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Chapter 10

Investigating Gene Function in Cereal Rust Fungi by Plant-Mediated Virus-Induced Gene Silencing

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Abstract

Cereal rust fungi are destructive pathogens, threatening grain production worldwide. Targeted breeding for resistance utilizing host resistance genes has been effective. However, breakdown of resistance occurs frequently and continued efforts are needed to understand how these fungi overcome resistance and to expand the range of available resistance genes. Whole genome sequencing, transcriptomic and proteomic studies followed by genome-wide computational and comparative analyses have identified large repertoire of genes in rust fungi among which are candidates predicted to code for pathogenicity and virulence factors. Some of these genes represent defence triggering avirulence effectors. However, functions of most genes still needs to be assessed to understand the biology of these obligate biotrophic pathogens. Since genetic manipulations such as gene deletion and genetic transformation are not yet feasible in rust fungi, performing functional gene studies is challenging. Recently, Host-induced gene silencing (HIGS) has emerged as a useful tool to characterize gene function in rust fungi while infecting and growing in host plants. We utilized *Barley stripe mosaic virus*-mediated virus induced gene silencing (BSMV-VIGS) to induce HIGS of candidate rust fungal genes in the wheat host to determine their role in plant–fungal interactions. Here, we describe the methods for using BSMV-VIGS in wheat for functional genomics study in cereal rust fungi.

Key words Virus-induced gene silencing, VIGS, Host-induced gene silencing, HIGS, Functional genomics, Wheat rust fungi, Barley stripe mosaic virus, *Puccinia* gene silencing

1 Introduction

Wheat production is severely affected by rust fungi, belonging to the genus *Puccinia*, despite continued efforts in understanding rust fungus epidemiology, in breeding for resistance, and chemical control [1]. Recent advances in structural genomics of *Puccinia* species have shed some light on various aspects of their complex lifestyle with their cereal hosts. Genome sequence data for the three *Puccinia* species that attack wheat, namely leaf or brown rust (*P. triticina*), stem or black rust (*P. graminis*) and stripe or yellow rust (*P. striiformis*), are now available [2–4]. The generation of these genomic resources, and their computational and comparative analyses

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have laid the groundwork allowing the prediction of a wide array of genes [2, 5–9]. However, the strict obligate biotrophic nature of rust fungi and their recalcitrance to genetic transformation precludes the application of most commonly available genetic methods to study the biological function of these genes. Recently, an RNA interference (RNAi)-based concept called host-induced gene silencing (HIGS) has emerged as an effective tool to characterize gene function in biotrophic fungi [10–13]. The concept behind this method is the downregulation of the target gene transcript in the colonizing fungus by the uptake of siRNAs/dsRNA produced by the host plant expressing hairpin RNA (hpRNA) specific to the targeted fungal gene sequence [10]. The silencing of genes that are vital for the pathogen can ultimately have a major effect on phenotypic outcomes, such as altered growth morphology or disease suppression. We have demonstrated that HIGS induced by *Barley stripe mosaic virus*-mediated virus-induced gene silencing (BSMV-VIGS) is a robust approach for high-throughput functional genomics analysis of candidate genes in rust fungi [11].

The VIGS system is a powerful forward and reverse genetics tool for creating transient gene knockdown phenotypes from which gene function can be inferred and is particularly useful for species which are difficult to transform genetically [14, 15]. The mechanism of VIGS is based on the fact that plants defend themselves against invading viruses which act as a trigger to induce RNA-mediated gene silencing [16]. By inserting a fragment of a gene of interest into the viral RNA genome, transcripts of this gene fragment are also targeted for degradation during the defence response of plant, resulting in the downregulation of the corresponding gene by sequence-specific posttranscriptional gene silencing [17]. This leads to a reduction or in some cases the complete abolition of target gene function, which in turn can result in phenotypic changes. Compared with other reverse genetics approaches for associating genes with traits, VIGS provides a quick functional assessment or validation of candidate genes. VIGS is well established for studying plant–pathogen interactions in dicotyledonous plants, but the development of new viral vectors based on BSMV has expanded its utility to monocotyledonous plants such as wheat [18, 19]. BSMV is a single-stranded RNA virus of the genus *Hordeivirus* that infects many monocot species important to agriculture [20]. It has a tripartite positive sense genome, consisting of three RNAs termed α, β, and γ which are required for infection [21]. The BSMV-VIGS system has been successfully implemented for functional characterization of genes required for disease resistance in wheat and barley [18, 19, 22, 23]. Recently, we utilized BSMV as a vector to induce RNAi in wheat leaves for silencing wheat leaf rust fungus *P. triticina* genes involved in pathogenesis [11]. In this chapter we provide the protocol for performing HIGS in rust fungi using BSMV-VIGS.

2 Materials

2.1 Construction of Recombinant BSMV γRNA Vector

1. BSMV γ vector DNA.
2. Restriction enzymes: PacI, NotI.
3. *Escherichia coli* (*E. coli*) DH5α transformation competent cells.
4. Ampicillin.
5. Agarose.
6. Plasmid DNA extraction kit.
7. Luria-Bertani (LB) media (liquid and agar plates): To prepare 1 l LB: Add 10 g bacto tryptone, 5 g yeast extract, and 10 g NaCl to 800 ml of distilled H_2O. Dissolve and adjust pH to 7.0 with NaOH. Adjust volume to 1 l and sterilize by autoclaving. This can be stored at room temperature. For solid media, add 15 g of Bacto agar per liter and autoclave.
8. 1 kb DNA ladder.
9. 10× Tris–borate–EDTA (TBE) gel electrophoresis buffer: To prepare 1 l 10× TBE: Dissolve 121.1 g Tris base, 61.8 g boric acid, and 7.2 g EDTA in 800 ml of RNAse-free H_2O. Make up to 1 l and autoclave. This can be stored for 6 months at room temperature. Dilute with sterile distilled H_2O (dH_2O) to make 1× working solution.
10. Gel DNA extraction Kit.
11. TE buffer (10 mM Tris–HCl, 1 mM EDTA).
12. T4 DNA Ligase.

2.2 Preparation of BSMV In Vitro Transcription Reactions

1. BSMV α, β, and γ plasmids.
2. Restriction enzymes: MluI, SpeI, and BssHII.
3. mMESSAGE mMACHINE® High Yield Capped RNA Transcription Kit.
4. RNase inhibitor.

2.3 Plant Inoculation with Viral Transcripts

1. Seeds of wheat (*Triticum aestivum*).
2. Square Dura Pots (3.5″) and germination trays.
3. Standard germination soil (substrate no. 1) and potting soil (no. 3) for plant growth.
4. 10× Glycine Phosphate (GP) buffer: Dissolve 18.77 g Glycine and 23.13 g of K_2HPO_4 (dipotassium phosphate) in 500 ml dH_2O and autoclave.
5. FES inoculation buffer. To prepare 250 ml FES: Dissolve 2.5 g sodium pyrophosphate, 2.5 g Bentonite , 2.5 g Celite in 50 ml of 10× GP buffer. Bring volume to 250 ml with ddH_2O and autoclave.
6. BSMV α, β, and γ in vitro RNA transcripts.

3 Methods

3.1 Preparation of BSMV Plasmids and Construction of Recombinant γ RNA Vector

1. Streak *E. coli* glycerol stocks carrying BSMV plasmids α, β, and γ on LB agar plates supplemented with ampicillin (100 mg/l) and culture overnight at 37 °C.
2. Isolate a single colony from each α, β, and γ plasmid plate and inoculate a 20 ml overnight LB culture containing Ampicillin (100 mg/l) at 37 °C with constant shaking (200–250 rpm).
3. Carry out plasmid extraction using a plasmid miniprep kit as per product instructions (*see* **Note 1**). Check the quality of each plasmid by running 1 μl of the eluted product on a 1% w/v agarose–TAE gel. Determine the concentration of each plasmid using a spectrophotometer (e.g., NanoDrop).
4. Select a candidate fungal gene (*see* **Note 2**) and PCR-amplify a segment of the gene-of-interest (GOI) using gene-specific forward and reverse primers harboring an NotI and a PacI restriction site, respectively (*see* **Note 3**).
5. Digest 5 μg of PCR-amplified product of the GOI with NotI enzyme. After digestion, run a sample on a 1.5% (w/v) agarose–TAE gel (*see* **Note 4**) along with a DNA ladder of appropriate size markers to confirm the expected size. Excise the desired DNA fragment from the gel and purify the DNA using the gel DNA extraction kit (*see* **Note 5**); elute the NotI digested PCR segment using TE buffer. Measure the concentration using a spectrophotometer and check the integrity and purity of the eluted fragment by running (1–2 μl) on a 1% agarose–TAE gel. Now, digest this fragment (1–5 μg) with the second (PacI) enzyme. Perform gel electrophoresis to analyze the result of this restriction digest reaction. Elute the DNA from the desired, excised gel band using the gel DNA extraction kit. Measure the concentration of the eluted DNA using a spectrophotometer and check the integrity by running (1–2 μl) on 1% agarose–TAE gel.
6. Similarly, treat the BSMV γ vector with PacI and NotI restriction enzyme to create compatible ends at the multiple cloning sites for cloning of the PacI- and NotI-digested fragment of the GOI.
7. Set up the ligation reaction using a molar vector to insert ratio of 1:3 (*see* **Note 6**). Ligate the restriction enzyme digested BSMV γ vector and segment of the GOI with 1 μl T4 DNA ligase and 2 μl 10× T4 DNA Ligase buffer overnight at 16 °C in a total volume of 20 μl.

8. Transform the ligated mixture into transformation competent *E. coli* DH5α as per the manufacturer instructions. Incubate at 37 °C for 1 h by shaking at 200 rpm. Spread the transformation mixture (10–100 μl) onto a LB agar plate supplemented with ampicillin (100 mg/l), and incubate at 37 °C overnight.
9. Next day, pick 10–15 colonies (*see* **Note 7**) and start 5 ml LB + ampicillin (100 mg/l) cultures for 16 h at 37 °C. Use 4 ml of bacterial culture to extract plasmid DNA and store the remaining at 4 °C under sterile conditions.
10. Set up a diagnostic restriction enzyme digest with Not1 and PacI to determine which plasmid contains the desired fragment of the GOI in the correct orientation (*see* **Note 8**). After identifying the correct construct, make a master plate using the remaining stored culture and carry out a large-scale preparation of plasmid DNA (*see* **Note 9**). Freeze your clone by adding 15% glycerol and storing at −80 °C.

3.2 Germinate Wheat Seeds

Germinate wheat seeds in 3.5″ Square Dura pots containing standard germination soil at 25 °C with 16 h light and 8 h dark period with 74 μmol/m^2s light intensity and 55–65% relative humidity. Label each pot with transcripts to be inoculated.

3.3 Preparation of In Vitro Transcripts

1. The α, β, and γ plasmid vectors are linearized by restriction enzyme digestion with MluI, SpeI, and BssHII, respectively, and used as templates for in vitro transcription using the mMessage and mMachine transcription kits, following the manufacturer instructions.
2. Run 1 μl of each of the digested plasmids on a 1% agarose–TAE gel to confirm that linearization is complete (*see* **Note 10**; Fig. 1). Having confirmed complete digestion, inactivate the reaction by heating at 65 °C for 20–30 min (*see* **Note 11**).
3. Treat each linearized plasmid reaction with RNAse inhibitor to prepare for in vitro transcription. Use 40 units RNAse inhibitor per 20 μl linearized plasmid reaction.
4. Set up the in vitro transcription reaction using the mMESSAGE mMACHINE® High Yield Capped RNA Transcription Kit following the manufacturer protocol and incubate at 37 °C for 2 h (*see* **Note 12**). Determine completion of transcription by running 1 μl of each reaction with 9 μl of RNase free H_2O and 10 μl of loading dye provided in the mMessage and mMachine transcription kit (*see* **Note 13**). A successful in vitro transcription reaction should yield intact bands and any smearing indicates degradation of the RNA transcripts (*see* **Note 14**).

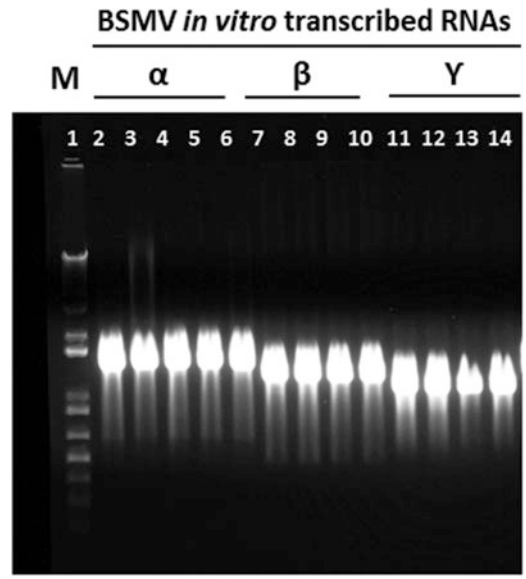

Fig. 1 Gel image of in vitro-synthesized RNA transcripts from linearized BSMV plasmid templates. *Lane 1*, 1 kb DNA ladder: *Lanes 2–6*, BSMVα in vitro-transcribed (IVT) RNAs; *Lanes 9–10*, BSMV β IVT RNAs; *Lanes 11–14*, BSMVγ IVT RNAs. 1 μl of IVT full-length RNA product loaded in each lane. Plasmids α, β, and γ linearized with restriction enzymes MluI, SpeI, and BssHII, respectively. Transcription reaction run on 1% agarose–TAE gel

3.4 Plant Inoculation with Viral Transcripts

1. For BSMV inoculation, combine the three transcripts (α, β, and γ) in equimolar ratio (1:1:1) using 1 μl of each in vitro-transcribed RNA in 22.5 μl of inoculation buffer (FES).
2. Apply the freshly prepared inoculum (transcript mix in FES buffer) on the first leaf of 10 day-old wheat seedlings using a pipette. Gently hold the base of the leaf with one hand and, while firmly holding it between the thumb and index finger of your other hand, rub the surface of the leaf with the inoculation mixture from the base to the tip in a single motion. Repeat the process one or two times as required (*see* **Note 15**).
3. Keep inoculated plants in the growth chamber at 25 °C with 16 h light–8 h dark cycle.

3.5 Symptom Observations and Fungal Inoculations

1. In wheat, BSMV symptoms can be seen as yellow mottling or small streaks on the leaves at 7–8 days post inoculation (dpi) (*see* **Note 16**; Fig. 2).
2. Once BSMV symptoms are observed, plants are challenged with rust urediniospores at 10 dpi and observed for disease or growth phenotype (*see* **Note 17**). Rust inoculations should be done away from the control plants. Thoroughly spray urediniospores suspended in Soltrol 170 on to the leaf surface using an airbrush.

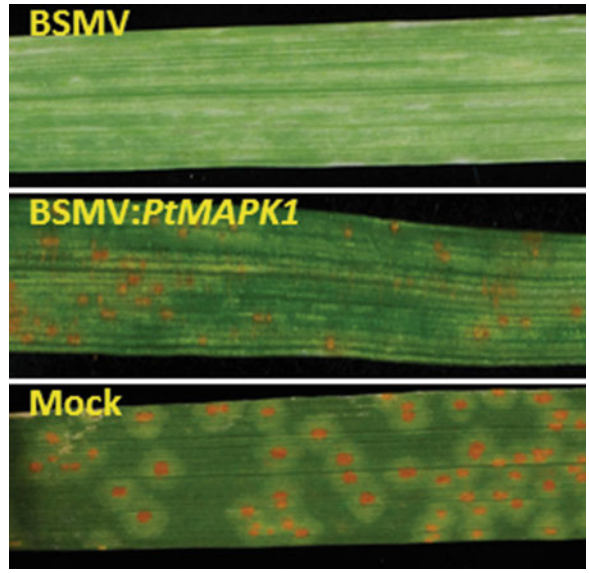

Fig. 2 Wheat plant inoculated with BSMV vector alone and derivative of recombinant γ vector carrying *P. triticina PtMAPK1* gene segment. Wheat plants inoculated with empty vectors showing typical BSMV symptoms of white mottling, spotting, and streaking in leaves (*upper panel*). Plants inoculated with BSMV vectors harboring *P. triticina* gene show disease suppression (*middle panel*) whereas FES treated (mock) controls are heavily infected with fungus (*lower panel*). Photographs were taken 10 days after fungal inoculation

3. Incubate plants in a dew chamber with near 100% relative humidity overnight. Next day, remove plants from the dew chamber and return to the growth chamber. For wheat leaf rust (*P. triticina*), inoculated plants will display discolored infected spots strating from 4 to 5 days post urediniospore inoculation, depending on the pathogen isolate used. For molecular analyses, rust fungus-challenged wheat tissues can be harvested at different time points as desired by experiments.

4 Notes

1. Avoid using RNase A during BSMV plasmid preparation as it may interfere with in vitro transcription. Any residual RNase will degrade the in vitro-transcribed RNAs produced from these plasmids.
2. HIGS relies on careful selection of fungal gene sequence as to avoid off-target RNAi effects. Different gene fragments can show variability in VIGS experiments. Fragments of 300–1500 bp are maintained in the γ-genome and have been

used successfully to induce VIGS. Silencing efficiency is reduced by shorter fragments, whereas longer inserted fragments run a strong risk of being lost from the recombinant virus.

3. It is recommended to use cDNA as template for the PCR amplification of the candidate fungal gene as it has no noncoding sequences. Alternatively, the fragment with the flanking restriction enzyme sites is synthesized.
4. Ethidium bromide used to stain nucleic acids in agarose gel is a carcinogen and should be handled carefully. Always wear disposable gloves when working with ethidium bromide. Anything coming in contact with it must be handled as a hazardous waste and disposed of accordingly.
5. PCR-amplified product can also be purified using any standard PCR Clean-UP System following the manufacturer instructions.
6. It is generally recommended to use 100 ng of total DNA in a standard ligation reaction. When setting up the ligation reaction, make sure to include a positive control (vector without insert) and a negative control (vector DNA without T4 DNA ligase) in parallel. This will provide information on how much background level of uncut or self-ligating recipient plasmid backbone is present. The plate with the ligated mixture should contain more colonies as compared with the control plates.
7. Pick colonies depending on the number of background colonies on the control plate. The higher the background, the more colonies need to be checked.
8. It is highly recommended that the selected positive clones are sequenced to confirm the presence of the correct insert.
9. All reagents and materials used should be nuclease-free to avoid degradation of RNAs during in vitro-transcript preparation.
10. Partial linearization will result in production of less viral RNA by T7 RNA polymerase. It is therefore important that complete restriction digestion is obtained for optimum results.
11. Alternatively, restriction enzyme digestion can be followed by DNA purification since any contamination in the digestion reaction may inhibit subsequent transcription. If using phenol–chloroform extraction, add one volume of phenol–chloroform–isoamyl alcohol (25:24:1) to the digested sample and vortex thoroughly by hand for 30 s. Centrifuge at room temperature for 5 min at 16,000 $\times$ *g* and carefully transfer the upper aqueous phase to a new tube. Precipitate the linearized plasmid by adding one tenth of a volume of ammonium acetate (5 M concentration) and two volumes of absolute ethanol and storing at −80 °C for 1 h. Collect the pellet by centrifuging at

16,000 × *g* for 15 min at 4 °C. Remove the supernatant and wash the DNA pellet with 70% ethanol, air-dry, and resuspend in TE to achieve a concentration of approximately 1 μg/μl.

12. Amplification of the transcribed RNA may require removal of any DNA by addition of 1 μl of DNase (supplied in the kit) and further incubation at 37 °C for 15 min.
13. The in vitro-transcribed product can be checked by running on standard 1% agarose–TAE gel. However, as you are dealing with RNA, make sure that the gel running buffer and electrophoresis unit is free of RNase contamination. Use gloves and filter-pipette tips when working with RNA.
14. Any smearing of the bands indicates degradation of the RNA transcripts. RNAs can be stored short term at −20 °C and for longer at −80 °C.
15. Do not damage the leaf by squeezing it too hard or applying too much force. Label each plant after inoculation to separate it from non-inoculated plants. When using two or more different constructs, make sure to change gloves after each application to prevent cross-contamination.
16. For BSMV-based VIGS in wheat, the apparent virus phenotype can usually be observed 10 days post-infection The timing of onset and region of initial silencing can vary between different genetic backgrounds. Since VIGS induces viral symptoms, an empty virus vector-infected plant has to be included as a negative control in each experiment.
17. If the target fungal gene is essential for the fungus, then HIGS will result in altered fungal growth in the host plant and/or a changed disease phenotype (*see* Fig. 2). The silencing phenotype obtained in experiments might indicate a possible function of the target gene. However, repeats to obtain reproducible phenotypes are often desirable. Silencing efficiency can differ from plant to plant even if all conditions are adjusted and standardized.

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