Virus-Induced Gene Silencing (VIGS) for Functional Characterization of Disease Resistance Genes in Barley Seedlings

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Abstract

With the recent advances in sequencing technologies, many studies are generating lists of candidate genes associated with specific traits. The major bottleneck in functional genomics is the validation of gene function. This is achieved by analyzing the effect of either gene silencing or overexpression on a specific phenotypic or biochemical trait. This usually requires the generation of stable transgenic plants and this can take considerable time. Therefore any technique that expedites the validation of gene function is of particular benefit in cereals, including barley. One such technique is Virus-Induced Gene Silencing (VIGS), which evokes a natural antiviral defense mechanism in plants. VIGS can be used to downregulate gene expression in a transient manner, but long enough to determine its effects on a specific phenotype. It is particularly useful for screening candidate genes and selecting those with potential for disease control. VIGS based on Barley Stripe Mosaic Virus (BSMV) is a powerful and efficient tool for the analysis of gene function in cereals. Here we present a BSMV VIGS protocol for simple and robust gene silencing in barley and describe it to evaluate the role of the hormone receptor *BRI1* (*Brassinosteroid Insensitive 1*) in barley leaf resistance to *Fusarium* infection.

Key words

Virus-Induced Gene Silencing (VIGS)
Post-Transcriptional Gene Silencing (PTGS)
Barley Stripe Mosaic Virus (BSMV)
Cereal
Barley
Silencing
Leaf detachment assay
Disease
Functional genomics
BRI1 (*Brassinosteroid Insensitive 1*)

1. Introduction

Virus-induced gene silencing (VIGS) is a simple and powerful tool that can be used to silence a gene in a relatively rapid time frame [1, 2]. VIGS is based on the natural phenomenon of RNA interference (RNAi), also known as post-transcriptional gene silencing (PTGS), that is induced as an antiviral response in plants [1]. PTGS is activated by the detection of double-stranded RNAs which are processed by the DICER enzyme into small interfering RNA (siRNA) molecules, 21–24 nucleotides in length [1, 3]. These siRNAs, in complex with ARGONAUTE proteins, form the RNA-induced silencing complex (RISC) that cleaves the target mRNA [4]. PTGS was first discovered in transgenic petunia plants in which the overexpression of chimeric chalcone synthase with the endogenous gene resulted in the reversible co-suppression of homologous genes [5, 6]. PTGS is now used widely to study gene function, either via the generation of stable RNA interference (RNAi) lines or by transiently expressing a fragment of the target gene using VIGS. The former has the advantage of the long-term availability of transgenic lines, while VIGS is more rapid, economic and eliminates the laborious transformation process. Indeed, VIGS is particular useful when used as a tool to screen and delineate the best target genes for a particular trait from a pool of candidate genes. The VIGS technique involves the insertion of a small fragment from the gene to be silenced into viral genome vectors. Subsequently corresponding viral RNAs are produced and applied to plants to induce the PTGS mechanism, which results in silencing of the target gene [7, 8, 9]. This technique has been applied in both monocots and dicots for the functional characterization of various genes involved in plant development and disease resistance [8, 9, 10].

Tobacco Mosaic Virus (TMV) derived VIGS vectors were the first gene silencing vectors employed for gene silencing in plants and these were used to silence the phytoene desaturase (*PDS*) gene in tobacco [8]. Potato Virus X was then developed which had a better silencing

ability, and was used to silence genes in both *Nicotiana benthamiana* and *Solanum lycopersicum* [1, 11]. Thereafter, other gene silencing vectors were developed from various plant viruses [12, 13, 14, 15]. The main disadvantages of these virus vectors were the narrower range of host plants that they could infect. For cereals, effective silencing vectors were developed from Barley Stripe Mosaic Virus (BSMV). BSMV is a positive-sense single strand RNA virus. Its tripartite genome consists of RNAs α, β, and γ [16]. The BSMV genome was modified as a gene silencing vector for use in barley and wheat [17].

BSMV vectors have been used as versatile tool to study disease resistance genes in wheat and barley. In wheat, Scofield et al. [18] reported the use of the BSMV system to silence the *lr21* (nucleotide binding leucine rich receptor) resistance gene in wheat, which conferred an increased susceptibility to leaf rust disease. BSMV-based silencing of *TaChlH* (magnesium chelatase subunit H) in wheat enhanced susceptibility to the causal agent of Septoria Tritici Blotch disease, *Zymoseptoria tritici* [19]. BSMV has also been used to confirm the role of genes in barley defense against pathogens. Silencing of barley *Sgt1* (suppressor of G-two allele of *Skp1*) and *Hsp90* (heat shock protein) compromised *Mla13*-mediated resistance toward the biotrophic mildew pathogen *Blumeria graminis* f. sp. *hordei* [20]. Silencing of barley chorismate synthase (*HvCS*), anthranilate synthase α subunit 2 gene (*HvASa2*), and chorismate mutase 1 (*HvCM1*) genes resulted in increased *B. graminis* penetration into epidermal cells of barley carrying mildew resistance locus a (*Mla*) [21]. BSMV-based silencing is not limited to seedlings but also can be used in adult plants to study the role of genes in disease resistance [18, 22, 23, 24].

The protocol we describe here has been successfully used in barley to study the role of *BRI1* in defense against *Fusarium culmorum*. This pathogen is responsible for yield loss in barley and other small-grains, attacking roots, stems and heads of cereal plants. In detached leaf assays, the silencing of *BRI1* using BSMV vectors in barley cv. Akashinriki enhanced *F. culmorum*-induced necrosis in leaves [22]. In this article we show the methodology used to silence the targeted gene in barley seedlings using BSMV vectors. The same protocol can be used to silence genes in barley heads and in wheat leaves and head tissue.

2. Materials

2.1. Plant and Growth Conditions

1. Barley seeds (cv. Akashinriki) (kindly provided by Dr. K. Sato, Barley Germplasm Centre, Okayama University, Japan).
2. Filter paper.
3. Circular Petri dish, 90 mm.
4. Leukopor (BSN medical, UK).
5. John Innes compost No-2.
6. Three inch diameter pots.
7. Plant growth room or chamber (22 °C, 16/8: light/dark, 70% humidity).

2.2. Amplification of Plant Gene Fragments and Cloning into BSMV Vectors

1. DNA extraction kit (E.Z.N.A.® HP Plant DNA Kit, USA, Cat. No. D2485-02).
2. *Escherichia coli* strain DH5α.
3. Platinum *Taq* DNA polymerase (Thermo Fisher Scientific, USA, Cat. No. 10966026).
4. dNTPs.
5. PCR machine.
6. Agarose.
7. Solis BioDyne 1 Kb marker (Solis BioDyne, Estonia, Cat. No. 07-12-00050).
8. Safeview (NBS-Biologicals, UK, Cat. No. NBS-SV1).
9. QIAquick PCR purification kit (Qiagen, Germany, Cat. No. 28104).
10. NanoDrop™ 1000 Spectrophotometer (Thermo scientific, USA).
11. pGEM-T easy cloning kit (Promega, UK, Cat. No. A1380).
12. Plasmid vectors: pα42 (pα), pβ42.sp1 (pβ), pSL038-1 (pγ), pSL039-PDS (pγPDS) [2].
13. Luria-Bertani (LB) liquid broth (Sigma-Aldrich, USA, Cat. No. L3152) and LB solid media containing 1.2% (w/v) agar.

14. Ampicillin (100 mg/mL stock concentration).
15. Shaking incubators for cultures.
16. Plasmid isolation kit (Qiagen, Germany, Cat. No. 27106).
17. Restriction enzymes: PacI, Sma1, MluI, SpeI.
18. Ethanol.
19. Sodium acetate.
20. Tris–Borate–EDTA 10× buffer.
21. X-Gal (Sigma-Aldrich, Cat. No. B4252) and Isopropyl thio β-D-galactopyranoside (IPTG) (Sigma-Aldrich, USA, Cat. No. I6758).

2.3. Digestion of BSMV Plasmids and In Vitro Transcription

1. BSMV vectors (α, β, γ and modified γ) (Kindly provided by Prof. Steven R. Scofield).
2. Plasmid isolation kit (Qiagen, Germany, Cat. No. 27106).
3. Sodium acetate.
4. Ethanol.
5. EDTA.
6. mMessage mMachine T7 in vitro transcription kit (Ambion, USA, Cat. No. AM1344).

2.4. Inoculation of Plant RNA and Buffers

1. 5× GP buffer (0.5 M glycine, 0.3 M K_2HPO_4 dibasic).
2. FES buffer (100 mL 5× GP buffer, 22 mM sodium pyrophosphate decahydrate, 27.7 mM bentonite, 166 mM celite, bring final volume to 500 mL *see* **Note 1**).
3. Glycine.
4. K_2HPO_4 dibasic.
5. Sodium pyrophosphate decahydrate.
6. Bentonite (Aldrich, USA, Cat. No. 28,523-4).
7. Celite (Fluka, USA, Cat. No. 22141).

2.5. Production of *Fusarium* Spores

1. *Fusarium culmorum* (Strain FCF200, kindly supplied by Dr. Paul Nicholson, John Innes Centre, Norwich, UK).
2. PDA (Scharlau, Spain, Cat. No. 01-483-500).
3. Incubator.
4. Cork borer.
5. Mung beans.
6. Autoclave.
7. Glass ware.

8. Cheese cloth (from any grocery store).
9. Mung bean broth: 20 g Mung beans were boiled in 250 mL of distilled water for 20 min. The liquid was passed through a cheese cloth and diluted to 1 L with distilled water. 25 mL of the broth was transferred to 100 mL flasks and autoclaved for 15 min at 121 °C.
10. Centrifuge.
11. Tween 20.
12. Hemocytometer (Hycor Biomedical, USA, Cat. No. H2-LN-87144-PK).
13. Microscope (Olympus CH30, UK).

2.6. Gene Silencing Confirmation

1. Tungsten beads (Qiagen, Germany, Cat. No. 69997).
2. Tissue lyser II (Qiagen, Germany, Cat. No. 85300).
3. RNeasy Mini Kit (Qiagen, Germany, Cat. No. 74104).
4. Oligo(dT) 12-18 Primer (Invitrogen, USA, Cat. No. 18418012).
5. SYBR Green Master Mix (Takara, Japan, Cat. No. RR420A).
6. qPCR machine (Agilent Mx3000P, USA, Cat. No. 401512).

2.7. Detached Leaf Assay and Image Analysis of Infected Area

1. Petri dishes (100 × 100 × 20 mm).
2. Plant agar (Duchefa, Netherlands, Cat. No. P1001).
3. Benzimidazole (Sigma-Aldrich, USA, Cat. No. 194123).
4. Glass Pasteur pipette.
5. Leukopor® (BSN medical, UK).
6. Growth room.
7. Camera (Nikon coolpix p500, Japan).
8. IMAGE-J software (NCBI).

2.8. Other Consumables

1. Gloves.
2. Sterile culture tubes (Falcon, USA, Cat. No. 352057).
3. Micro-pipettes (Gilson, France).
4. Centrifuge tubes (Sarstedt, Germany, Cat. No. 72.695.500).
5. Gel imaging system (Fusion *Fx*, Vilber lourmat, Denmark).
6. Gel apparatus (Helixx Mupid-exU, USA).
7. Autoclaved ddH_2O.
8. Dry heating block (Thermo Fisher Scientific, USA, Cat. No. 11-473-70).

2.9. Software and Databases

1. Primer3 software (version 0.4.0; http://frodo.wi.mit.edu/primer3/).
2. Ensembl plants database (http://plants.ensembl.org/index.html).
3. GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

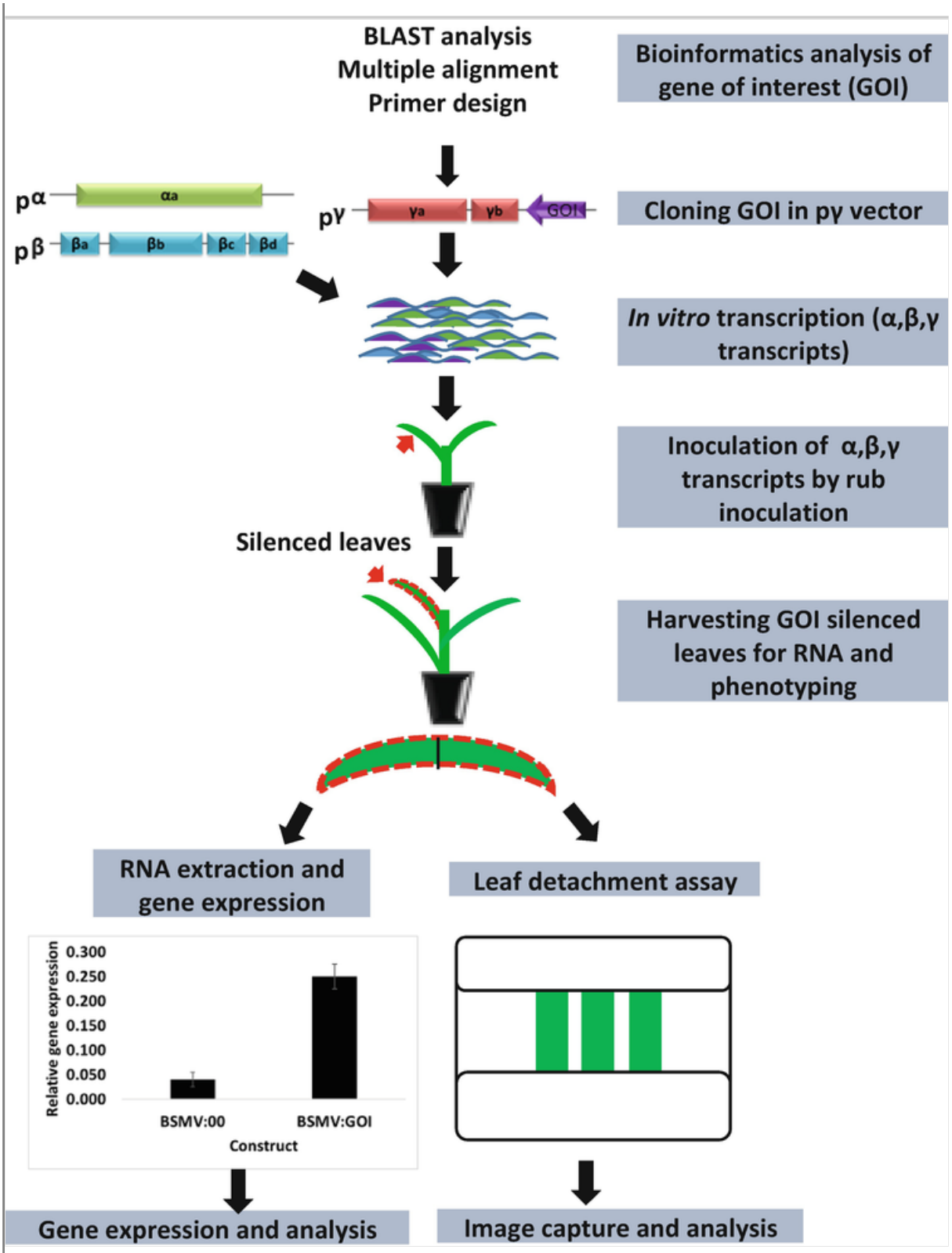
3. Methods

3.1. Overview of VIGS Metholology

1. Figure 1 shows an overview of the VIGS methodology. It overviews the steps involved from fragment selection through to the production of the RNA used to inoculate plants with the BSMV virus encoding the fragment that targets the specific gene for silencing.

Fig. 1

Schematic representation of VIGS methodology and the detached leaf assay for the functional characterization of disease resistance genes in cereals. The tripartite genome (α,β,γ) of Barley Stripe Mosaic Virus (BSMV) was cloned into plasmids. A fragment of the gene of interest (GOI) was amplified from barley genomic DNA and subsequently cloned into the pγ vector. The plasmids carrying α, β, γ genomes were linearized and their respective transcripts were produced by in vitro transcription. Transcripts were suspended in FES buffer and rub-inoculated onto the first leaf of seedlings. The third leaf of inoculated plants was harvested and used for both gene expression studies and a detached leaf assay, in order to, respectively, evaluate the efficacy of gene silencing and role of the GOI in disease resistance. Empty vector treatment (BSMV:00) and gene of interest silenced plants (BSMV:GOI)



3.2. Seed Germination and Plant Growth Conditions

1. Barley seeds were placed in 9 cm diameter Petri dishes containing two pieces of filter paper soaked with 6 mL of sterile double-distilled water (ddH_2O).
2. Plates were sealed with Leucopore tape and placed in the dark, for 2 days at 4 °C in order to stratify the seed, followed by 2 days incubation at 21 °C to facilitate germination.
3. Etiolated seedlings were transferred to 3 in. pots containing John Innes compost No 2; two seedlings per pot.
4. Plants were grown in a contained glasshouse where the temperature is 22 °C with a 16 h light/8 h dark photoperiod and 70% relative humidity.

3.3. Preparation of Fungal Conidial Production

1. Mycelium from glycerol stocks of *F. culmorum* FCF 200 (−80 °C) was subcultured on potato dextrose agar (PDA) plates and incubated at 25 °C for 7 days in the dark.
2. For conidia (asexual spore) production, three to four PDA plugs of 10 mm diameter were taken from these PDA cultures using a cork borer and these were used to inoculate 25 mL of Mung bean broth.
3. The flasks were kept in shaking incubator at 200 rpm, 25 °C for 5 days in dark.
4. The conidial suspension was passed through two layers of sterile cheese cloth into 50 mL centrifuge tubes and the filtrate was centrifuged at room temperature, 5000 rpm. The liquid was discarded and the spore pellet was washed three times with sterile ddH_2O.
AQ2
5. Conidia were resuspended in 0.02% (v/v) Tween20 and the concentration was determined using disposable hemocytometer (KOVA) slides.
6. The conidial concentration was adjusted to 1×10^6 spores/mL using 0.02% Tween20.

3.4. Selection of the Gene Target Regions for Both VIGS Fragments and VIGS Validation via qRT-PCR

1. A short exonic fragment of 120–500 bp is sufficient to initiate gene silencing by PTGS in plants [2]. Insert length influenced stability but not efficiency of VIGS and silencing is transient in most cases [25].
2. If the user aim is to silence an entire family of genes, then the gene silencing fragments need to be homologous to the most conserved exonic region of the gene family sequences. When a specific gene is the target, the most variable region should be used for gene silencing. The silencing fragments should be in the range of 120–500 bp in length for better stability in plants [18, 25, 26] and note that genes with a minimum homology of 85% are likely to be downregulated by VIGS [8, 26].
3. The gene fragment must target an exon. If amplifying the target sequence from gDNA extracts, it is also important to verify that the primers do not span introns. Therefore, it is important to compare genomic and mRNA gene sequence to locate introns.
4. At the gene fragment selection stage, one must try and ensure the fragment does not have any off target gene silencing potential. Thus it is important to analyze by BLAST analysis of the gene fragment sequence against the barley genome to determine its homology with other sequences (*see* **Note 2**).
5. The chosen fragment should not contain an Mlu1 restriction site, as this enzyme is later used to cut the vector into which the fragment is cloned. Also the silencing fragments should not contain the restriction sites that are used for cloning into γ vector.
6. The efficacy of VIGS was verified using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. At the gene fragment design stage, it is important to concurrently select the primers to be used for qRT-PCR in order to ensure that they do not overlap with the gene silencing fragment.
7. Its good practice to use two non-overlapping independent gene fragments to make sure a phenotype is due to the specific silencing of the target gene by using VIGS.
8. The empty vector γ was used as a negative control for the BSMV VIGS experiments and BSMV carrying a 185-bp fragment of the barley phytoene desaturase (*PDS*) gene which causes photo bleaching when silenced was used as positive control [17].

3.5. Amplification of Gene Silencing Fragments, Sequencing, and Digestion

1. Genomic DNA of barley cv. Akashinriki was isolated using the E.Z.N.A.® HP Plant DNA Kit and the concentration was measured using a Nano Drop. Concentration of the genomic DNA was then adjusted to 30 ng/μL with ddH_2O. Two independent, non-overlapping gene fragments of the barley *BRI1* (*Brassinosteroid Insensitive 1*) (Gen bank ID: AB109215.1) were amplified from genomic DNA using gene-specific primers sets BRI1-A or BRI1-B (listed in Table 1). Pac1 and Sma1 restriction site sequences were, respectively, added to the 5′ end of the forward and reverse primers.
2. PCR was performed in a 10 μL reaction containing 0.5 U Taq DNA polymerase and 1× PCR buffer (Invitrogen, UK), 1.5 mM $MgCl_2$, and 125 mM of each of forward and reverse primers (BRI1-A/B) with 1.25 units of Taq DNA polymerase with 30 ng barley genomic DNA. PCR was performed in a Peltier thermal Cycler DNA engine and the PCR program consisted of an initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s and a final extension step at 72 °C for 5 min.
3. 5 μL of PCR product was visualized on a 0.8% (w/v) agarose gel and remaining 45 μL of PCR product was purified using QIAquick PCR purification kit according to the manufacturer instructions, with the final product being eluted into 30 μL of sterile ddH_2O (*see* **Note 3**).

4. The concentration of DNA in the eluent was checked using the NanoDrop.

5. The fragments were cloned into the pGEM-T easy vector using the pGEM-T Easy cloning kit and following the manufacturer's instructions.

6. 10 µL of the ligation mixture was transformed into 20 µL of high efficient *E. coli* DH5α competent following the manufacturer's instructions. The transformed cells were spread on LB-agar plates containing 100 µg/mL of Ampicillin, 20 mg/mL X-gal, and 100 mM of IPTG. The plates were incubated overnight at 37 °C.

7. The clones were screened for inserts via colony PCR, using a pinhead amount of cells suspended in 5 µL of ddH_2O as template. The PCR mix comprised of 10 µL reaction containing 0.5 U Taq DNA polymerase and 1× PCR buffer (Invitrogen, UK), 1.5 mM $MgCl_2$, and 125 mM of each of M13 forward and reverse primers (Table 1) in a total volume of 25 µL. The PCR program consisted of an initial denaturation step at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 68 °C for 45 s, and a final extension step at 72 °C for 5 min.

8. The PCR product was visualized on a 0.8% agarose gel, and the positive clones were inoculated into 5 mL of LB broth supplemented with 100 µg/mL (w/v) of ampicillin, and cultures were incubated in an orbital shaker at 200 rpm, 37 °C, overnight.

9. Plasmids were extracted from the cultures using the QIAprep plasmid extraction kit according to manufacturer's instructions. The final elution was performed in a 30 µL of sterile ddH_2O.

10. pGEM-T clones carrying gene fragments were sequenced using M13 sequencing primers at Macrogen Inc. (Korea) (*see* **Note 4**).

11. Verified plasmids carrying gene fragments were double digested with Pac1 and Sma1. The digestion reaction consists of 2 µg of vector, 1× NEB cut smart buffer, 20 units of Pac1 and 20 units of Sma1 and ddH_2O to a final volume of 50 µL. The reaction mixture was first incubated at 37 °C for 2 h and then at 25 °C for 2 h. Restriction digestion was performed on a dry heating block.

12. The digested product was run on a 1% (w/v) agarose gel, and the required insert was excised from the gel and purified using the QIAquick Gel Extraction Kit according to manufacturer's instructions. The final elution was performed in 30 µL sterile ddH_2O and eluent was stored at −20 °C (*see* **Note 5**).

Table 1

Primers used

Primer code	Primer sequence (5′ to 3′)	Application* AQ3
BRI1-A for	CGATTAATTAAGCGGAGGCAGAAGAATGA	Cloning VIGS construct BSMV:BRI1-A
BRI1-A rev	CGACCCGGGGTCACCCTGGCCACTCAC	
BRI1-B for	CGATTAATTAAGTGAGTGGCCAGGGTGAC	Cloning VIGS construct BSMV:BRI1-B
BRI1-B rev	CGACCCGGGTGGATGATGTGCGGAATG	
BRI1:RT for	CAACGATGCTCAAGGTGATG	BRI1-specific qRT-PCR primers
BRI1:RT rev	CCGGTGGTCATCTTCCTAAT	
RNAH:RT for	GCACAGGGAATCGTCAAAGT	RNAH-specific qRT-PCR primers
RNAH:RT rev	TCAAAACAACACAACATCGAAGT	
pSL038 for	TGATGATTCTTCTTCCGTTGC	Sequencing primers for pSL038
pSL038 rev	TGGTTTCCAATTCAGGCATCG	
M13 for	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing primers for pGEM-T
M13 rev	AGCGGATAACAATTTCACACAGGA	

3.6. Cloning of the Gene Silencing Fragments into the pSL038-1 that Encodes the BSMV Gamma Genome

1. Barley stripe mosaic virus (BSMV) has a tripartite RNA genome, and the three genome fragments from strain ND18 were cloned into ampicillin-resistant plasmids (a modified gamma-b genome) [17].

2. The BSMV gamma genome vector pSL038-1 was double digested with the restriction enzymes Pac1 and Sma1 in same way as

described above in Subheading 3.5, **step 11** (*see* **Note 6**).

3. The digested sample was purified using QIAquick Gel Extraction Kit by following the manufacturer's instructions, and the final elution was performed in 30 μL sterile RNase-Free water.

4. The purified VIGS fragments were ligated into the Pac1 and Sma1 digested pSL038-1 vector. The ligation mixture comprised 1 μg of linearized vector, 0.5 μg of purified insert, 1× ligation buffer, 25 units of T4 ligase and the final volume was made up to 10 μL using sterile ddH_2O. The samples were incubated at 16 °C, overnight. All the ligation mixture was transformed into *E. coli* DH5α by following the manufacturer's instructions. The transformed cells were spread on LB-agar plates containing 100 μg/mL of Ampicillin. The plates were incubated at 37 °C, overnight.

5. The resulting colonies were screened for inserts by colony PCR as described in Subheading 3.5, **step 7** with an exception that the primers used were specific to pSL038-1 (Table 1). The verified clones were sent for sequencing at Macrogen Inc. (Korea) using the pSL038-1 primers.

6. The verified clones containing either pSL038-1-BRI1-A (pγBRI1-A) or pSL038-1-BRI1-B (pγBRI1-B) were stored in 20% glycerol at −80 °C.

3.7. Preparation of BSMV Vector Plasmids

1. *E. coli* clones carrying ampicillin-resistant plasmids encoding either the BSMV RNA genomes α, β, γ (pα, pβ, and pγ), pγPDS [18], pγBRI1-A, or pγBRI1-B were cultured.

2. Glycerol stocks of each clone was streaked on LB-ampicillin (100 μg/mL, w/v) plates and incubated at 37 °C, overnight. A single colony was picked and inoculated in 5 mL LB broth supplemented with 100 μg/mL (w/v) of ampicillin and incubated overnight at 200 rpm, 37 °C.

3. Plasmid isolation was performed using Qiagen QIAprep Miniprep Kit as per the protocol; RNaseA was excluded from the buffer P1 in the first step and isolated plasmids were stored at −20 °C (*see* **Note 7**).

3.8. Linearization and Purification of BSMV Vector Plasmids

1. The plasmids pα, pγ, pγPDS, pγBRI1-A, and pγBRI1-B were linearized with the restriction enzyme Mlu1, while pβ was linearized with Spe1. All the restriction enzyme digestions were performed with NEB enzymes, following the manufacturer's instructions. The digestion reaction consists of plasmid DNA (2 μg), 1× NEB cut smart buffer, 20 units of either Mlu1 or Spe1; the final volume was adjusted to 50 μL using RNase-free water (*see* **Note 8**).

2. Plasmid linearization was verified by visualizing the digestion products (5 μL) via agarose gel electrophoresis (1% w/v agarose gel). Once complete digestion was confirmed, enzymes were heat-inactivated by incubating the reaction at 65 °C for 20 min.

3. The linearized plasmids were purified by ethanol precipitation. The digested plasmids mixture was mixed with 0.1× volume of 3 M sodium acetate pH 5.2 and 2× volumes of 100% (v/v) ethanol. Precipitation was facilitated by incubating the mixture at −20 °C for 15 min.

4. The precipitation reactions were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet was washed with 750 μL of 70% (v/v) ethanol and centrifuged for 10 min at 13,000 rpm, 4 °C.

5. The pellet was air-dried and dissolved in 20 μL of RNase-free water. The concentration of DNA was determined using the NanoDrop.

3.9. Preparation of Viral RNA by In Vitro Transcription

1. RNA was transcribed from the viral genomes using the purified linear plasmids as template and the mMessage mMachine™ T7 in vitro transcription kit, following manufacturer's instructions. Reactions contained linearized plasmid DNA (0.5 μg) 1× buffer, 5 units of in vitro transcription enzyme mix, 1× NTP/CAP and RNase-free water to a total volume of 20 μL.

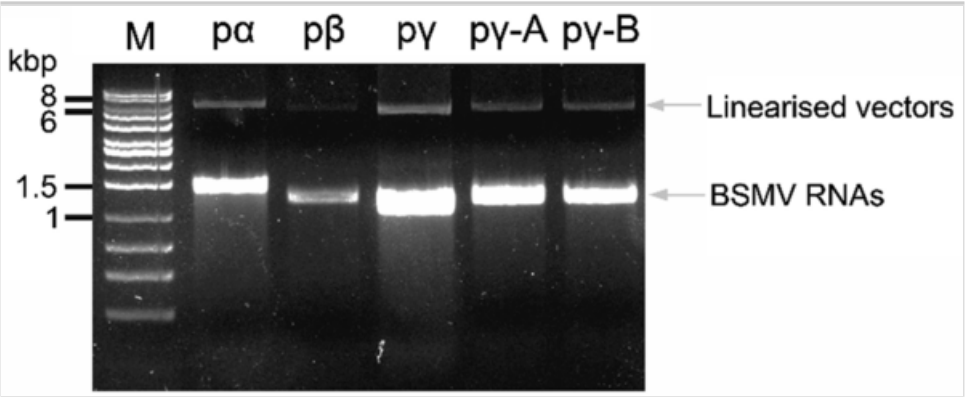
2. The samples were incubated at 37 °C for 2 h and the quality of transcribed RNA was visualized by running 1 μL of each in vitro transcription reaction (diluted to 10 μL with RNase-free 6× gel loading buffer) through a 1% (w/v) agarose gel (Fig. 2). All the RNA samples were stored at −80 °C until further use (*see* **Note 9**).

3. For each plant to be treated, 0.35 μL of each RNA transcript (α, β, and relevant γ genome transcript) was required (based on the number of plants to be treated the reaction volume for in vitro transcription can be scaled up) (*see* **Note 10**).

Fig. 2

Agarose gel electrophoresis of in vitro transcription products using linearized BSMV vectors pα, pβ, pγ, pγBRI1-A (pγ-A), and pγBRI1-B (pγ-B). DNase treatment was not performed on the samples; the linearized BSMV vectors can be seen above the RNAs,

both highlighted with arrows. (M) Solis BioDyne 1 Kb marker



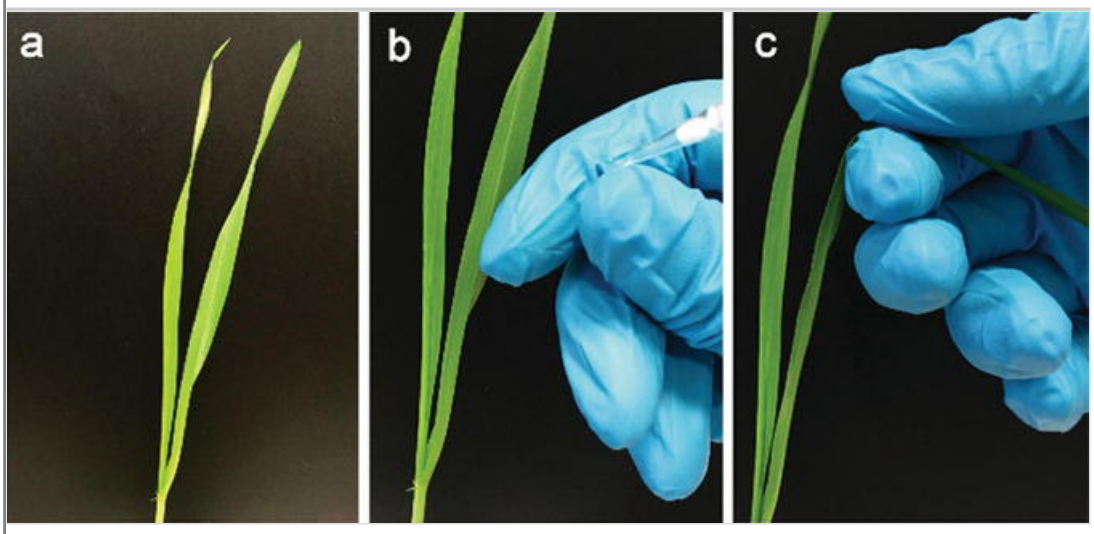
3.10. Inoculating Leaves with the BSMV Transcripts

1. Plants were inoculated with FES buffer containing a 1:1:1 transcript mixture of the α, β, and relevant γ genome transcript (γ: negative control; γPDS: positive control; γBRI1-A or γBRI1-B: targeting *BRI1* for silencing). FES buffer served as a mock treatment. For each plant, VIGS inoculum was prepared by mixing 9 μL of FES Buffer with 0.35 μL each of the α, β genome transcripts and the relevant γ-based genome transcript (*see* **Note 11**).

2. The VIGS inoculum was applied to the first leaf of 10-day-old seedlings. 10 μL of transcript mixture was pipetted onto the index. Keeping the leaf in between thumb and index finger, the transcript mixture was applied by pulling the finger tips gently (to prevent any major damage to leaf) from the base toward the tip and repeat this three times (Fig. 3 and *see* **Note 12**).

Fig. 3

Steps in the inoculation of barley cultivar Akashinriki with viral transcripts. (**a**) 10-day-old seedling. (**b**) Application of viral transcripts mixture in FES buffer to the index finger and thumb (10 μL). (**c**) Inoculation of viral transcripts mixture to the leaf by gently pulling from base to tip, keeping the leaf in between index finger and the thumb



3.11. Validation of the Efficacy of the VIGS Platform Using *PDS* as a Positive Control Target Gene

1. During the infection BSMV moves systematically in seedling leaves and at 14 days post-viral inoculation, the visual symptoms of *PDS* gene silencing were evident on the third leaf of seedlings [26].

2. Silencing of *PDS* resulted in the photo bleaching of emerging leaves with a maximum photo bleaching observed in the third leaf (Fig. 4).

Fig. 4

Silencing of phytoene desaturase (*PDS*) in barley seedlings resulted in photobleaching of newly emerging leaves. Codes: Mock (FES, VIGS buffer negative control), BSMV:00 (mock virus treatment), BSMV:PDS (positive control)

3.12. Confirmation of Gene Silencing by qRT-PCR

1. Gene silencing was quantified by qRT-PCR of RNA extracts, using primers specific to *BRI1* (BRI1:RT, Table 1) and relative to that of the RNA helicase housekeeping gene (RNAH:RT, Table 1) (*see* **Note 13**).

2. Total RNA was extracted from the VIGS-treated leaf segments at 14 days post viral infection. The frozen leaf samples were ground using a TissueLyser by adding two tungsten beads (1 min at 30 Hz). RNA was extracted from the ground leaf tissue using the RNeasy Mini Kit, according to the manufacturer's instructions (*see* **Note 14**).

3. cDNA was prepared from the extracted RNA following the protocol described by Ansari et al. [27] with the minor variation that the primer used was oligo $dT_{12–18}$ (Invitrogen) and the resulting cDNA was diluted to 200 μL using sterile ddH_2O.

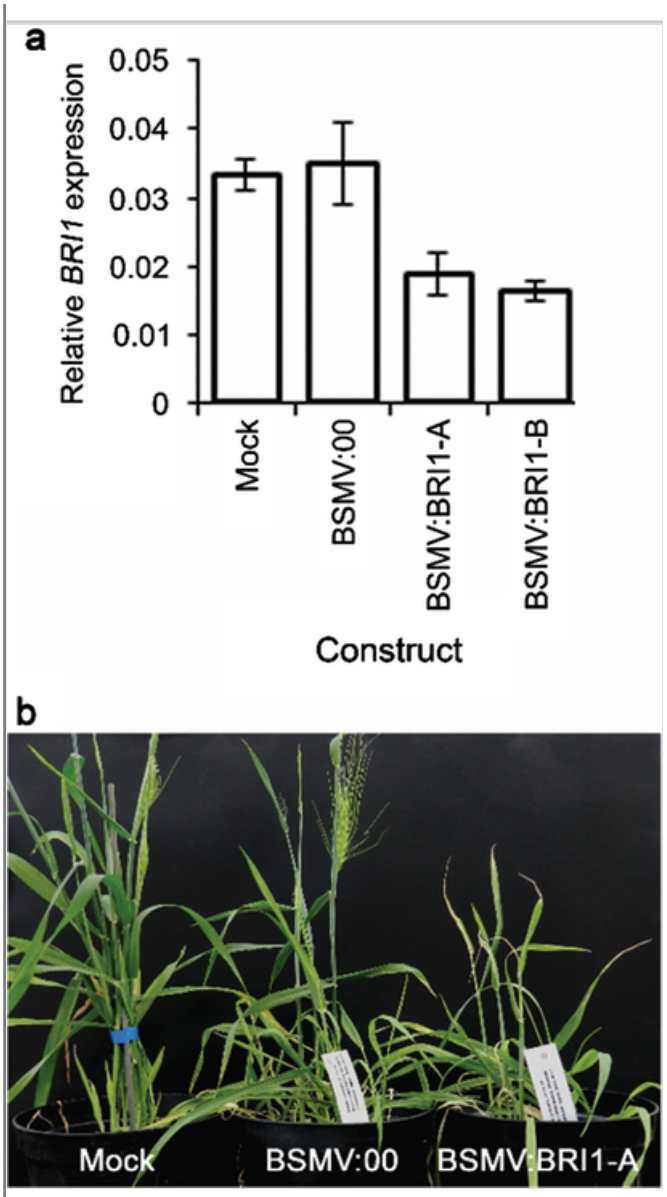
4. PCR reactions contained 2.5 μL of diluted cDNA, 12.5 μL Premix Ex Taq™ (Takara, Japan), and 100 nM each of forward and reverse primers in a total volume of 25 μL. Independent PCRs were concurrently conducted for the housekeeping and target genes.

5. PCR reactions were conducted in a Stratagene Mx3000™ qRT-PCR machine (Stratagene, USA) and the program consisted of 1 cycle of 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 1 cycle of 95 °C for 60 s.

6. The cycle threshold (Ct) values obtained by qRT-PCR were used to calculate the relative gene expression using the formula $2^{-(\text{Ct target gene} - \text{Ct housekeeping gene})}$ (Fig. 5), as described previously [28].

Fig. 5

VIGS of *BRI1* in barley cultivar Akashinriki. Plants were treated with Mock (FES VIGS buffer), VIGS constructs BSMV:00 (empty vector), or BSMV:BRI1-A/-B (two independent constructs targeting *BRI1* gene). (**a**) Graph showing the relative accumulation of *BRI1* transcripts, calculated using quantitative qRT-PCR ($2^{-(\text{Ct } BRI1 - \text{Ct } RNA\ helicase)}$). (**b**) Silencing of *BRI1* resulted in stunting of plant growth



3.13. Using a Detached Leaf Assay to Determine the Effect of VIGS of *BRI1* on Barley Response to the Pathogen *Fusarium culmorum*

1. At 14 days post-viral inoculation, the third leaf was harvested and an 8 cm segment was prepared for use in the detached leaf assay, and the remaining leaf material was flash frozen in liquid N_2 and stored at −80 °C prior to RNA extraction.

2. Square Petri dishes (100 mm × 100 mm × 20 mm) containing 1% (w/v) plant agar, pH 5.7 supplemented with 0.5 mM benzimidazole were prepared and a rectangle of agar (5 cm) was removed from the center of the plates to prevent excessive fungal growth.

3. Leaf segments were placed in each plate, the cut ends resting on the agar and the adaxial side facing upward. The removed agar rectangle was cut into two strips. These strips were used to overlay, and thus hold in place and prevent dehydration, of the cut edges of the leaf segments (*see* **Note 15**).

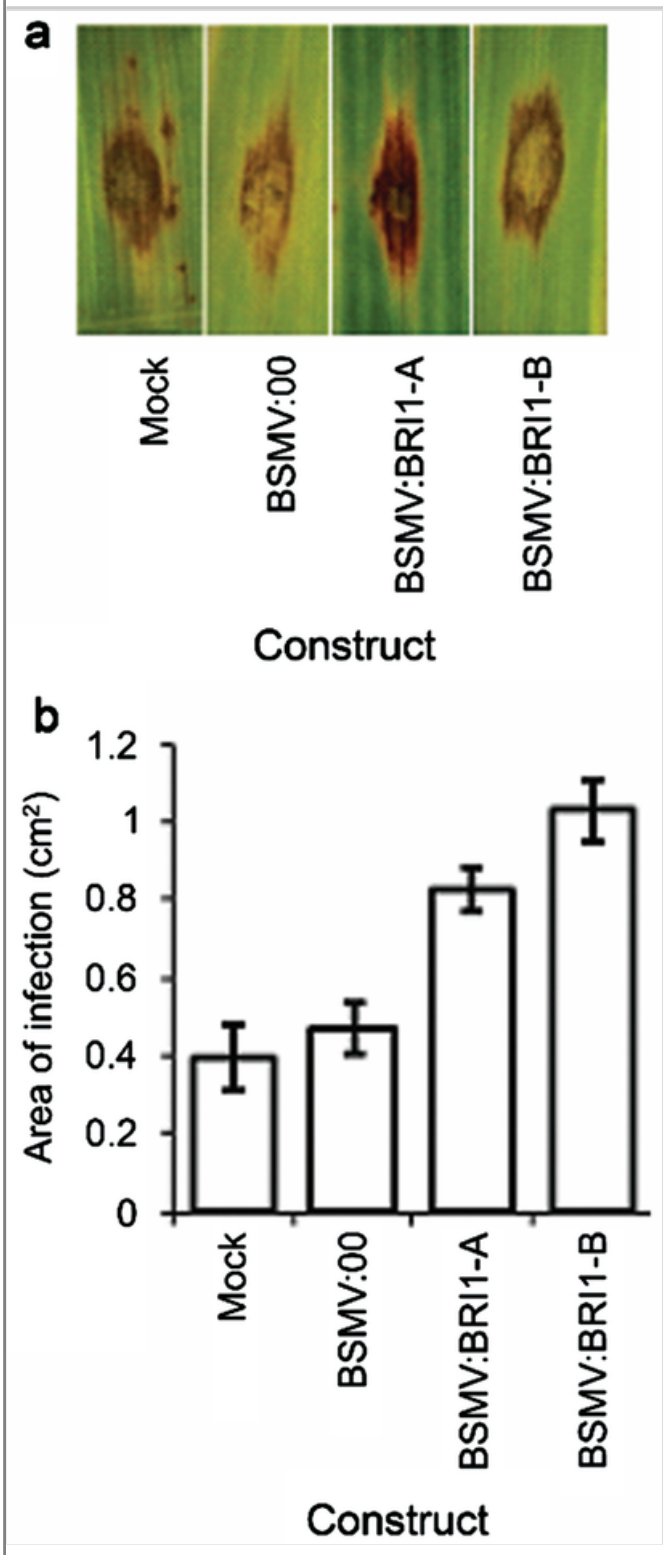
4. The center of each leaf section was punctured with a glass Pasteur pipette. Wounds were treated with a 5 μL droplet containing either 0.02% Tween20 (mock treatment) or 5 μL of conidia of *F. culmorum* strain FCF 200 (1×10^6 spores mL^{-1} in 0.2% Tween20). Plates were taped with porous Leukopor® (BSN medical, UK). Plates were incubated in a controlled environmental room at 22 °C with a 16/8 h light/dark regime and 70% relative humidity (*see* **Note 16**).

5. For each virus/*Fusarium* treatment combination, a total of eight leaf sections were treated per technical replicate and there were at least three biological replicates of the experiment.

6. After 3 days leaves with symptoms were observed and photographed with a scale bar next to plates. The necrotic leaf area was measured using Image J software [29]. The *BRI1*-silenced leaf segments developed significantly larger necrotic lesions upon *F. culmorum* infection than did those treated with the negative control BSMV:00 viral treatment (Fig. 6).

Fig. 6

Effect of VIGS of *BRI1* in the barley cultivar Akashinriki on *Fusarium culmorum* infection of seedling leaves as assessed in a detached leaf experiment. Plants were treated with Mock (VIGS FES buffer), VIGS constructs BSMV:00 (empty vector) or BSMV:BRI1-A/-B (two independent constructs targeting *BRI1* gene). (**a**) Phenotype of leaf segments showing necrosis upon infection with *F. culmorum*, at 3 days post inoculation. (**b**) Quantification of necrosis using ImageJ software



4. Notes

1. FES buffer can be aliquoted into 50 mL volumes and autoclaved (121 °C for 20 min); these aliquots can be stored at room temperature under sterile conditions.

2. It is highly recommended to test potential off target gene silencing by qRT-PCR on a closely related gene that shares some homology (less than 85%) with the VIGS gene fragment of the targeted gene. This will prove that a potential phenotype due to VIGS is due specifically to the targeted gene.

3. PCR products were run on a gel to check specificity before purification with the QIAquick PCR purification kit.

4. It is highly recommended to sequence the plasmids to confirm the correct fragment was cloned into pGEM-T vector before subcloning into pSL038-1.

5. During the gel extraction of the gene fragment, it's important to avoid any residual ethanol from the wash buffer (Buffer PE) in the QIAquick column. Before the elution step, we recommend to let the column stand 10 min to evaporate any remaining ethanol. Residual ethanol in the eluate may interfere with the ligation of the gene fragment into pSL038-1.

6. Use pGEM-T with the gene fragment insert as a positive control in restriction digestion; the release of the fragment from this vector will act as a check to confirm that the double restriction worked.

7. For all the VIGS plasmids isolation, it is recommended to omit any RNase from your plasmid isolation protocol; residual RNaseA will interfere with the in vitro transcription process.

8. While performing the in vitro transcription reaction, always use RNase-free water and RNase-free filter tips to minimize any chances of RNase contamination. While running the agarose gel for RNA visualization, use only buffers prepared with DEPC-treated water and clean the gel tank with 3% (v/v) H_2O_2.

9. The agarose gel will indicate the quality of the RNA. A smear in the in vitro transcription product indicates degradation of the RNA. If the samples were not treated with DNase, there will be a band at approximately 6 Kbp (linearized vector) and a bright band of RNA at approximately 1.5 Kbp (Fig. 2).

10. As the only difference between the 1:1:1 transcript mixtures (α, β, γ) is the relevant γ genome transcript, therefore one needs more α and β transcript than of the specific γ constructs.

11. Before the application of viral transcripts, the plants should be well watered to aid recovery from the mechanical damage caused by the abrasive material present in FES buffer. The abrasive material causes damage to the leaf surface to facilitate entry of the viral transcripts into the plants. Be gentle on the leaves when applying the VIGS transcripts. It is important to compare FES treatment with BSMV:00 treatment to evaluate the influence of the viral infection on your disease assessment.

12. Always wear gloves before applying the viral transcripts and change the gloves between each treatment to prevent any cross contamination of the transcripts.

13. Housekeeping genes need to be checked for constitutive expression under the specific disease conditions and in the tissue being assessed.

14. Prior to investing effort in the phenotyping procedure, it is best to test the efficacy of the gene silencing via qRT-PCR.

15. In plates containing the detached leaves, it is advised to include a few mL of sterile water at the base to ensure high humidity in the plate, this is essential for the germination of fungal conidia.

16. It is better to avoid using a cultivar that is sensitive to the BSMV pathogen.

17. BSMV can enhance pathogen susceptibility and therefore this technique may not be suitable for studying genes that have small effects on disease resistance.

AQ4
AQ5

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