.5 mL 10% glycerol and aliquot 40 µL per 1.5 mL microcentrifuge tube.
10. Freeze the aliquots in liquid nitrogen and store at −80°C.

3.1.2. Cloning of Gene Fragments into the VIGS Vector pTV00 to form pTV-GOI

A fragment of the GOI to be silenced must be cloned in the polylinker of pTV00 (see detailed description at http://www.plantsci.cam.ac.uk/research/baulcombe/methods.html). Best results may be obtained if the fragment is inserted in an antisense orientation after choosing appropriate unique cloning sites. Because of enzyme buffer compatibility (assuming that you are using enzymes from New England Biolabs), *Bam*HI and *Sal*I sites are preferred for cloning GOI into pTV00, but other combinations of restriction sites can also be used if *Bam*HI or *Sal*I sites cannot be avoided on the GOI fragment (unique sites in the polylinker are *Spe*I, *Bam*HI, *Sma*I, *Xma*I, *Hind*III, *BspD*I, *Cla*I, *Acc*I, *Apa*I, and *Kpn*I consecutively).

1. Find a region of sufficient size (150–400 bp; Note 1) in the GOI cDNA sequence that does not contain the chosen restriction sites. If only one member of a gene family should be silenced, the fragment having the least identity with other family members should be chosen (a 23 or more than 23 nt continuous sequence perfect match may induce silencing of the other homologues [12]; even constructs designed from the UTR regions are possible). For silencing of homologous genes, choose the region with the highest identity. In our experience, it is also possible to silence two

independent genes by tandem insertion of two 150–200 bp sequences specific for each GOI.

2. The primers should carry a 5' GC rich sequence (AGCT) followed by the restriction site of one of the cloning enzymes and sufficient nucleotides for annealing to the GOI (about 21–25 nucleotides)
3. PCR amplify the desired GOI fragment with the primers using cDNA prepared from plant tissues expressing the GOI. Preferably, use a proofreading polymerase and follow the manufacturer's instructions for optimal performance. Pipette a 50 μL reaction and divide it into 10 μL aliquots. Run each aliquot at a different annealing temperature in the range of 55–65°C.
4. Run your PCR products along with a molecular mass standard on a 1% agarose gel. Excise PCR fragments of the expected size and extract the DNA with an appropriate kit (e.g., MN NucleoSpin Extract II, http://www.mn-net.com). About 200 ng of PCR fragment will be needed for further cloning. If the amount of the PCR fragment is too low, repeat the PCR at the optimal annealing temperature. If the PCR result is unsatisfying at all annealing temperatures, design a new primer pair.
5. Digest, in separate reactions, 200 ng of pTV00 vector and 200 ng of the PCR fragment with the restriction enzymes according to the manufacturer's instructions.
6. Run both digestions using a 1% agarose gel; excise the 5.5 kb pTV00 band and the digested PCR fragment. Extract DNA.
7. Set up a 20 μL ligation reaction with approximately 50 ng of digested pTV00 and 100 ng digested PCR fragment. Ligate overnight at 16°C.
8. Transform an electro- or chemical-competent *E. coli* strain carrying lacIq (e.g., TOP10, http://www.invitrogen.com) with the ligation mixture (1-2 μL). Add warm

(37°C) S.O.C medium to cells and incubate cells in a 37 °C shaker at 200 rpm for 30-60 min.

9. Plate 50 – 200 μL of the transformed cells on LB plates containing 50 mg/L kanamycin and incubate the bacteria at 37°C overnight.
10. Pick a few single colonies (e.g. 4) and inoculate each of them into 15 mL culture tubes containing 5 mL of LB liquid medium (50 mg/L kanamycin). Shake overnight at 200 rpm at 37°C.
11. Isolate plasmid DNA using a standard kit (e.g., MN NucleoSpin Plasmid).
12. Digest 1 μL of each plasmid with appropriate restriction enzymes and run the digestion products on a 1% agarose gel. Plasmids that contain both the correct pTV00 and GOI fragment are used to confirm the correct sequence of the insert by sequencing with the primers: TRV FOR 5'-GCTGCTAGTTCATCTGCAC-3'; TRV REV 5'-GCACGGATCTACTTAAAGAAC-3'.
13. One plasmid with the correct sequence is used to transform *A. tumefaciens* electro-competent cells as described in the following section.

3.1.3. Transformation of *A. tumefaciens* GV3101 by Electroporation

1. Pre-warm 1 mL of S.O.C. per construct to 28°C.
2. Slowly thaw on ice one aliquot (40 μL) of competent *A. tumefaciens* GV3101 cells.
3. After thawing, mix the cells with 50 ng of plasmid DNA from a correct pTV-GOI clone.
4. Fill the mixture into an ice-cold electroporation cuvette with a gap of 1 mm.
5. Perform electroporation using an electroporator (e.g., Bio-Rad MicroPulser, http://www.bio-rad.com) following the instrument instruction.
6. Add 1 mL of pre-warmed S.O.C. immediately after pulse.

7. Mix the cells with the S.O.C., pipette the suspension into a 1.5 mL microcentrifuge tube, and incubate for 30 min at 28°C at 200 rpm.
8. Plate 50 µL on a LB agar plates containing 50 mg/L kanamycin; meanwhile plate 10 µL to another plate in order to avoid that too many colonies may appear.
9. Incubate the plates for 48 h at 28°C.
10. Isolate a single colony. Use this colony to inoculate 3 mL of liquid LB medium containing 50 mg/L kanamycin. Grow the bacteria in a shaker at 200 rpm and 28°C for about 20 h until they reach an OD_{600} of about 1.
11. Prepare a glycerol stock by mixing 0.5 mL of this culture with 0.22 mL of 50% glycerol, freeze in liquid nitrogen and store at −80°C. The culture or the glycerol stock can be used as starting culture for the VIGS procedure.

3.3. Plant Inoculation

3.3.1. Plant growth

1. Prepare fresh sterilization solution containing 2% (w/v) of dichloroisocyanuric acid sodium salt and 0.005% Tween-20 in H_2O.
2. Incubate 50-100 *N. attenuata* seeds (Note 2, 3) in 1 mL of sterilization solution for 5 min using 1.5 mL centrifugation tubes; shake occasionally. The following steps should be carried out under a sterile hood.
3. Decant solution and wash seeds in 1 mL sterile H_2O. Repeat decanting and washing steps at least three times.
4. Add 1 mL of diluted smoke solution and 10 µL of 0.1 M GA_3 stock and incubate seeds for 1 h (Note 4). Shaking occasionally or inclining the tubes to provide a larger surface area of seed exposure during incubation yields more even germination and greater germination efficiency.

5. Decant smoke/ GA_3 solution, wash seeds three times with sterile H_2O, and distribute 30–40 seeds/plate with 0.6% agar or phytagel-supported Gamborg's B5 cultivation media.
6. Allow plates to dry briefly until any water transferred with seeds has evaporated, then close with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL) and transfer to a growth chamber (16 h light/8 h dark, 26°C, 150-200 μmol $s^{-1}m^{-1}$ PAR).
7. After 10 days carefully remove seedlings from agar and transfer to low-nutrient soil in small Teku plastic pots.
8. After 10 days in Teku pots, transfer seedlings to 1 L pots in soil. Place pots in groups of 10–11 into flat plastic trays with a ca. 5.5 cm rim; this is convenient for the VIGS inoculation. Transfer to a climate chamber with a constant temperature of 22°C and 16 h day/8 h night light regime, ca. 65% relative humidity, and medium light intensity (200-250 μmol $s^{-1}m^{-1}$ PAR).

3.3.2. Inoculation of *N. attenuata* seedlings by syringe infiltration

1. Plants should be inoculated at 4–6 days post-potting, namely, 24–26 days post-germination. It is very important that plants are in the right stage because with older plants, VIGS has been found to be less efficient (Note 5).
2. The whole experiment is carried out in a climate chamber with a constant temperature of 22°C (Note 6) and 16 h day/8 h night light regime, ca.65% relative humidity, and medium light intensity (200-250 μmol $s^{-1}m^{-1}$ PAR).
3. In parallel with plant growth, streak *A. tumefaciens* cultures pTV00 (empty vector control; Note 7), pTV-GOI, pTV-PDS (as the positive control), and pBINTRA6 onto YEP or LB agar plates with 50 mg/L kanamycin. Allow to grow for 2 days at 28°C. These plates can be stored at 4°C for approximately 1 month.

4. Prepare sterile Erlenmeyer flasks and enough YEP medium. For every 1 mL of inoculation solution, you will need 2.5 mL of liquid YEP culture with pTV-GOI plus 2.5 mL pBINTRA6 liquid YEP culture; calculate 1 mL inoculation solution per plant. Do not fill flasks to more than 50% of their maximum volume to allow sufficient gas exchange while shaking cultures.
5. On day 3 after potting, check the size of the plants. If the plants are too small, postpone the VIGS experiment for 1–2 days; ideally, the leaf size should be 2–3 cm in length during infiltration (Note 5). If the size is sufficient, start small overnight pre-cultures of *A. tumefaciens* GV3101 carrying viral vectors in 15 mL culture tubes with YEP + kanamycin (50 mg/L) at 28°C and 200 rpm. The pre-culture can be used with 1/30-1/10 dilutions for making the larger cultures.
6. Water plants in the morning of the day that you want to VIGS, or the day before. Open stomata are important for the efficient bacteria inoculation into the leaves.
7. On day 4 after potting (or later depending on plant growth), add pre-cultures (final dilution 1/30 to 1/10) to Erlenmeyer flasks containing suitable amount of YEP medium (with 50 mg/L kanamycin) and incubate at 28°C and 200 rpm. These need to grow at least 5–6 h to reach the correct OD (0.4-0.6). Alternatively, you can inoculate a 1:1,000 aliquot of your pre-culture into the prepared Erlenmeyer flasks and grow the cultures overnight (about 16 h).
8. When the culture reaches an OD_{600} of 0.4–0.6, spin down the bacteria at 4,000 × g for 10 min. You can centrifuge 40 mL at a time in 50 mL Falcon tubes.
9. Discard the supernatants and resuspend the pellets by shaking (do not vortex) in the inoculation solution, which contains 5 mM $MgCl_2$ and 5 mM MES. The volume of the inoculation solution should be 1/5 the volume of your culture, so that the final OD_{600} of the inoculation solution is 2.0–3.0.

10. For every construct, make a final inoculation solution by mixing equal volumes of resuspended *Agrobacterium* carrying pTV-GOI construct and pBINTRA6. To avoid contamination, add suspensions of *Agrobacterium* containing pTV-GOI construct into aliquots of *Agrobacterium* with pBINTRA6.
11. Perform inoculations using a 1 mL syringe without a needle: pressure-inject *Agrobacterium* suspended in inoculation solution into 3 leaves per plant, with 1–4 inoculations on the underside of each leaf. You should try to saturate at least 75% of each inoculated leaf (the leaf turns dark green). It is easiest to inoculate plants during the light period when the stomata are open.
12. Cover the whole tray of plants with a plastic bag or with an upside-down tray (make sure the upside-down tray is tightly closed against the tray holding the plants to provide high humidity for establishment of *A. tumefaciens* transformation), and leave the lights off for 2 days.
13. Remove the plastic bags or trays and switch on the lights.
14. The viral spread is always accompanied by a characteristic leaf phenotype: after about 10–12 days, newly developing leaves are wrinkled, and leaves which mature post-inoculation often appear lighter than the oldest rosette leaves which were not VIGSed. You should discard plants that do not develop wrinkled leaves by day 16 post-inoculation as they will not show gene silencing. VIGSed plants should also be smaller than non-VIGSed plants grown under the same conditions. The wrinkled-leaf phenotype will lessen as plants start to elongate.
15. Bleaching of the leaves on the PDS positive control should appear at approximately 10–14 days post-inoculation. Experiments should begin once clear and even bleaching is visible in pTV-PDS positive control plants; tissues which were already established

at the time of inoculation will not bleach, and the corresponding tissues should be avoided in experiments with GOI-silenced and EV control plants.

4. Notes:

1. GOI fragments at a size of about 300 bp provide effective gene silencing; fragments shorter than 150 bp may result in a weak silencing effect, while fragments longer than 300 bp increase the probability of unspecific silencing of genes with short sequence homologies.
2. *N. attenuata* seeds can be obtained upon request from Prof. Ian T. Baldwin (baldwin@ice.mpg.de), Department of Molecular Ecology, Max Planck Institute for Chemical Ecology in Jena, Germany.
3. RNAi stably silenced lines of one gene also can be used as starting material for a successful VIGS of another independent target GOI.
4. *N. attenuata* seeds require smoke cues to break dormancy and germinate. Treatment with smoke solution is not required for other tobacco species like *N. benthamiana*; however, it is still recommended to use the GA_3 solution for increased and synchronized germination in other tobacco species.
5. Proper plant age is critical for the establishment of VIGS. Older plants are usually more resistant to *A. tumefaciens* infection, while younger plantlets may not survive damage to the leaves caused during infiltration. Plants that we normally use for infiltration have about 6-9 leaves with leaf blades 2–3 cm long.
6. Low temperature is important for proper VIGS establishment, but the temperature should permit normal development of plants and larvae. We find the best compromise is to establish VIGS at 22°C.
7. pTV00 should be used as a negative control to exclude nonspecific phenotypic effects of VIGS as virus infection is known to change hormone and metabolic homeostasis in

plant cells. In most cases virus-infected plants will contain higher levels of the pathogenesis-associated hormone salicylic acid.

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