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Virus-Induced Gene Silencing and Transient Gene Expression in Soybean (Glycine max) Using Bean Pod Mottle Virus Infectious Clones

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Abstract

Virus-induced gene silencing (VIGS) is a powerful and rapid approach for determining the functions of plant genes. The basis of VIGS is that a viral genome is engineered so that it can carry fragments of plant genes, typically in the 200 to 300 base pair size range. The recombinant viruses are used to infect experimental plants, and wherever the virus invades, the target gene or genes will be silenced. VIGS is thus transient, and in the span of a few weeks, it is possible to design VIGS constructs and then generate loss-of-function phenotypes through RNA silencing of the target genes. In soybean *(Glycine max)*, the *Bean pod mottle virus* (BPMV) has been engineered to be valuable tool for silencing genes with diverse functions and also for over-expression of foreign genes. This protocol describes a method for designing BPMV constructs and using them to silence or transiently express genes in soybean.

Keywords

gene expression, RNA silencing, soybean, viral vector, virus-induced gene silencing

Comments

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Virus-induced gene silencing (VIGS) is a powerful and rapid approach for determining the functions of plant genes. The basis of VIGS is that a viral genome is engineered so that it can carry fragments of plant genes, typically in the 200 to 300 base pair size range. The recombinant viruses are used to infect experimental plants, and wherever the virus invades, the target gene or genes will be silenced. VIGS is thus transient, and in the span of a few weeks, it is possible to design VIGS constructs and then generate loss-of-function phenotypes through RNA silencing of the target genes. In soybean *(Glycine max),* the *Bean pod mottle virus* (BPMV) has been engineered to be valuable tool for silencing genes with diverse functions and also for over-expression of foreign genes. This protocol describes a method for designing BPMV constructs and using them to silence or transiently express genes in soybean.

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INTRODUCTION

Virus-induced gene silencing (VIGS) methods have been developed that enable rapid silencing or expression of genes in soybean *(Glycine max).* This protocol describes the use of infectious cDNA clones of *Bean pod mottle virus* (BPMV) that have been engineered to accept inserts of foreign gene fragments. BPMV is a bi-partite RNA virus that requires two genomic RNAs, RNA1 and RNA2, for replication and spread throughout the plant host. The RNA2 component has been modified to accept foreign inserts, such as plant gene fragments. BPMV RNA1 and recombinant RNA2 clones are mixed and then biolistically bombarded into soybean leaves to initiate systemic infections that also lead to silencing of the soybean target gene(s). There are different versions of the BPMV RNA2 clones that can be chosen based on the experimental goals (Fig. 1). Here, we describe

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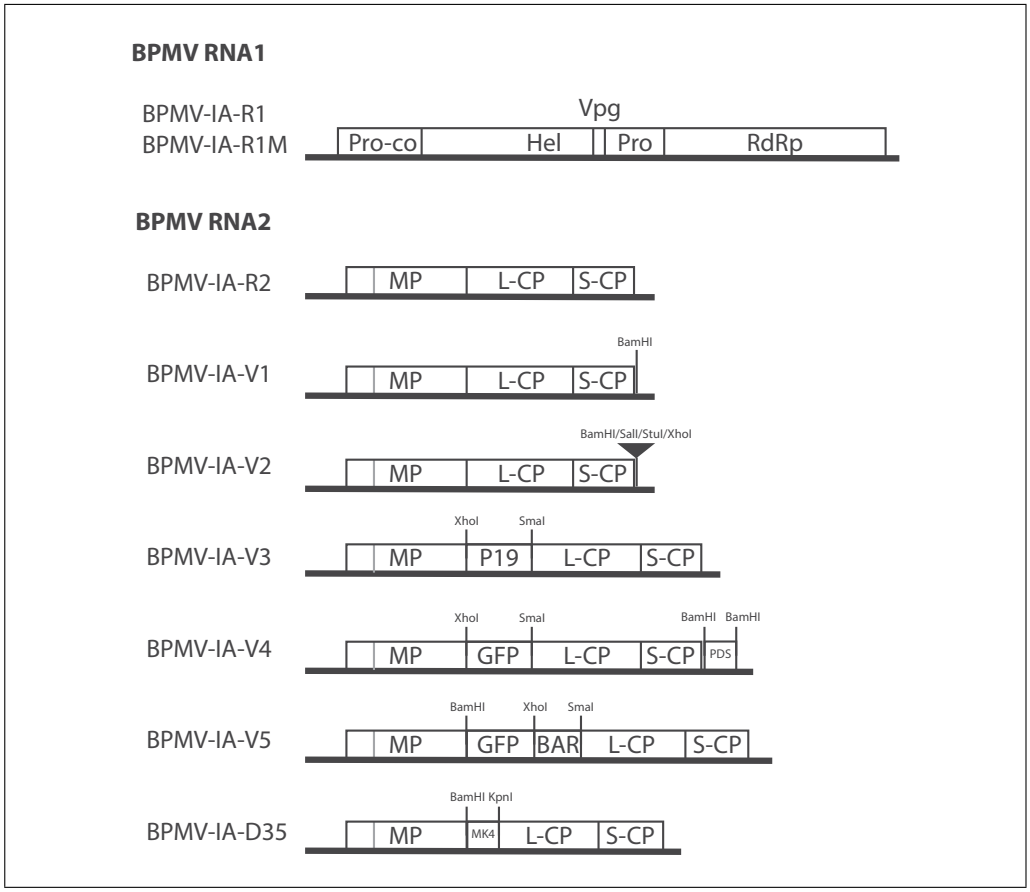

Figure 1 Schematic representation of BPMV viral vector sequences. Restriction enzyme sites for cloning are identified. Functions of the viral open reading frames are indicated as follows: Pro-co, proteinase co-factor; Hel, helicase; VPg, genome linked viral protein, Pro, proteinase; RDRP, RNA dependent RNA polymerase; MP, movement protein; L-CP, large subunit of coat protein; S-CP, small subunit of coat protein. The vectors are named according to Zhang et al. (2010), and some of the base vectors carry inserts. The inserts are as follows: P19, 19 kDa protein from *Tomato bushy stunt virus*; GFP, green fluorescent protein; BAR, bialaphos resistance protein; small caps PDS, fragment of the soybean *Pds* gene; small caps MK4, fragment of the soybean MAP kinase 4 gene.

construction of BPMV clones carrying soybean gene fragments for VIGS or open reading frames for expression, procedures for biolistic inoculations, storing infected tissue, and rub-inoculation of new plants with sap of plants that were biolistically inoculated earlier.

BASIC PROTOCOL 1

CONSTRUCTION OF BPMV CLONES FOR VIGS APPLICATIONS

This basic protocol describes the steps needed to construct a BPMV RNA2 that can be used only for VIGS of soybean genes. The RNA2 used is pBPMV-IA-V2 [(Zhang et al., 2010), Fig. 1], which carries its cloning site after the stop codon in the viral open reading frame. Thus, a protein cannot be made by the foreign insert. The cloning site incorporates the following restriction enzyme sites: *Bam*HI, *Sal*I, *Stu*I, and *Xho*I, which enable directional cloning of inserts. In this protocol, we provide an example of directional cloning using BamHI and XhoI. Generally, an insert cloned in the antisense orientation in this vector is more effective at inducing silencing of the target gene when compared the same insert cloned into the vector in the sense orientation. The steps in this protocol can be grouped into five main stages: (1) preparation of the RNA2 vector plasmid for ligation, (2) preparation of the

soybean gene fragment for ligation into the BPMV vector, (3) ligation, 4) transformation into *E. coli*, and (5) identification of correct clones and their preparation for biolistic inoculation.

Materials

Plasmid DNA of the infectious BPMV RNA2 clone, pBPMV-IA-V2 (Zhang et al., 2010)
TOP10 Electrocompetent *E. coli* cells and SOC broth
LB broth and plates containing 100 μg/ml ampicillin
Qiagen Plasmid Midi-Prep Kit
*Bam*HI, *Xho*I (*Sal*I and/or *Stu*I, if necessary), 10× restriction buffer, and bovine serum albumin (BSA) (Promega)
Calf intestinal alkaline phosphatase (CIAP)
6× Loading dye (see recipe)
1% agarose gel
1-kb ladder
Qiagen QIAquick Gel Extraction Kit
Soybean genomic DNA or cDNA
Forward and reverse target gene primers diluted to 10 μM (Support Protocol 1)
Qiagen QIAquick PCR Purification Kit
Taq DNA polymerase including 10× PCR buffer and $MgCl_2$ (Invitrogen)
T4 DNA ligase and 10× ligation buffer
MCS primers: MCS_F 5′-CTACAGTTTTTGACATTCTCC-3′; MCS_R 5′-ATAGACAGAGCATACTCAACG-3′

Electroporator
Electroporation cuvettes (0.1 cm gap)
37°C shaking incubator
Nanodrop spectrophotometer
Vortex mixer
Thermal cycler
0.2-ml PCR tubes
15- and 50-ml Falcon tubes
20-μl pipet tips, sterile

Preparation of the BPMV RNA2 vector plasmid for ligation

1. Transform pBPMV-IA-V2 into TOP10 *E. coli* by electroporation. Add 1 ml SOC and transfer to a 15-ml Falcon tube. Incubate 1 hr at 37°C with gentle shaking (200 rpm). Plate 50, 100, and 200 μl of cells on LB plates containing ampicillin (100 μg/ml). Invert the plates and incubate overnight at 37°C.

2. The next morning, pick a single colony and inoculate it into 5 ml LB broth containing ampicillin (100 μg/ml) in a 50-ml Falcon tube, grow for 6 hr at 37°C with shaking at 200 rpm.

3. Transfer 1 ml of culture into 200 ml LB + ampicillin (100 μg/ml) and grow for 18 hr at 37°C with shaking at 250 rpm.

4. Prepare the pBPMV-IA-V2 plasmid by midi-prep. We use the Qiagen Plasmid Midi-Prep Kit. Quantify DNA yield using a Nanodrop spectrophotometer.

5. Digest 5 μg of pBPMV-IA-V2 with *Bam*HI and *Xho*I in a 100 μl reaction as follows (note that the enzymes are added twice):

Sterile water	to volume
10× Restriction buffer	1× final concentration
Dilute BSA (0.1 mg/ml)	1.0 μl – mix by vortexing
pBPMV-IA-V2 DNA	5 μg
*Xho*I (10 U/μl)	2.5 μl
*Bam*HI (10 U/μl)	2.5 μl – mix gently
Incubate at 37°C for 2 hr, then add	
*Xho*I (10 U/μl)	2.5 μl.
*Bam*HI (10 U/μl)	2.5 μl – mix gently

Continue to incubate at 37°C for another 2 hr, or overnight at room temperature. Heat inactivate restriction enzymes for 15 min at 65°C.

6. Treat the digested vector with CIAP. Set up the following reaction:

pBPMV-IA-V2 digestion reaction	105 μl
10× CIAP buffer	1× final concentration
Dilute CIAP (0.01 units/μl)	8.0 μl – mix gently
Incubate at 37°C for 30 min, and then add	
Dilute CIAP (0.01 units/μl)	5 μl – mix gently.

Continue to incubate at 37°C for another 30 min.

CIAP can be added 30 min before taking samples from 37°C or simultaneously along with restriction enzymes.

At this step, it is possible to store the digested product overnight at −20°C before proceeding to the clean-up step.

7. Gel purify the digested pBPMV-IA-V2 product. Add 6× loading dye and run the digestion reaction on a 1% agarose gel alongside a 1-kb ladder and undigested vector DNA as a control to confirm that digestion is complete. The linear digested product is ~7.8 kb. Cut this band from the gel using a razor blade, place the gel slice in a 1.5-ml microcentrifuge tube, and then extract the DNA from the agarose gel slice. We follow the protocol supplied with the Qiagen QIAquick Gel Extraction Kit. Determine the final concentration of the eluted DNA using a Nanodrop spectrophotometer.

Typical yield ranges from 30 to 40 ng of DNA in 27 μl final volume. The pBPMV-IA-V2 is now ready for ligation with PCR products of soybean gene fragments that are also cut with BamHI and XhoI. These PCR products are prepared in steps 8 to 12. For best results, it is recommended to use freshly gel-purified products in ligations. Therefore, it is recommended that steps 1 to 7 be performed in parallel with steps 8 to 12.

Preparation of the soybean gene fragment for ligation into digested pBPMV-IA-V2

8. Use PCR to amplify soybean target sequences for insertion into pBPMV-IA-V2. Set up the following 50-μl reaction in 0.2-ml PCR tubes:

Genomic DNA template (50 ng/μl)	1 to 2 μl

Use soybean genomic DNA unless introns would be present in the desired product. If this is a problem, use soybean cDNA.

9. Thaw and vortex (5 sec) the 10× PCR buffer, $MgCl_2$, dNTPs, and primers (see Support Protocol 1 for primer design). Make a master mix of:

Sterile water	to volume
10× PCR Buffer	1× final concentration
50 mM $MgCl_2$	1.5 mM final concentration
10 mM dNTPs	0.1 mM final concentration
10 μM Forward Target Gene Primer	0.5 μM final concentration
10 μM Reverse Target Gene Primer	0.5 μM final concentration
Vortex for 2–3 sec, then add	
5 U/μl *Taq* DNA Polymerase	0.25 U/10 μl final concentration.

Mix gently and quickly spin down, transfer 49 μl of master mix into PCR tubes containing template (step 8). Tap the tubes to mix. Give a quick spin to ensure the sample is on the bottom of well.

10. Place the tubes in a thermal cycler programmed as follows:

1 cycle:	2 min	94°C (initial denaturation)
35 cycles:	30 sec	94°C (denaturation)
	30 sec	50° to 68°C (depends on the annealing temperature of the primers)
	30 sec	72°C (extension)
1 cycle:	5 min	72°C (final extension)
Final step:		10°C (until taken out of the machine).

11. Add 5 μl of 10× loading dye to the PCR reaction and run 10 μl on a 1% agarose gel to confirm product size and yield.

12. Clean up the PCR product (we use the Qiagen QIAquick PCR Purification kit), and determine DNA yield by Nanodrop spectrophotometer. Typical yield is ~1 μg of DNA in 27 μl final volume.

13. Digest the PCR products to create sticky ends that are compatible with the vector backbone that was prepared in steps 1 to 8. The example here is a directional cloning using *Bam*HI and *Xho*I. Set up the following 40-μl reaction:

PCR product	27 μl
Water	to volume
10× Restriction Buffer	4.0 μl
BSA (0.1 mg/ml)	0.4 μl
Mix by vortexing for 2–3 sec, then add	
*Xho*I (10 U/μl)	2.0 μl
*Bam*HI (10 U/μl)	2.0 μl
Mix gently and then incubate for 2 hr at 37°Cr, then add	
*Xho*I (10 U/μl)	2.0 μl
*Bam*HI (10 U/μl)	2.0 μl
Mix gently and then incubate for 2 hr at 37°C.	

Heat inactivate restriction enzymes for 15 min at 65°C.

Continue with clean up or store the digested PCR products overnight at −20°C.

14. Purify the digested PCR product. We use the Qiagen QIAquick PCR Purification Kit. Determine DNA concentration using a Nanodrop spectrophotometer. Typical DNA concentrations after digestion and PCR purification range from 10 to 15 ng/μl.

At this point, the pBPMV-IA-V2 vector backbone (steps 1 to 8) and the PCR product (steps 9 to 14) have both been digested to have compatible ends.

Ligation and transformation into E. coli

15. Set up the 10-μl ligation reaction as follows. We typically use 100 ng of the vector DNA and an 8:1 molar ratio of insert:vector. For a ~300-bp insert, you will need 31 ng of insert DNA. The amount of insert to be used can be calculated as follows:

ng Vector DNA × kb size of insert/kb size of vector × molar ratio of insert/vector = ng of insert

Vector DNA (~100 ng)	X µl
Insert DNA (0.3 kb, ~31 ng)	Y µl
10× ligation buffer	1× final concentration, thaw and vortex prior to use, check for precipitates.
Sterile water	to volume
Mix by pipetting up and down, then add	
T4 DNA ligase (1 U/µl)	1.0 µl
Mix by pipetting up and down	
Incubate for 3 hr at room temperature or overnight at 4°C.	

The ligation can be confirmed by PCR at this step and also after transformation. To confirm by PCR, proceed to step 16, or to use it in transformation, proceed to step 19.

16. To check the ligation reaction by PCR, set up the following 10-µl reactions.

Ligation reaction	1.0 µl into 0.2-ml PCR tubes
Make the following master mix:	
Sterile water	to volume
10× PCR buffer	1× final concentration
50 mM $MgCl_2$	1.5 mM final concentration
10 mM dNTP mix	0.1 mM final concentration
MCS_F primer (10 µM)	0.5 µM final concentration
MSC_R primer (10 µM)	0.5 µM final concentration
Vortex for 2–3 sec, then add	
Taq DNA polymerase (5 U/µl)	0.25 U/10 µl final concentration
Mix gently, quickly spin down contents of tube, and then transfer 9 µl into PCR tubes.	

These MCS primers flank the cloning site in pBPMV-IA-V2 and can be used for colony PCR and sequencing to confirm insert orientation and sequence. These primers have an annealing temperature of 58°C.

17. Run PCR reactions using the following thermal cycle profile:

1 cycle:	2 min	94°C (initial denaturation)
35 cycles:	30 sec	94°C (denaturation)
	30 sec	58°C (annealing)
	30 sec	72°C (extension)
1 cycle:	5 min	72°C (final extension)
Final step:		10°C (hold until samples are removed from the thermal cycler)

18. Run the PCR products on an agarose gel as follows:

    a. Add 3 µl loading dye to the PCR products.
    b. Load products onto a 1% agarose gel.
    c. Run for 1.5 hr at 90 V.

We recommend including undigested vector as a control. A band of 250 bp is expected if there is no insert, but if ligation is successful a band in the range of 500 bp will also be observed. Proceed to transformation if the correct bands are present.

Transform the ligation reaction into E. coli

19. On ice, thaw tubes of competent cells (50 µl).

In this protocol, we use electroporation of commercially prepared One Shot TOP10 Electrocomp E. coli from ThermoFisher Scientific.

20. Add 1 µl of ligation reaction to the competent cells, gently pipet up and down to mix.

21. Pipet the mixture between metal plates of a pre-chilled 0.1-cm electroporation cuvette and keep on ice.
22. Wipe moisture from cuvette and place in an electroporator; pulse according to manufacturer's protocol for *E. coli.*
23. Immediately add 1 ml of SOC to cuvette.
24. Transfer contents of cuvette into a 15-ml Falcon tube. Place the tube at an angle in a shaker-incubator and shake at 200 rpm for 30 min to 1 hr at 37°C.
25. Spread 25 μl, 100 μl, and 200 μl of transformation onto LB plates containing 100 μg/ml ampicillin and incubate the plates over night at 37°C.

Identification of correct clones and their preparation for biolistic inoculation

26. Perform a 20 μl colony PCR to confirm the presence of pBPMV-IA-V2 carrying the correct insert. Prepare the following master mix containing:

Sterile water	to volume
10× PCR buffer	1× final concentration
50 mM $MgCl_2$	1.5 mM final concentration
10 mM dNTPs	0.1 mM final concentration
10 μM primer MCS_F	0.5 μM final concentration
10 μM primer MCS_R	0.5 μM final concentration.

Vortex 2–3 sec to mix, then add 0.1 μl *Taq* DNA polymerase (5 U/μl).
Mix gently and transfer 20 μl to PCR wells.

27. Pick single colonies from transformation plates with a sterile 20-μl pipet tip. Lightly touch the tip onto an LB plate containing ampicillin (100 μg/ml), then put tip into PCR well. Once all colonies are picked, use a 20-μl pipet to mix by pipetting up and down with each tip. Discard tips into biohazard container.
28. Briefly spin the PCR plate to ensure contents are in the bottom of the well.
29. Run the following PCR program:

1 cycle:	10 min	94°C (initial denaturation)
35 cycles:	1 min	94°C (denaturation)
	30 sec	58°C (annealing)
	1 min	72°C (extension)
1 cycle:	5 min	72°C (final extension)
Final step:		10°C (hold until samples are removed from thermal cycler).

30. Check PCR products for correct size by agarose gel electrophoresis as follows:
    a. Add 3 μl of loading dye to PCR products
    b. Load products on a 1% agarose gel
    c. Run for ~1.5 hr at 90 V.

    Correctly sized products will be in the size range of ~500 bp; empty vector is approximately 250 bp. Proceed with plasmid preps for clones that are correct.
31. Prepare a midi-prep of the pBPMV-IA-V2 clone carrying the desired insert(s) as described above in steps 2 to 4.

    The plasmid DNA is ready for sequencing to confirm the identity of the insert and for biolistic inoculation described in Basic Protocol 2. The MCS_F and MCS_R primers can be used for sequencing across the cloning site.

INOCULATION OF SOYBEAN PLANTS USING BIOLISTICS

The BPMV infectious clones can be efficiently inoculated into soybean plants by the use of a biolistic method to introduce plasmid DNA of the infectious clones into soybean cells. Because the virus is bipartite, it is necessary to bombard seedlings with both the RNA1 and RNA2 DNA clones to initiate infection. The protocol described here was developed for the PDS-1000/He particle delivery system manufactured by Bio-Rad (Hercules, CA) (See Video 1.) All steps are performed at room temperature unless indicated otherwise.

Materials

1-μm Gold microcarriers (see recipe)
Spermidine master mix (see recipe)
pBPMV-IA-R1M [Infectious cDNA of BPMV RNA1, see Zhang et al., 2010)]
pSMV101A (expresses the *Soybean mosaic virus* helper component proteinase to promote transcription of BPMV cDNA clones in bombarded cells)
pBPMV-IA-V2 or pBPMV-IA-2D5C for controls (Zhang et al., 2010)
pBPMV-IA-V2 clones prepared in Basic Protocol 1
100% isopropanol
70% isopropanol
Soybean seedlings at 10 to 14 days after germination with fully expanded unifoliate leaves (Fig. 2A): place plants in the dark 24 hr before bombardment
Drierite desiccant

Vortex mixer
Sonicating water bath
Pipets and pipet tips
Microcentrifuge
Macocarriers and macrocarrier holders (Bio-Rad)
PDS-1000/He particle delivery system (Bio-Rad)
1100 psi rupture disks (Bio-Rad)
High pressure helium tank
Forceps
Stopping screens (Bio-Rad)
Leaf support platform (Fig. 2B)
Course metal screen (Fig. 2C)
Spray bottle
Clear plastic dome
50-ml polypropylene tubes
Cheesecloth
Lyophilizer
Kim wipes

Coat gold particles with DNA

1. Place the tubes containing the plasmid DNAs [pBPMV-IA-R1M, pBPMV-IA-V2 (empty vector as control or clone prepared in Basic Protocol 1), and pSMV101A], freshly prepared spermidine master mix, and prepared gold microparticles on ice.

2. To a microcentrifuge tube add the following components

   2.5 μg pBPMV-IA-R1M construct
   2.5 μg pBPMV-IA-V2 construct (empty vector as control or clone prepared in Basic Protocol 1)
   0.25 μg pSMV101A.

3. Place the tubes on a vortex mixer set at lowest speed.

   It is useful to have a vortex mixer that can hold multiple tubes.

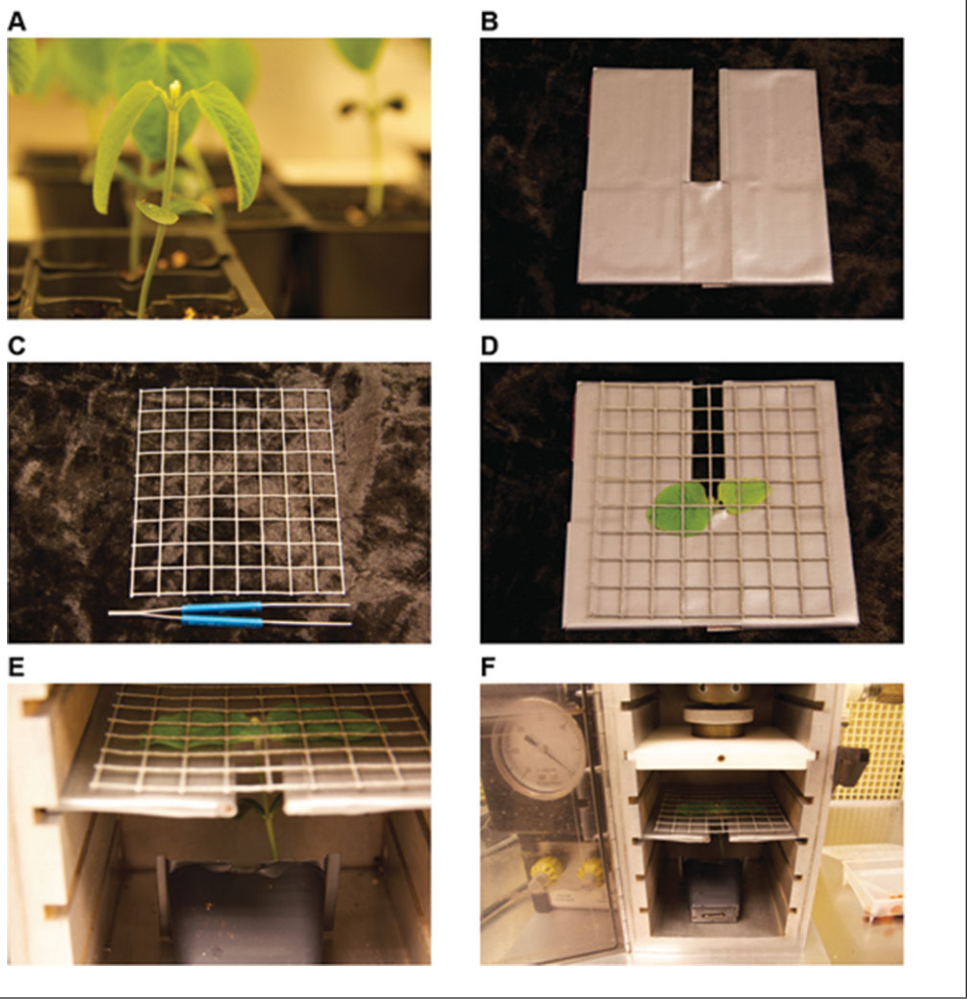

Figure 2 Preparation of soybean plants for biolistic bombardment. (**A**) Dark-treated soybean seedlings with fully expanded primary leaves. (**B**) Leaf support platform. (**C**) Metal screen. (**D**) Depiction of soybean seedling being supported by platform and held flat with the metal screen. (**E**) Close-up image of soybean seedling positioned in the biolistic chamber. (**F**) Image of chamber just prior to biolistic inoculation.

4. Add 25 μl of gold to each tube containing BPMV plasmid DNAs. Using a cut pipet tip, sonicate and pipet gold so that it is well mixed. Keep the tubes vortexing as much as possible while adding the gold, continue to vortex for 1 to 2 min.
5. Add 85 μl of the spermidine master mix to the tubes containing the plasmid DNAs plus the gold, continue to vortex for 1 to 2 min.
6. Centrifuge the tubes for 15 sec at 13,000 × *g*, room temperature, to pellet the gold, remove the supernatant.
7. Add 85 μl of 70% isopropanol to wash the pellet, flick the tubes with finger to mix.
8. Centrifuge the tubes for 15 sec at 13,000 × *g*, room temperature, to pellet the gold, remove the supernatant.
9. Add 85 μl of 100% isopropanol to pellet, DO NOT mix.
10. Centrifuge the tubes for 15 sec at 13,000 × *g*, room temperature, to pellet the gold, remove the supernatant.

11. Resuspend the pellets in 25 μl of 100% isopropanol. Close the tubes immediately to avoid evaporation. This is enough for 3 bombardments.

    The tubes can sit on the bench while preparing macrocarriers, which are plastic disks that the gold (microcarrier) suspensions will be loaded onto in step 14 below.

Prepare the macrocarriers

12. Place the macrocarrier disks into the macrocarrier holders. Need three holders per construct.
13. Sonicate and pipet the samples up and down to mix. The gold can be difficult to resuspend after the DNA is bound.
14. Spread 8 μl of the gold microcarrier suspension evenly onto the center of each macrocarrier. Close the tube between each macrocarrier to avoid evaporation, and sonicate the tube between each macrocarrier to reduce clumping.

Biolistically inoculate the soybean seedlings

15. Label the plants with a tag for each construct.
16. Open the valve on the helium tank and tighten the regulator.
17. Switch the power on to the biolistic chamber and the vacuum pump.
18. Remove the microcarrier launch assembly and the rupture disk retaining cap.
19. Add enough 100% isopropanol to cover the rupture disks.
20. Use forceps to place a rupture disk into the disk retaining cap and tighten onto the fitting in the chamber. Make sure it is tight!
21. Place a stopping screen into the microcarrier launch assembly.
22. Place a loaded macrocarrier holder with **gold side down** onto the microcarrier launch assembly.
23. Slide the microcarrier launch assembly plate under the rupture disk retaining cap.
24. Slide the leaf support into the chamber, we usually use the 4th slot from the top (Fig. 2F).
25. Place the plant in the chamber with leaves on top of support, place a wire screen on top of the leaves to hold them in place (Fig. 2D). Gently position the plant so that the unifoliate leaf surface is in the center of the chamber (Fig. 2E,F).
26. Close the chamber door, and de-pressurize the chamber until it reaches 25 inches Hg, then hold the vacuum.
27. Press and hold the fire button. When the pressure reaches ~1100 PSI, the rupture disk will burst and make an audible "pop."
28. Switch off the fire button, vent the chamber, remove the microcarrier launch assembly and discard the stopping screen, remove the disk retaining cap and discard the remainder of the rupture disk.
29. Remove the plant from the chamber and mist with water using a spray bottle.
30. Repeat these steps until all plants are bombarded, continue to spray plants with water during bombardment.

    You may not always be able to tell if the plant has been bombarded. Sometimes there are holes at the bombardment site. Other times, you can see the wire imprint on the leaves. Prior to bombarding the first plant, it is a good practice to perform a test fire with a

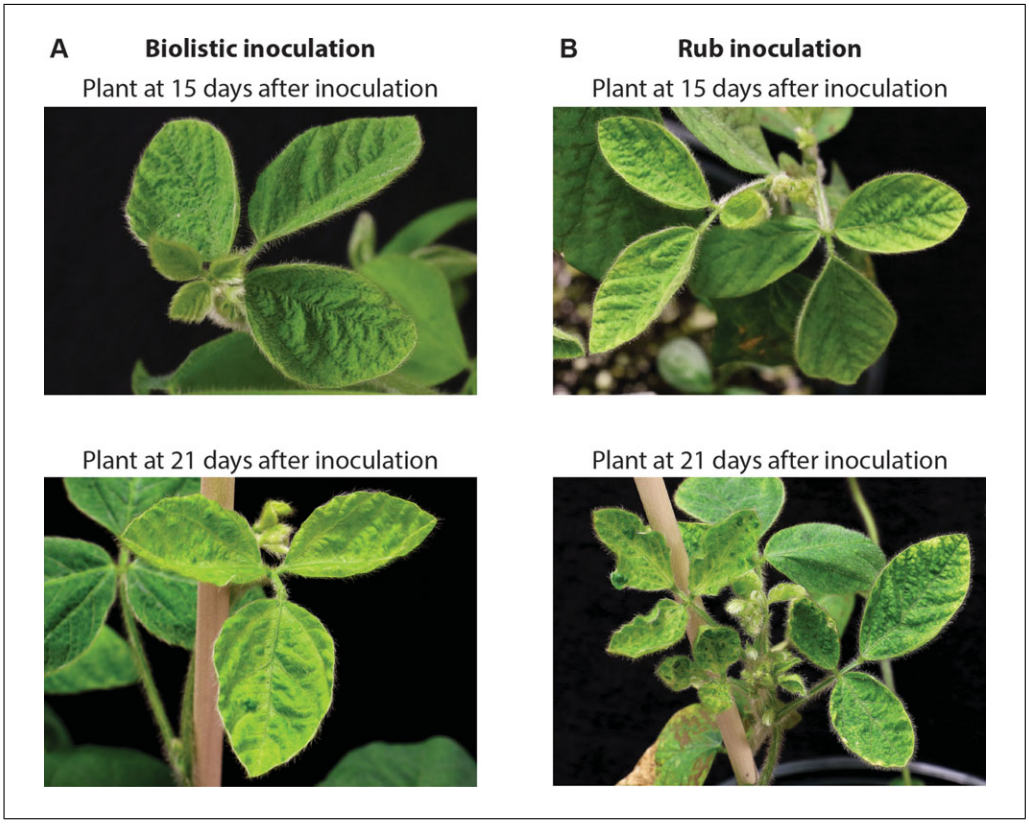

Figure 3 Examples of symptoms of soybean plants after biolistic or rub inoculation. (**A**) Soybean plants at 15 (top) and 21 (bottom) days after biolistic inoculation. (**B**) Soybean plants at 15 (top) and 21 (bottom) days after rub inoculation. Different plants are shown in each panel to provide the reader some sense of the symptom variation that might be expected.

rupture disk but no loaded macrocarrier or plant to ensure that the system is operating as expected.

Growth and care for the inoculated plants

31. Cover the plants with a clear plastic dome for 24 hr to keep the humidity elevated. They should be placed back into the dark.

32. The next day, transplant into larger pots (6- to 8-in. in diameter).

33. Place the bombarded plants into a growth chamber set to no warmer than 22°C with a 16 hr day length and 18°C night.

    The maximum temperature should not exceed 25°C.

34. Continue growing for at least three weeks. Symptoms of viral infection (blistering accompanied by mottled patches of dark and light green) should appear in ~2 to 3 weeks if biolistic inoculation was successful (Fig. 3A). However, the symptoms will vary in their severity depending on the gene that is being silenced and the expected phenotype due to loss of function of the targeted genes(s).

    At this time, plants may be used for experiments, or they can be used as a source of tissue that is collected and stored for future rub inoculations. One bombarded plant can be used to make inoculum for many experimental plants, which is often preferable for VIGS experiments.

Harvesting tissue for storage

35. At four to five weeks post-bombardment, harvest all leaves with viral symptoms and place into 50-ml polypropylene tubes or place leaves between layers of cheesecloth (one construct per layer). Use four layers of cheesecloth to separate different constructs and place corresponding label on each layer.

36. Lyophilize the tissue overnight to dry completely.

37. Place the tissue in a new 50-ml tube with drierite desiccant in the bottom and store indefinitely at −20°C for future rub inoculations.

Place a wadded Kimwipe on top of the drierite to keep it separated from leaf tissue.

ALTERNATE PROTOCOL

CONSTRUCTION OF BPMV CLONES FOR GENE EXPRESSION APPLICATIONS

The BPMV vector system can also be used to express foreign genes. This requires the use of different vectors and cloning strategies to insert protein-coding sequences into the open reading frame of BPMV RNA2. The recombinant clones are constructed in a way that enables insertion of foreign sequences while conserving the viral open reading frame, and this is confirmed by sequencing the clones. This protocol is for gene expression using the BPMV-IA-D35 RNA2, but other published clones, such as BPMV-IA-V3 and BPMV-IA-V5 (Fig. 1) can also be used for gene expression. Our experience is that open reading frames of up to 1.5 kb can be inserted into BPMV, but we have been unsuccessful when attempting to express larger proteins such as GUS, which is encoded by an ~1.8 kb open reading frame. It is important to remember that these clones can also induce gene silencing when they carry sequences that share identity with soybean genes. Therefore, this must be taken into consideration if the goal is to express soybean genes with BPMV in soybean.

Materials

Plasmid DNA of the infectious BPMV RNA2 clone, pBPMV-IA-D35
TOP10 Electrocompetent *E. coli* cells
Qiagen Plasmid Midi-prep kit
Sterile water
*Bam*HI, *Kpn*I, and 10 restriction buffers and bovine serum albumin (BSA) (Promega)
Loading dye
1% agarose gel
1-kb DNA ladder
Template DNA or cDNA for amplifying insert sequence
Primers to amplify foreign coding sequence (Support Protocol 2)
Taq DNA polymerase
Qiagen QIAquick PCR Purification Kit
LB broth and plates containing 100 μg/ml Ampicillin
SOC broth
T4 DNA ligase and 10× ligation buffer
Oligonucleotide primer: R2-1548 F (5′-CAAGAGAAAGATTTATTGGAGGG A-3′); This primer flanks the cloning site in pBPMV-IA-D35 and can be used for colony PCR with a gene-specific reverse primer and sequencing to confirm insert orientation and sequence (Zhang et al., 2010)

Electroporator
Electroporation cuvettes
Vortex mixer

Thermal cycler
Nanodrop spectrophotometer

Prepare vector for ligation with PCR products

1. Transform pBPMV-IA-D35 into TOP10 *E. coli* using electroporation, add 1 ml of SOC, and transfer to a 15-ml Falcon tube. Incubate 1 hr at 37°C with gentle shaking (200 rpm). Plate 50, 100, and 200 μl of cells on LB plates containing ampicillin (100 μg/ml). Invert plates and incubate overnight at 37°C.

2. Make a midi-prep of the plasmid DNA as described in steps 2 to 4 of Basic Protocol 1.

3. Digest 2.5 μg of pBPMV-IA-V2 with *Bam*HI and *Kpn*I in a 100 μl reaction as follows:

Sterile water	75.7 μl
10× restriction buffer	10.0 μl
Dilute BSA	1.0 μl – mix by vortexing
pBPMV-IA-D35 DNA	8.3 μl – mix gently
*Bam*HI (10 U/μl)	2.5 μl
*Kpn*I (10 U/ μl)	2.5 μl – mix gently
Incubate for 2 hr at 37°C, and then add	
*Bam*HI (10 U/μl)	2.5 μl
*Kpn*I (10 U/μl)	2.5 μl – mix gently
Continue to incubate another 2 hr at 37°C.	

At this step, it is possible to store the digested product overnight at −20°C before proceeding to the clean-up step. The star activity of BamHI can necessitate sequential digestion of the vector in the buffers optimized for BamHI and KpnI.

4. Clean up the digested pBPMV-IA-D35 product as follows:

   a. Add loading dye and then run the digestion reaction on a 1% agarose gel alongside a 1-kb ladder and undigested vector DNA as a control to confirm that digestion is complete.

      The linear digested product is ~7.8 kb, and an additional 307 base pair band corresponding to the soybean MPK4 gene fragment that is being replaced should also be visible.

   b. Cut the larger band from the gel and then extract the DNA from the agarose gel slice. We use the Qiagen QIAquick Gel Extraction Kit.
   c. Determine the final concentration of the eluted DNA.

      The pBPMV-IA-D35 is now ready for ligation with PCR products that are also cut with BamHI and KpnI. These PCR products are prepared in steps 5 to 9.

5. Use PCR to amplify foreign sequences for insertion into pBPMV-IA-D35. An example PCR reaction is provided here:

DNA template	1 to 2 μl

DNA concentration must be adjusted depending on the type of template DNA.

Thaw and vortex (5 sec) the buffer, $MgCl_2$, dNTPs, and primers (see Support Protocol 2 for primer design). Make a master mix of:

Sterile water	to volume
10× PCR Buffer	1×
50 mM $MgCl_2$	1.5 mM final concentration
10 mM dNTPs	0.1 mM
10 μM Primer A	0.5 μM
10 μM Primer B	0.5 μM
Vortex, then add	
Taq DNA Polymerase (5 U/μl)	0.25 U/10 μl.

6. Mix gently and quickly spin down, transfer 48 to 49 μl of master mix into wells containing template. Tap the tubes to mix. Give a quick spin to ensure sample is on bottom of well. Place the tubes in a thermal cycler programmed as follows:

1 cycle:	2 min	94°C (initial denaturation)
35 cycles:	30 sec	94°C (denaturation)
	30 sec	58°C (annealing)
	30 sec	72°C (extension)
1 cycle:	5 min	72°C (final extension)
Final step:		10°C (until taken out of the machine).

7. Add 5 μl of 10× loading dye to the PCR reaction and run 10 μl on a 1% agarose gel to confirm product size and yield.

8. Clean up the PCR product (we use the Qiagen QIAquick PCR Purification kit), and determine DNA yield using a Nanodrop spectrophotometer. Nominal yield is ~1 μg of DNA in 27 μl final volume.

9. Digest the PCR products to create sticky ends that are compatible with the vector backbone that was prepared in steps 1 to 4. The example here is a directional cloning using *Bam*HI and *Kpn*I. Set up the following reaction:

PCR product	27 μl
Water	4.6 μl
10× restriction buffer	4.0 μl
BSA (0.1 mg/ml)	0.4 μl
Mix by vortexing for 2-3 sec	
*Bam*HI	2.0 μl
*Kpn*I	2.0 μl
Mix gently and then incubate for 2 hr at 37°C, then add	
*Bam*HI	2.0 μl
*Kpn*I	2.0 μl
Mix gently and then continue to incubate for another 2 hr at 37°C.	
Heat inactivate the restriction enzymes for 15 min at 65°C.	

Continue with clean up or store the digested PCR products overnight at −20°C.

10. Purify the digested PCR product. We use the Qiagen QIAquick PCR Purification Kit. Determine DNA concentration using a Nanodrop spectrophotometer.

At this point, you now have pBPMV-IA-D35 vector backbone (steps 1 to 4) and the PCR product (steps 5 to 9) that have been digested to have compatible ends. They can be ligated in the following steps.

11. Perform the ligation reaction, transformation of competent *E. coli*, and colony PCR to identify clones carrying inserts as described in Basic Protocol 1 steps 15 to 30.

Note that the colony PCR should use oligonucleotide primer R2-1548 F combined with the reverse gene-specific primer or the forward and reverse gene specific primers. Following colony PCR, no band is expected in either reaction if there is no insert (empty vector). If the construct is correct, then a band that is the size of the insert + 270 bp bp will be observed if using R2-1548 F and the gene-specific reverse primers, or the insert if using the forward and reverse gene specific primers.

12. Prepare midi-preps of the pBPMV-IA-D35 clone carrying the desired insert(s) as described in Basic Protocol 1 steps 2 to 4.

The plasmid DNA is ready for sequencing to confirm the identity and orientation of the insert and for biolistic inoculation described in Basic Protocol 2. The oligonucleotide primer R2-1548 F primer can be used to sequence across the cloning site to confirm reading frame and insert sequence.

PRIMER DESIGN FOR AMPLIFYING SOYBEAN GENE FRAGMENTS TO CLONE INTO pBPMV-IA-V2

SUPPORT PROTOCOL 1

This support protocol describes the steps needed to design primers to be used for cloning soybean gene fragments into the pBPMV-IA-V2 vector.

Additional Materials *(also see Basic Protocol 1)*

Soybean target gene list
Computer, monitor, and internet access

1. Obtain soybean gene sequences from Soybase (*http://www.soybase.org*) or Phytozome (*http://www.Phytozome.net*).
2. Design primers from the coding sequence (CDS) or transcript sequence. Double-check the genomic sequence to note intron locations for size.

   If the primer pair spans an intron, then it would be necessary to use cDNA as a template instead of genomic DNA.
3. Align sequences and check for *Bam*HI and *Xho*I cut sites. The sequence should not have either of these, because they will be added to the primers for cloning into the vector.
4. Design primers in conserved sequence regions following these general rules:
   a. Preferentially design from the 3′ most exon of the gene.
   b. The PCR products should be in the 200 to 300 bp size range.
   c. 18 to 22 bp length, avoid long runs of a single base, 54°C to 58°C T_m [using the 2(A + T) + 4(G + C) method], GC content of 40% to 60%, keep duplex formations and hairpins to a minimum.
   d. For maximal silencing, the sequence of the construct should be cloned in the anti-sense orientation in the BPMV-IA-V2 construct to maximize silencing.
   e. Sense orientation will work but anti-sense typically induces greater silencing.
   f. To ensure the anti-sense orientation, always use the CDS or transcript sequence.
5. Add the restriction enzymes (RE) as follows:

*Xho*I to the Forward primer	5′-CTCGAG . . . ..-3′
*Bam*HI to the Reverse primer	5′-GGATCC . . . ..-3′

6. Add 3 to 4 base pairs to the 5′ ends, before the restriction enzyme sites. The addition of a combination of A and T is usually best to keep overall melting temperature lower.

   The final forward primer will contain the XhoI site: 5′-atatCTCGAG[GENE OF INTEREST sequence]-3′. The final reverse primer will contain the BamHI site: 5′-aaatGGATCC[GENE OF INTEREST sequence]-3′.

SUPPORT PROTOCOL 2

PRIMER DESIGN FOR AMPLIFYING OPEN READING FRAMES TO CLONE INTO pBPMV-IA-D35

This support protocol describes the steps needed to design primers to be used for cloning soybean gene fragments into the pBPMV-IA-D35 vector.

Additional Materials *(also see Basic Protocol 1)*

Foreign gene sequences
Computer, monitor, and internet access

1. Design primers from the foreign coding sequence (CDS). For the forward primer, the 5′ end typically starts at the ATG start codon of the foreign sequence if the entire protein is to be expressed. Since the foreign open reading frame will be placed within the context of the viral open reading frame, it is not essential to have an ATG codon. For the reverse primer, design it so that the resulting PCR product will not include the stop codon of the foreign open reading frame. It is essential that no stop codons are introduced with the foreign sequence.
2. Check the sequences to be inserted for *Bam*HI and *Kpn*I cut sites. The sequence should not have either of these, because they will be added to the primers for cloning into the vector. If one or both is present, then it will be necessary to remove them by site-directed mutagenesis.
3. Follow these primer design rules: 18 to 22 bp length, avoid long runs of a single base, 54° to 58°C T_m [using the 2(A + T) + 4(G + C) method], GC content of 40% to 60%, keep duplex formations and hairpins to a minimum.

   It may not be possible to adhere to all these rules, because primer sites are constrained by the sequences at the termini of the foreign coding region.

4. Add the restriction enzyme (RE) sites as follows:

*Bam*HI to the Forward primer	5′-GGATCC . . . . .-3′
*Kpn*I to the Reverse primer	5′-GGTACC[plus one base] . . . . .-3′

   It is necessary to add an extra base between the KpnI sequence and the gene-specific sequence, because the KpnI sequence is not in frame with the viral open reading frame. The additional base will restore the open reading frame.

5. Add 3 to 4 base pairs to the 5′ ends, before the restriction enzyme sites. The addition of a combination of A and T is usually best to keep overall melting temperature lower

   For example, the final forward primer will contain the BamHI site: 5′-atatGGATCC[GENE OF INTEREST sequence starting with the first codon]-3′. Final reverse primer will contain the KpnI site: 5′-aaatGGTACC[plus one extra base][GENE OF INTEREST sequence excluding the stop codon]-3′.

6. Use primer design software and an open reading frame prediction to confirm that the resulting PCR product will not disrupt the viral open reading frame when it is inserted with *Bam*HI and *Kpn*I.

SUPPORT PROTOCOL 3

RUB INOCULATION USING LYOPHILIZED BPMV-INFECTED SOYBEAN LEAVES

It is essential to repeat VIGS experiments multiple times. While it is possible to do this via the biolistic inoculation protocol, the method is time and resource intensive, which limits the throughput. The ~300 bp target gene inserts are usually stable in BPMV RNA2 clones for at least one passage to new plants. Therefore, infected tissue can be harvested from plants that were biolistically inoculated, and this tissue can be stored indefinitely

and used to rub inoculate experimental plants. Rub inoculation is simple and it greatly streamlines the VIGS procedure, and since it is nearly 100% efficient, there is more consistent silencing from plant to plant (see Video 2).

Additional Materials *(also see Basic Protocols 1 and 2)*

Lyophilized tissue from plants infected with recombinant BPMV constructs from Basic Protocol 2
50 mM potassium phosphate buffer, pH 7.0
~10-day old soybean seedlings with fully expanded unifoliate leaves
Carborundum (320 grit) (silicon carbide powder), an abrasive dusted onto soybean leaves just before inoculation

Disposable gloves
Mortar and pestle

1. Grind 25 to 50 mg of dried tissue in 1 to 2 ml of 50 mM potassium phosphate buffer, pH 7.0.
2. Use a cut-off pipet tip to transfer slurry into a 1.5 ml microcentrifuge tube.
3. Spin briefly to clarify the liquid, this leaf sap solution will serve as the inoculum.
4. Lightly dust the soybean leaves with carborundum by gently shaking or tapping the flask as it is inverted over the leaves.

   To make a simple duster, pour several grams of carborundum into a 125-ml Erlenmeyer flask. Cut and layer squares of cheesecloth, and drape them over the top of the flask. Secure the layers of cheesecloth to the mouth of the flask with string or rubber bands, so that the cheesecloth does not come off when the flask is inverted. Cover with foil when not in use to keep free of contaminants.

   The layers of cheesecloth on the flask may need to be adjusted to produce the desired flow of carborundum.
5. Pipet 15 to 20 μl of the leaf sap solution onto the surface of leaves.
6. Use a gloved-finger to rub the drop of inoculum over the leaf surface.

   If leaves appear visibly damaged after rubbing, then the inoculation was too aggressive. Use a new glove each time you switch to a new construct to avoid cross contamination.
7. After all leaves are inoculated, gently rinse inoculated leaves with tap water.
8. Place plants at 20° to 22°C and grow for approximately 3 weeks to observe symptoms (Fig. 3B).

   The vigor of the plants is vital to the outcome of the experiments. Check the soil daily to determine if water is needed, and fertilize at one week intervals with an appropriate fertilizer solution.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps.

Gold carriers for biolistic use

Place 60 mg of gold in a 2-ml tube
Add 1 ml of 100% ethanol
Mix by flicking with a finger, incubate 10 min at room temperature

Centrifuge for 30 sec at 13,000 × *g*, room temperature, to pellet the gold; discard ethanol supernatant

Add 1 ml of sterile water, invert and flick tube to mix

Centrifuge at 13,000 × *g* for 30 sec to pellet the gold; discard the water and repeat this wash step once

Add 1 ml sterile water and let sit until use

Loading dye

30% glycerol
0.25% (w/v) bromphenol blue
0.25% (w/v) xylene cyanol FF
Filter sterilize
Store at 4°C

Spermidine master mix

Add 90 μl of sterile water to a tube containing 10 μl of 1 M spermidine, and mix by vortexing to make a 0.1 M spermidine solution.

To a new tube add the following per reaction:

50% glycerol	50 μl
2.5 M $CaCl_2$	25 μl
0.1 M spermidine	10 μl

Mix by vortexing and place on ice until use. Discard any remaining diluted spermidine.

Prepare enough for 1 extra sample immediately before use.

COMMENTARY

Background Information

Viral vectors are powerful tools for silencing plant genes to test their functions and for transiently expressing genes of interest (Becker, 2013). At least seven different viral vectors have been demonstrated to be useful or potentially useful in soybean for gene silencing and/or gene expression (Whitham et al., 2015). Of these, vectors derived from *Bean pod mottle virus* have been the most widely used. Recombinant, infectious clones of *Bean pod mottle virus* were first shown to be useful for gene silencing and gene expression in 2006 (Zhang and Ghabrial, 2006). This infectious clone fused the T7 RNA polymerase promoter to the 5′ ends of the BPMV RNA1 and RNA2 sequences, and carried a cloning site between the cistrons encoding movement protein (MP) and large coat protein (L-CP) subunit. T7 polymerase is used to generate in vitro RNA transcripts that are rub-inoculated onto soybean leaves (Kachroo and Ghabrial, 2012). The position of the cloning site between the MP and L-CP cistrons necessitates conserving the viral open reading frame so that all BPMV proteins are produced, which are required for systemic infections. This cloning site can be used for gene fragments (no stop codons) needed for silencing soybean genes or for insertion of full open reading frames for gene expression. It is possible to target sequences for silencing that contain stop codons by using site-directed mutagenesis to alter stop codons to preserve the viral open reading frame (Rao et al., 2013).

This protocol is based on another set of BPMV vectors that were developed subsequently by Zhang et al. (Zhang et al., 2010, 2013). These vectors place the BPMV RNA1 and RNA2 cDNAs under control of the *Cauliflower mosaic virus* 35 S promoter and the nopaline synthase terminator. The plasmid DNA of these recombinant BPMV clones can be directly inoculated onto soybean and other legumes to initiate infections. In the plant cell, the 35 S promoter drives transcription of the viral RNA, which expresses BPMV proteins, replicates, and moves systemically. The plasmid DNA can be introduced into plant cells using biolistics, as we have described, or by rub inoculation (Zhang et al., 2010, 2013; Pflieger et al., 2014).

The vectors developed by Zhang et al. (Zhang et al., 2010) utilize different cloning sites and strategies to further expand the capabilities of the BPMV vector system (Fig. 1). The BPMV-IA-V1 and BPMV-IA-V2 (Basic

Protocol 1) were designed specifically for VIGS. The cloning site for foreign inserts was placed immediately after the stop codon of the open reading frame of RNA2. This position is advantageous, because the reading frame is irrelevant. Therefore, it is possible to clone any sequence into the vector without concern for stop codons, which opens the possibility to target non-coding sequences like untranslated regions. Another advantage that was observed by Zhang et al. (Zhang et al., 2010) is that sequences in the anti-sense orientation seem to silence genes more effectively. It is straightforward to make the anti-sense constructs, which often contain stop codons, using the cloning site located after the viral stop codon. A third cloning site in the 5′ untranslated region of the virus was also recently shown to be useful for inserting fragment for VIGS (Ali et al., 2014), but that site has not been incorporated into the vector set described here.

Other vectors developed by Zhang et al. (Zhang et al., 2010) include BPMV-IA-V3, which is primarily for gene expression. This clone carries the P19 open reading frame from *Tomato bushy stunt virus* that can easily be replaced with sequences from genes of interest. BPMV-IA-D35 has been used for silencing and for gene expression (Liu et al., 2011). It contains a different cloning site from BPMV-IA-V3, and it carries a fragment of the soybean MAP kinase 4 (MK4, Glyma16g03670) that induces a very obvious VIGS phenotype. The MK4 fragment is easily replaced by gene fragments for VIGS or open reading frames for viral gene expression. BPMV-IA-V4 is similar to BPMV-IA-V3 in that is carries a cloning site between the MP and L-CP that is used for gene expression, but it also has a second BamHI cloning site immediately after the viral stop codon, which is identical to the BPMV-IA-V1 cloning site. This is intended to be a dual purpose vector that can be used for simultaneous gene expression and gene silencing. BPMV-IA-V5 was designed to enable the simultaneous expression of two open reading frames, and this was demonstrated to work in principle with GFP and the herbicide resistance gene BAR. In our experience, there is a size limitation for inserts in the BPMV vector that is in the range of 1.5 kb, so the multiple reading frames must be small.

This protocol is focused on the use of biolistics to introduce the viral plasmids into soybean cells. However, it is possible to inoculate the plasmids directly onto soybean by means of rub-inoculation (Zhang et al., 2010). In addition, the rub-inoculation of BPMV plasmid DNA has also been shown to be effective in another legume host, *Phaseolus vulgaris* cultivar Black Valentine (Pflieger et al., 2014). For some widely used viral vectors (*e.g., Tobacco rattle virus* and *Potato virus X*), agrobacterium infiltration works very well (Dinesh-Kumar et al., 2003; Lu et al., 2003). This has not been deeply explored for BPMV in soybean, because of the inefficiency of agrobacterium transformation and the difficulties in reliably and reproducibly infiltrating soybean leaves.

Critical Parameters and Troubleshooting

The condition of the plants and their growth conditions are critical. The plants should be healthy and on a regular fertilization schedule. They should be placed in the dark for 24 hr before inoculation. The maximum temperature in which they are grown should not exceed 25°C if being used for VIGS. Control insects and other pathogens, because some of these induce responses in soybean plants that resemble viral infection, and certain beetles such as the bean leaf beetle (*Cerotoma trifurcata*) can transmit the virus. In our experience, cross-contamination due to accidental transmission while tending plants is not a major concern, and this unlikely possibility can be further reduced by segregating infected plants from other soybean plants.

For the biolistic inoculation, it is imperative to properly prepare the gold microcarriers and mix them well to prevent clumping prior to pipetting onto the macrocarrier disks. The BPMV plasmid DNA stocks should be high quality and with a concentration not less than 150 ng/μl.

Biolistically inoculated plants may not exhibit strong viral symptoms. Enzyme-linked immunosorbent assays (ELISAs) can be performed to confirm viral infection. We have successfully used the commercially available Agdia PathoScreen BPMV kit for ELISA (PSA 46400/0480).

It is important to keep in mind that BPMV is a successful pathogen of soybean, and as such, it may affect the physiology of the plant and its interactions with the environment or other organisms. Also, the virus behaves differently in different soybean genotypes. Therefore, it is imperative that pilot experiments and controls are performed to ensure that the virus does not interfere with the phenotype that is being assessed. The system can be optimized by trying different soybean genotypes, adjusting temperature and lighting, or by using different versions of the BPMV RNA1. BPMV-IA-R1

induces milder symptoms that can be difficult to see versus the BPMV-IA-R1M. BPMV-IA-R1 is also less aggressive, which likely will also result in less thorough silencing of the target gene(s).

BPMV VIGS is effective in a variety of tissues (Juvale et al., 2012). VIGS has been demonstrated to occur in leaves, petioles, stems, flowers, and roots. Studying traits in each tissue type requires appropriate experimental design, controls, and optimization. For example, a detailed protocol on the use of VIGS to identify genes involved in soybean cyst nematode resistance has been developed (Kandoth et al., 2013).

The ~300 bp inserts are generally stable in the BPMV vectors, but if silencing of target genes is not observed, then the insert stability should be tested. If the insert is stable but silencing is not observed, then it is also possible to try a different gene fragment.

If using the system for gene expression, keep in mind that there is a size limitation. While this has not been carefully tested, we have not been able to express the GUS open reading frame which is about 1.8 kb. The largest insert that we have expressed to date is on the order of 1.5 kb.

Anticipated Results

By three weeks after biolistic or rub-inoculation, symptoms of viral infection are expected on the third and fourth trifoliate leaves. Quantitative reverse-transcriptase PCR or Northern blots should demonstrate that the target gene(s) is successfully silenced. Most genes in soybean have from two to four nearly identical copies, so these may be silenced as well. At this time, it is appropriate to perform assays on silenced tissues to test the role of the silenced gene.

Time Considerations

The recombinant RNA2 clones take approximately a week to design and build.

The biolistic inoculation is performed in a single day using plants that were placed in the dark the prior day.

Soybean plants are optimal for inoculation at the time that their unifoliate leaves are fully expanded, which is typically at about 10 to 14 days after sowing.

Following inoculation, the systemic infections are expected to occur within 2 to 3 weeks. This can depend on environmental conditions and insert sequence and size. The tissue can be harvested for lyophilization and storage at four to five weeks after bombardment, and can be stored indefinitely.

The rub inoculation using infected leaf material as inoculum can be prepared in a few minutes, and it takes less than a minute per plant. As with the biolistic inoculation, it is recommended to dark treat the soybean seedlings for 24 hr prior to inoculation.

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