Barley Stripe Mosaic Virus-Virus Induced Gene Silencing (BSMV-VIGS) Protocol

Outlined Protocol

i. Germinate and plant wheat seed.
ii. Prepare BSMV plasmids. Determine quality and concentration of plasmid via agarose gel and NanoDrop respectively.
iii. Linearize plasmids via appropriate restriction endonuclease. Check completion of digestion reaction via agarose gel.
iv. Prepare BSMV RNA inoculum from plasmids via *in vitro* transcription using RNA cap structure analog and T7 RNA polymerase. Check completion of transcription reaction via agarose gel.
v. Inoculate 2nd leaf of 10 day old seedlings with BSMV transcripts.
vi. Observe viral infection 8-9 days post BSMV inoculation.
vii. Observe silencing (if visual silencing marker is used) 14 days post BSMV inoculation.
viii. Inoculate with *Puccinia triticina* at point of silencing; 14 days post BSMV inoculation.
ix. Collect tissue for RNA isolation at point of silencing; 14 days post BSMV inoculation.
x. Isolate RNA from sampled tissue via TRIZOL extraction. Digest DNA in RNA samples via DNase I reaction.
xi. Observe *Puccinia triticina* infection 10 days post *Puccinia triticina* inoculation.
xii. Synthesize cDNA via SuperScript III reverse transcriptase.
xiii. Perform Quantitative Real Time Polymerase Chain Reaction (QRT-PCR).

Detailed Protocol

1. **Germinate and Plant Wheat Seed**:
    a. Germinate *Puccinia triticina* resistant (WGRC7) and *Puccinia triticina* susceptible (WICHITA) winter wheat seeds on filter paper in lab. WGRC7 is approximately 24 hours delayed in germination time compared to WICHITA. Select seeds with root radicle at similar point of emergence to equilibrate maturity of plants to be used in inoculations.
    b. Plant germinated seed 4 plants per 5" AZ pot on trays such that plants can be bottom-watered. Label each pot with genotype and transcripts to be inoculated with.
2. **Preparation of BSMV Plasmids:** (estimate for each 20 plants to be inoculated need to prepare 1.25 μg of each α, β, and γ purified plasmid plus 1 μL to run on gel)
    a. Streak plates form each plasmid *E. coli* glycerol stock (LB agar media + 100 mg/L carbinecilin). Incubate plates upside down at 37˚C overnight.
    b. Isolate single colony from each plasmid plate and inoculate a 15 mL overnight culture (LB +100 mg/L carbinecilin) for each plasmid, incubate at 37˚C overnight with shaking at 225-250 rpm.
    c. Purify plasmids using QIAGEN QIAprep Centrifuge Miniprep Kit as per product protocol omitting the addition of RNase A to buffer P1 as this may interfere with *in vitro* transcription.
        i. Determine quality of each plasmid via separation of 1μL of plasmid on 1% w/v agarose gel (circular α, β, and γ migrate at approximately 4000-5000 bp).
        ii. Determine concentration of each plasmid using NanoDrop. Accurate determination of plasmid concentration is necessary for subsequent steps in protocol. The plasmid must by at a concentration of 144-5000 ng/μL for linearization.
3. **Linearize Plasmid via Restriction Endonucleases:** (estimate for each 20 plants to be inoculated need to prepare 8 μL of linearization reaction or 1 μg of linearized plasmid of each α, β, and γ plasmids plus 1 μL to run on gel)
    a. Perform linearization in 20 μL reactions containing:
        2.5 μg plasmid
        6 units of enzyme
        2 μL 10X enzyme buffer
        Nuclease-free H_2O to bring volume to 20 μL

Plasmid	Enzyme	Incubation Temp (˚C)
pα	*Mlu*I	37

pβ	*Spe*I	37
pγ	*Mlu*I	37
pγ-Lr21	*Bss*HII	50
pγ-PDS	*Bss*HII	50

b. Incubate at activation temperature for 90 min; incubate at heat inactivation temperature for 20 min.
c. Check completion of linearization via separation of 1µL product on 1% w/v agarose gel (linear α, β, and γ migrate at approximately 6000-8000 bp).
d. Treat linearized plasmid reaction with RNase inhibitor to prepare for *in vitro* transcription. Use 40 units (1 µL) RNase inhibitor per 20 µL linearization reaction.

4. **Perform *In Vitro* Transcription:** (estimate for each 20 plants to be inoculated need to prepare 20 µL of each α, β, and γ transcripts plus 1 µL to run on gel)
   a. Perform reverse transcription as per product protocol using RNA cap structure analog / rNTP mix and T7 RNA polymerase.
      i. Combine cap analog and rNTP's:
         25 A_{260} units cap analog
         1 mmol rGTP
         2 mmol rATP
         2 mmol rUTP
         2 mmol rCTP
      ii. Perform *in vitro* transcription in 20 µL reactions containing:
         2 µL T7 10X buffer
         1 µL T7 RNA polymerase
         2 µL cap / rNTP mix
         7 µL linearized plasmid
         8 µL nuclease-free H_2O
      iii. Incubate at 37˚C for 2 hours.
   b. Determine completion of transcription on 1% w/v agarose gel by sampling 1 µL of each reaction 2 hours into transcription reaction while continuing incubation until gel has been visualized. There should be a faint band at approximately 10,000 bp and a bright band at approximately 3,000 bp. Any smearing of the smaller band indicates degradation of the RNA transcript.
   c. Combine transcripts α:β:γ in 1:1:1 or 20µL:20µL:20µL ratio and store at -80˚C until inoculation. For each 20 plants to be inoculated need 60 µL of total transcript.

5. **Inoculate 2nd Leaf Of 10 Day Old Seedlings (10 days post planting) With BSMV:**
   a. Prepare sterile inoculation buffer:
      <u>GP Buffer (500 mL)</u>
      18.77 g glycine
      26.13 g K_2HPO_4 dibasic
      Bring to 500 mL with ddiH_2O
      <u>FES Buffer (500 mL)</u>
      100 mL GP buffer
      5 g sodium pyrophosphate decahydrate
      5 g bentonite
      5 g celite
      Bring to 500 mL with ddiH_2O
      Aliquot into 50 mL volumes and autoclave
   b. Use 200 µL wide opening pipette tips.
   c. Organize plants into groups of 20 to be inoculated with the same transcripts.
   d. Inoculate plants keeping transcripts on ice and maintaining RNase-free technique.
      i. Mix FES buffer well by shaking. Add 440 µL of FES to the 60 µL of transcript. Mix buffer and transcripts in pipette tip until homogenous.

ii. Aspirate 23 μL inoculum and place on index finger. Dab inoculum between index finger and thumb 3 times. Holding the plant steady with free hand, draw 3rd leaf between index finger and thumb 3 times starting at the base of the leaf and extending to the tip of the leaf each time. Should hear a squeak.
iii. Mark the plant with toothpick once inoculated.
iv. For each of the remaining 19 plants in group use 23 μL of inoculum. Mix inoculum in pipette tip before each inoculation as components of the FES buffer may settle out of solution.
v. Change gloves and pipette tips for each group of plants, inoculate remaining plants.

6. **Observe Viral Infection Symptoms:**
   a. Viral infection symptoms should be visible on the 3rd leaf 8-9 days post BSMV inoculation.
   b. The leaf that was inoculated (2nd leaf) will appear wounded and will die. The new leaf (3rd leaf) will display infection symptoms visible as yellow modeling or streaking of leaf tissue.
7. **Observe Gene Silencing:** (if visual silencing marker is used) Plants inoculated with PDS should display visible photobleaching on new leaf tissue 14 days post BSMV inoculation. Photobleaching is distinct from viral symptoms in appearance and is denoted by white streaking on leaf.
8. **Inoculate with *Puccinia triticina*:**
   a. Once photobleaching is observed (14 days post BSMV inoculation) inoculate plants with *Puccinia triticina*. Rust inoculation should be done away from control plants.
      i. Suspend ~400μL^3 spores in 10 mL Soltrol 170 immediately prior to inoculation.
      ii. Thoroughly spray spores suspension on to leaf surface using airbrush and fixed air line.
   b. Incubate plants in dew chamber overnight at chamber settings of water 29˚C, wall 11˚C, to create air temperature of 15˚C with high humidity.
   c. Remove plants from chamber and return to greenhouse.
   d. Mist air above inoculated plants each day for several days post *Puccinia triticina* inoculation.
   e. Inoculated plants should display infection spots on 3-4 days post *Puccinia triticina* inoculation.
9. **Collect Tissue for RNA Isolation:**
   a. Once photobleaching is observed and plants are to be inoculated with *Puccinia triticina*, sample 50-100 mg of tissue (~1 inch of young tissue) in 1.5 mL RNase-free tube, flash-freeze in liquid nitrogen.
   b. Store at -80˚C until RNA isolation.
10. **Isolate RNA for QRT PCR**:
   a. Grind tissue in tube with small volume of liquid nitrogen using a sterilized glass rod using a new rod for each sample.
   b. Add 1 mL of TRIZOL to tissue.
   c. Vortex to mix.
   d. Incubate at room temperature for 5 min.
   e. Add 200 μL 100% chloroform.
   f. Invert to mix ~15 times.
   g. Incubate at room temperature for 3 min.
   h. Centrifuge at 12,000 x g at 4˚C for 15 min.
   i. Transfer upper phase (should be clear!!) to new 1.5 mL RNase-free tube containing 500 μL of 100% isopropanol (use room temperature isopropanol to precipitate less salt).
   j. Incubate at room temperature for 10 min.
   k. Centrifuge at 12,000 x g at 4˚C for 10 min.
   l. Pour off supernatant.
   m. Allow RNA to dry at room temp ~5 min.
   n. Elute in 15 μL RNase-free H_2O.
   o. Randomly select 10% of samples to run on 1% w/v agarose check gel to assess the quality of RNA. Four distinct fairly sharp bands should be visible.

p. Check and record precise concentration of each sample via NanoDrop. Dilute RNA to concentration of 1 μg/μL.
q. Store at -80˚C.

11. **Synthesize cDNA:**
    a. Clean up RNA via DNase I digestion as per product protocol.
        i. Perform DNA digestion in 10 μL reactions containing:
            1 μL (1μg) RNA
            1 μL DNase I
            1 μL 10X DNase I buffer
            7 μL nuclease-free H_2O
        ii. Vortex to mix, centrifuge and incubate at room temperature for 15 min.
        iii. Add 1 μL 25 mM EDTA to stop reaction.
        iv. Incubate at 65˚C for 10 min to inactivate enzyme.
    b. Perform reverse transcription using SuperScript III reverse transcriptase as per product protocol.
        i. To each 10 μL DNase I digestion reaction add:
            1 μL Olig(dT) WL175 (50μM)
            1 μL 2.5 mM dNTP mix
        ii. Vortex to mix and incubate at 65˚C for 5 min.
        iii. Centrifuge to collect.
        iv. To each 13 μL reaction add:
            4 μL First Strand Buffer
            1 μL 0.1M DTT
            1 μL (40 units) HPRI RNase inhibitor
            1 μL SuperScript III reverse transcriptase
        v. Mix in pipette tip.
        vi. Incubate at 50˚C for 60 min.
        vii. Incubate at 70˚C for15 min.

12. **Reverse Transcriptase Quantitative PCR (RT-qPCR):**
    a. Perform RT-qPCR amplification analysis using gene specific primers. Test primer amplification efficiency using four 1/5 serial dilutions of a single cDNA sample. Mix water and cDNA first then prepare PCR master mix. Prepare 3 replicates of 25 μL for each dilution.
        i. Prepare reactions in 25 μL reactions containing:
            12.5 μL 2X Sybr Green Supermix
            1 μL forward primer 5-10 pmol/μL (5-10 mM)
            1 μL reverse primer 5-10 pmol/μL (5-10 mM)
            1 μL cDNA
            9.5 μL nuclease-free H_2O

            Alternatively prepare 3 replicates of 15 μL for each dilution. Some primer pairs tolerate the smaller reaction mix while others do not.
        ii. Prepare reactions in 15 μL reactions containing:
            7.5 μL 2X Sybr Green Supermix
            0.6 μL forward primer 5-10 pmol/μL (5-10 mM)
            0.6 μL reverse primer 5-10 pmol/μL (5-10 mM)
            0.6 μL cDNA
            5.7 μL nuclease-free H_2O
        iii. Seal plate with film being careful not to touch the surface. Spin plate to remove any bubbles at the liquid surface.
        iv. Run the primer efficiency test using the thermocycling profile:
            Cycle 1 (1x) 95˚C 5 min
            Cycle 2 (40x) 95˚C 30 sec
            55˚C- 60˚C 30 sec
            72˚C 50 sec

Cycle 3 (1x) 95˚C 1 min
Cycle 4 (1x) 55˚C 1 min
Cycle 5 (81x) 55˚C 10 sec (increase set point after Cycle 2 by 0.5˚C)

b. Once primer efficiency is established perform cDNA concentration test starting with the cDNA dilution that resulted in Ct of 25 in primer efficiency test. Dilute a small volume of each cDNA sample preparing 2 replicates for each cDNA using a housekeeping gene.
   i. Prepare reactions in 25 µL reactions containing:
      12.5 µL 2X Sybr Green Supermix
      1 µL forward primer 5-10 pmol/µL (5-10 mM)
      1 µL reverse primer 5-10 pmol/µL (5-10 mM)
      1 µL cDNA
      9.6 µL nuclease-free H_2O
      Alternatively prepare 3 replicates of 15 µL for each dilution. Some primer pairs tolerate the smaller reaction mix while others do not.
   ii. Prepare reactions in 15 µL reactions containing:
      7.5 µL 2X Sybr Green Supermix
      0.6 µL forward primer 5-10 pmol/µL (5-10 mM)
      0.6 µL reverse primer 5-10 pmol/µL (5-10 mM)
      0.6 µL cDNA
      5.7 µL nuclease-free H_2O
   iii. Seal plate with film being careful not to touch the surface. Spin plate to remove any bubbles at the liquid surface.
   iv. Run the primer efficiency test using the thermocycling profile:
      Cycle 1 (1x) 95˚C 5 min
      Cycle 2 (40x) 95˚C 15 sec
      55˚C- 60˚C 15 sec
      72˚C 50 sec
      Cycle 3 (1x) 95˚C 1 min
      Cycle 4 (1x) 55˚C 1 min
      Cycle 5 (81x) 55˚C 10 sec (increase set point after Cycle 2 by 0.5˚C)
   i. Dilute cDNA to that of the lowest Ct set.

c. Perform transcript abundance analysis for each gene of interest. Prepare replicates for each cDNA.
   v. Prepare reactions in 25 µL reactions containing:
      12.5 µL 2X Sybr Green Supermix
      1 µL forward primer 5-10 pmol/µL (5-10 mM)
      1 µL reverse primer 5-10 pmol/µL (5-10 mM)
      1 µL cDNA
      9.7 µL nuclease-free H_2O
      Alternatively prepare 3 replicates of 15 µL for each dilution. Some primer pairs tolerate the smaller reaction mix while others do not.
   vi. Prepare reactions in 15 µL reactions containing:
      7.5 µL 2X Sybr Green Supermix
      0.6 µL forward primer 5-10 pmol/µL (5-10 mM)
      0.6 µL reverse primer 5-10 pmol/µL (5-10 mM)
      0.6 µL cDNA
      5.7 µL nuclease-free H_2O
   vii. Seal plate with film being careful not to touch the surface. Spin plate to remove any bubbles at the liquid surface.
   viii. Run the primer efficiency test using the thermocycling profile:
      Cycle 1 (1x) 95˚C 5 min

Cycle 2 (40x) 95˚C 30 sec
55˚C- 60˚C 30 sec
72˚C 50 sec
Cycle 3 (1x) 95˚C 1 min
Cycle 4 (1x) 55˚C 1 min
Cycle 5 (81x) 55˚C 10 sec (increase set point after Cycle 2 by 0.5˚C)

RT-qPCR Primer Sequences:
Act1F: AAATCTGGCATCACACTTTCTAC
Act1R: GTCTCAAACATAATCTGGGTCATC
PDSF: TGTCTTTAGCGTGCAAG
PDSR: GATGATTTCGGTGTCACT
Lr21(2)F: GAACGAAGATGACGAACAA
Lr21(2)R ATGAGCCGGTACTAAAGGTC