Chapter 8
An Improved Virus-Induced Gene Silencing (VIGS) System in Zoysiagrass

Yi Xu, Jin Zhang, Jinping Zhao, Junqi Song, and Qingyi Yu

Abstract Virus-induced gene silencing (VIGS) is a powerful RNA-silencing technique for transient gene manipulation and functional verification. However, the procedure of its application among dicot and monocot species varies based on types of vectors, plant susceptibility, as well as inoculation methods. Here, we described a simple yet efficient tobacco rattle virus (TRV)-based VIGS system for functional analysis of genes in zoysiagrass (*Zoysia japonica* cv. "Zenith"), an important warm-season turfgrass species, using *Nicotiana benthamiana* as an intermediate host. VIGS of the *PDS* reporter gene resulted in a characteristic photo-bleaching phenotype in majority of the inoculated leaves up to 70% reduction of expression of the endogenous *phytoene desaturase* (*PDS)* gene in zoysiagrass. Our protocol provides a fast and efficient toolbox for high-throughput functional genomics in zoysiagrass species, which could potentially be applied to other warm-season turfgrass species.

Keywords Virus-induced gene silencing · Tobacco rattle virus · Zoysiagrass · *Agrobacterium tumefaciens* · Functional genomics · Phytoene desaturase · Post-transcriptional gene silencing

8.1 Introduction

Since its emergence, virus-induced gene silencing (VIGS) has been providing a powerful approach for plant genetic and functional characterization, in a timely manner. VIGS is basically taking advantage of post-transcriptional gene silencing (PTGS), which in plants is dependent upon a relatively high degree of nucleotide homology between RNA transcript and target gene sequence (Ding 2000; van den Boogaart et al. 1998). In brief, VIGS mechanism is co-opted to target host mRNAs by including a fragment of target gene into a modified viral genome. The viral replication of chimeric double-stranded intermediates, including the target gene fragment, are produced and then recognized by RNA-induced silencing complex (RISC) as

Y. Xu · J. Zhang · J. Zhao · J. Song · Q. Yu (✉)
Texas A&M AgriLife Research and Extension Center at Dallas, Dallas, TX, USA
e-mail: qyu@ag.tamu.edu

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foreign invaders in plant. Subsequently, the Dicer-like proteins process them into small interfering RNAs (siRNAs), which serve as specific templates to target any transcripts with highly similar sequences for degradation (Baulcombe 1999). Therefore, it is designed to manipulate target gene function utilizing the power of plant–virus interaction, without genome modification in plant itself.

The VIGS system has been successfully established in tobacco by suppressing phytoene desaturase (PDS) gene (Kumagai et al. 1995). Many plant DNA and RNA viruses have been modified as VIGS vectors and used to investigate gene functions. In eudicot species, VIGS has been reported to be adapted to tomato, Arabidopsis, petunia, potato, cassava, tobacco, soybean, vine, cotton, rose, apricot, almond, and sweet cherry, etc. (Burch-Smith et al. 2006; Chen et al. 2004; Faivre-Rampant et al. 2004; Fofana et al. 2004; Liu et al. 2002a, 2013; Kawai et al. 2016; Muruganantham et al. 2009; Nagamatsu et al. 2007; Qu et al. 2012; Tang et al. 2010; Tian et al. 2014; Turnage et al. 2002; Zhang and Ghabrial 2006; Zhao et al. 2020a, b, c). The most widely used VIGS vector is based on tobacco rattle virus (TRV), due to its vigorous viral replication and wide host spectrum (Burch-Smith et al. 2006; Liu et al. 2002b). In comparison to the many choices of VIGS systems adapted for dicots, only a few VIGS systems have been established for monocot species. Four RNA viruses, barley stripe mosaic virus (BSMV), brome mosaic virus (BMV), bamboo mosaic virus, foxtail mosaic virus (FoMV), and one DNA virus, rice tungro bacilliform virus (RTBV), were modified for VIGS in several monocot species (Ding et al. 2006; Holzberg et al. 2002; Liou et al. 2014; Liu et al. 2016; Meng et al. 2009; Pacak et al. 2010; Purkayastha et al. 2010; Tai et al. 2005; Yuan et al. 2011). BMV-based vectors have been extensively used for VIGS in some monocot species, but are not suitable for others, or not for all cultivars within a single host species (Ding et al. 2006; Pacak et al. 2010). Considering the advantages of higher viral activity and wider host range using TRV-based VIGS system, it is not uncommon for researchers to incorporate VIGS vector that was designed for eudicot species when they need to develop a better VIGS system for monocot plants. Recently, efforts have been put to use TRV-based VIGS system in monocot species. For instance, Singh et al. (2013) first reported their success of using TRV-based VIGS on gladiolus, and provided optimized protocol. In another report, Zhang et al. (2017) reported a rapid and whole-plant level gene silencing phenotype in both wheat and maize, using the TRV-based VIGS system. Therefore, TRV-based VIGS system has been proven to be fast, convenient, and efficient in such two monocot plants, which will also inspire future studies on other monocot species.

Zoysiagrass (*Zoysia* spp.) is one of the important warm-season turfgrass species. Due to its superior morphological characteristics and turf performance, zoysiagrass has been extensively used in home lawns, golf courses, sports fields, recreational parks, and other land surfaces (Patton et al. 2004). Besides its commercial use, zoysiagrass is also an ideal plant material to explore growth regulation and responses to environmental stresses in perennial monocot species (Huang et al. 2014). Efforts have been taken for studying gene functions by using polyethylene glycol (PEG)-mediated

direct gene transfer into protoplast (Inokuma et al. 1998), or by following the traditional plant tissue culture and Agrobacterium-mediated transformation in zoysiagrass. Toyama et al. (2003), found that zoysiagrass tissue culture could produce up to four types of calluses. Among them, only type 3 (yellow, compact, and friable) callus was suitable for Agrobacterium-mediated transformation. The selection criteria for such callus type requires extensive experience, and may also be subjective. A newer version of Agrobacterium-based transformation was recently reported in zoysiagrass, using stolon nodes as transforming materials (Ge et al. 2006). The callus formation process was bypassed, and the transformation period was shortened from 5 months to 3 months. However, the transformation frequency in this system was only up to 6.8%, which is not suitable for high-throughput functional genomic study. On the other side, VIGS assays using DNA viruses have also been exploited. For example, a rice tungro bacilliform virus (RTBV)-based VIGS system could lead to an average of 30.5–42.4% reduction of the *PDS* gene expression in half of the inoculated zoysiagrass plants (Zhang et al. 2016).

In this chapter, we described a protocol to use the leaf sap from TRV-infected *N. benthamiana* leaves to inoculate zoysiagrass. This improved VIGS method showed that the TRV-based system is able to suppress *PDS* reporter gene expression up to 70% in the inoculated zoysiagrass plants, for at least 5 weeks. Therefore, our protocol provides a fast and efficient toolbox for high-throughput functional genomics in zoysiagrass species, which may also have the potential to be applied to other warm-season turfgrass species.

8.2 Materials

8.2.1 Amplification of Target Gene

1. Zoysiagrass (*Zoysia japonica*) cultivar "Zenith" seeds (Pennington Seed Company, Madison, GA)
2. *Nicotiana benthamiana* seeds
3. Plastic nursery pots, trays, and tray covers
4. TRIzol reagent (Thermo Fisher Scientific, Waltham, MA)
5. TURBO DNA Free Kit (Thermo Fisher Scientific, Waltham, MA)
6. Microcentrifuge
7. Liquid nitrogen
8. Mortar and pestle
9. Chloroform
10. Isopropanol
11. Ethanol
12. High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA)
13. Polymerase chain reaction (PCR) primers

14. GoTaq Master Mix (Promega, Madison, WI)
15. Thermal cycler
16. Gel electrophoresis system
17. Agarose
18. Ethidium bromide
19. TBE buffer (10X stock solution): 0.9 M Tris-borate, 20 mM EDTA, pH 8.0
20. DNA loading buffer (6X): 30% glycerol, 0.25% bromophenol blue, store at 4 °C.

8.2.2 Cloning Target Gene into Vector

1. PEG/$MgCl_2$ solution: 40% PEG 8000, 30 mM $MgCl_2$
2. TRV1 (19) and TRV2 vector (pYY13, Dong et al. 2007; Sha et al. 2014)
3. *Escherichia coli* cells (DB3.1 and DH5α)
4. Water bath or incubator
5. SOC growth medium (Thermo Fisher Scientific, Waltham, MA)
6. Incubating orbital shaker
7. Luria-Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Bring to 1 L with pure water, adjust pH to 7
8. Kanamycin stock solution (50 mg/mL, 1000X): 0.5 g kanamycin. Bring to 10 mL with pure water, sterile filter, store at −20 °C
9. Petri dish
10. Resuspension buffer (P1): 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0), store at 4 °C
11. NaOH/SDS solution (P2): 0.2 N NaOH, 1% SDS
12. Potassium Acetate solution (P3): 3 M KOAc (pH 6.0), store at 4 °C
13. Restriction nuclease: PstI and buffer (New England Biolabs, Ipswich, MA)
14. T4 DNA polymerase and buffer (New England Biolabs, Ipswich, MA)
15. dATP and dTTP
16. Dithiothreitol.

8.2.3 Agrobacterium Transformation

1. *Agrobacterium tumefacien* cells (GV3101)
2. Liquid nitrogen
3. Rifampicin stock solution (50 mg/ml, 1000X): 0.5 g rifampicin. Bring to 10 mL with pure water, sterile filter, store at −20 °C.

8.2.4 Inoculation

1. 10 mM $MgCl_2$
2. 10 mM MES (pH 5.6)
3. 200 μM acetosyringone
4. Seedlings of *N. benthamiana* and zoysiagrass "Zenith"
5. 1 mL needleless syringe
6. Silicon carbide (~400 mesh)
7. Scotch-Brite heavy duty scour pad
8. Latex gloves.

8.3 Methods

8.3.1 Preparation of Plant Materials

1. Sow seeds of *Z. japonica* cv. "Zenith" and *N. benthamiana* in plastic pots filled with soil (see Note 1). Set greenhouse/growth chamber parameters as follows: 28/25 °C day/night temperature, 60/70% day/night humidity, 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Perform regular greenhouse irrigation and fertilization as needed. For the first week, trays need to be covered in order to facilitate seed germination.
2. A first batch of zoysiagrass "Zenith" plants can be prepared without *N. Benthamiana*, from which total RNA will be extracted.

8.3.2 Amplification of Target Gene Fragment (TGF)

1. Sequence fragments (around 200 ~ 400 bp) of target genes in zoysiagrass were identified from our *Z. japonica* genome assembly (unpublished data) (*see* Note 2).
2. PCR primers were designed in order to amplify TGF. To use ligation-independent reaction (LIC), LIC2 and LIC1 sequences were added in front of forward primer and reverse primer, respectively (Dong et al. 2007; Zhao et al. 2016).
3. RNA extraction using TRIZol reagent was performed for zoysiagrass plant leaf powder, which was first flash frozen in liquid nitrogen, and then ground using mortar and pestle.
4. Total RNA was treated with TURBO DNA Free kit to remove any genomic DNA contamination, and then was transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit.
5. PCR amplification was performed to obtain TGF using cDNA as template.
6. Check the quality and quantity of PCR product by gel electrophoresis in a 1% agarose gel to ensure that it was specifically amplified and was the expected size.

7. Purify PCR product with the following procedures: (a) Add equal volume of PEG/$MgCl_2$ solution, PCR product and pure water, mix well; (b) Centrifuge for 20 min at 16,000 g; (c) Discard supernatant (*see* Note 3); (d) Resuspend the pellet in 70% ethanol; (e) Centrifuge for 20 min at 16,000 g; (f) Discard supernatant and air-dry the pellet for 15–20 min; (g) Dissolve the pellet with appropriate amount of pure water.

8.3.3 Cloning into TRV2-LIC Vector

1. Digest TRV2-LIC vector with PstI, and check digestion result by gel electrophoresis in a 1% agarose gel.
2. Set up T4 DNA polymerase reactions as described in Table 8.1. Mix well, and incubate at 22 °C for 30 min, followed by 70 °C for 20 min, and hold at 4 °C.
3. Set up LIC reaction as follows: Mix 5 μL of T4-treated vector and 5 μL of T4-treated PCR product together, incubate at 65 °C for 30 min, and decrease to 22 °C at 0.2 °C/s, and then incubate at 16 °C overnight (*see* Note 4).
4. Transform the LIC reaction product into *E. coli* competent cells. Thaw 100 μL of DH5α cells on ice, and then add LIC reaction product. Mix gently when pipetting to avoid agitation. Incubate on ice for 30 min. Heat shock cells for 30 s in a water bath at 42 °C and chill on ice for at least 2 min.
5. Add 1 mL of SOC growth medium, and shake for 1 h at 37 °C.
6. Centrifuge briefly to collect cells, and resuspend in 200 μL of LB medium. Spread cells on LB plates containing 50 μg/mL kanamycin. Incubate plates at 37 °C overnight.
7. Pick 3–5 colonies on the plates, and grow in 10 mL LB medium containing 50 μg/mL kanamycin until log phase.
8. Extraction of plasmid from E. coli. Collect cells from 1.5 mL E. coli culture by centrifuging for 2 min at 10,000 g. Discard supernatant and add 200 μL of ice-cold P1 solution, resuspend cells by vigorous vortexing (no cell clumps shall be observed). Add 200 μL of P2 solution, and briefly invert tube for 3–5 times until the solution becomes viscous and clear. Add 200 μL of ice-cold P3

Table 8.1 T4 DNA polymerase treatment

Component	Volume	Component	Volume
Digested TRV2 vector	2.50 μL (~50 ng)	Purified PCR product	2.50 μL (~50 ng)
10x NEB buffer 2.1	0.50 μL	10x NEB buffer 2.1	0.50 μL
100 mM dTTP	0.25 μL	100 mM dATP	0.25 μL
1 M DTT	0.05 μL	1 M DTT	0.05 μL
T4 DNA polymerase	0.10 μL	T4 DNA polymerase	0.10 μL
ddH_2O	1.60 μL	ddH_2O	1.60 μL
Total	5.00 μL	Total	5.00 μL

solution and briefly invert 5–8 times until no more precipitate accumulates, and solution becomes less viscous.
9. Centrifuge for 10 min at 15,000 g. Carefully transfer supernatant into a new tube by pipetting. Avoid any white precipitate to be transferred.
10. Add 2.5–3 volume of absolute ethanol pre-chilled at −20 °C, invert several times.
11. Centrifuge for 10 min at 12,000 g. Discard supernatant and air-dry pellet.
12. Dissolve pellet in appropriate amount of pure water.
13. The presence of TGF in TRV2-LIC vector can be verified by PCR and gel electrophoresis, as well as Sanger sequencing, using either gene-specific or vector-specific primers.

8.3.4 Preparation of Agrobacterium Used in VIGS

1. Transform TRV1, TRV2 (as empty vector control), and TRV2 containing TGF (TRV2-TGF) into *Agrobacterium tumefacien* strain GV3101. Thaw 100 μL of GV3101 cells on ice, add 50–100 ng TRV1, TRV2 or TRV2-TGF into cells by pipetting. Mix with pipetting and avoid agitation. Incubate on ice for 30 min.
2. Chill cells in liquid nitrogen for 5 min.
3. Heat shock cells in a water bath at 37 °C for 5 min. Chill on ice for at least 2 min.
4. Add 1 mL of SOC growth medium, and shake for 2 h at 28 °C.
5. Centrifuge briefly to collect cells, and resuspend in 200 μL LB medium. Spread cells on LB plates containing 50 μg/mL kanamycin and 50 μg/mL rifampicin. Incubate plate at 28 °C for at least 2 d.
6. Pick 3–5 colonies on the plates, and grow in LB medium containing 50 μg/mL kanamycin and 50 μg/mL rifampicin until log phase.
7. Extraction of plasmid from GV 3101 (*see* Sect. 8.3.3 Steps 8–12)
8. Confirm TRV1, TRV2 and TRV2-TGF constructs by either PCR or Sanger sequencing.
9. For long-term storage, add 500 μL of 50% sterile glycerol solution to 500 μL of log-phase GV3101 culture containing TRV1, TRV2 or TRV2-TGF vector, respectively. Mix well and store at −80 °C.

8.3.5 Agro-Infiltration of N. Benthamiana

1. One day before agro-infiltration, grow the transformed Agrobacterium GV3101 strains containing TRV1, TRV2, and TRV2-TGF respectively in 20 mL of LB medium with 50 μg/mL kanamycin and 50 μg/mL rifampicin overnight.
2. Harvest cells by centrifugation for 2 min at 10,000 g. Discard supernatant.
3. Resuspend cells in infiltration buffer containing 10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone and adjust to an OD600 of 1.0.

4. Incubate cell suspension for at least 3 h at room temperature.
5. Mix TRV1 and TRV2 (as empty vector control), TRV1 and TRV2-TGF in a 1:1 ratio, respectively.
6. Infiltrate *N. benthamiana* with cell mixture (TRV1 + TRV2, or TRV1 + TRV2 − TGF) into the abaxial leaf surface, using 1 mL needleless disposable syringe (*see* Note 5).
7. Transfer infiltrated *N. benthamiana* plants into a growth chamber with the following settings: 22/20 °C day/night temperature, 70% humidity, 200 μmol m^{-2} s^{-1} PAR to facilitate virus infection.
8. Leaves from TRV1 + TRV2 and TRV1 + TRV2 − TGF-infected *N. benthamiana* plants were harvested for subsequent inoculation into zoysiagrass. VIGS was monitored using a reporter gene (e.g. *PDS*) as positive control. Leaves from TRV1 + TRV2 − PDS-infected *N. benthamiana* plants that exhibited photobleaching phenotypes, indicative of suppression of the endogenous *PDS* gene, were used for inoculation into zoysiagrass.

8.3.6 Inoculation of Zoysiagrass

1. Collect VIGS-infected *N. benthamiana* leaves containing TRV1 + TRV2 and TRV1 + TRV2 − TGF, respectively.
2. Grind leaves using mortar and pestle, add a bit of silicon carbide (400 mesh) to facilitate abrasion during inoculation.
3. Cut Scotch-Brite heavy duty scour pad into thumbnail-size pieces. Dip one piece in leaf zap, and gently rub both sides of zoysiagrass leaf blade for five to eight times, with the direction from base to tip (*see* Note 6).
4. Cover inoculated zoysiagrass in the dark for 24 h at room temperature.
5. Remove cover and transfer zoysiagrass to growth chamber described in Sect. 8.3.5 Step 7.
6. Normally, the phenotype of VIGS will occur in 3 weeks.
7. Photograph the plants with silencing phenotype and collect tissues for subsequent experiments.

8.3.7 Evaluation of Gene Silencing

Here, we cloned a fragment of zoysiagrass *PDS* gene, as a reporter gene, into the TRV2 vector. The TRV1 + TRV2 − PDS-infiltrated *N. benthamiana* plants showed photobleaching phenotype, mostly on young leaves (Fig. 8.1). We then used photobleached leaf sap to inoculate zoysiagrass (Fig. 8.2). After three weeks, most of the

Fig. 8.1 Virus-induced gene silencing of the zoysiagrass *PDS* gene in *N. benthamiana* (Photos were taken at 14 d after agro-infiltration, left: control; right: silenced plant)

Fig. 8.2 Mechanical inoculation of zoysiagrass leaf blades with leaf sap collected from *N. benthamiana* plants expressing TRV1 + TRV2-*PDS*

newly developed leaf blades from inoculated zoysiagrass showed chlorosis phenotype (Fig. 8.3). RT-PCR analysis using primers corresponding to different regions of *PDS* gene confirmed the expression of the *PDS* gene in zoysiagrass was reduced up to 70%, at 5 weeks after inoculation (Fig. 8.4). Therefore, the test of *PDS* reporter gene in TRV-based VIGS system has validated our method as a fast and efficient tool for gene manipulation in zoysiagrass.

Fig. 8.3 Virus-induced gene silencing of the *PDS* gene in zoysiagrass (Photos were taken at 35 d after inoculation for empty vector control [left] and silenced zoysiagrass [right])

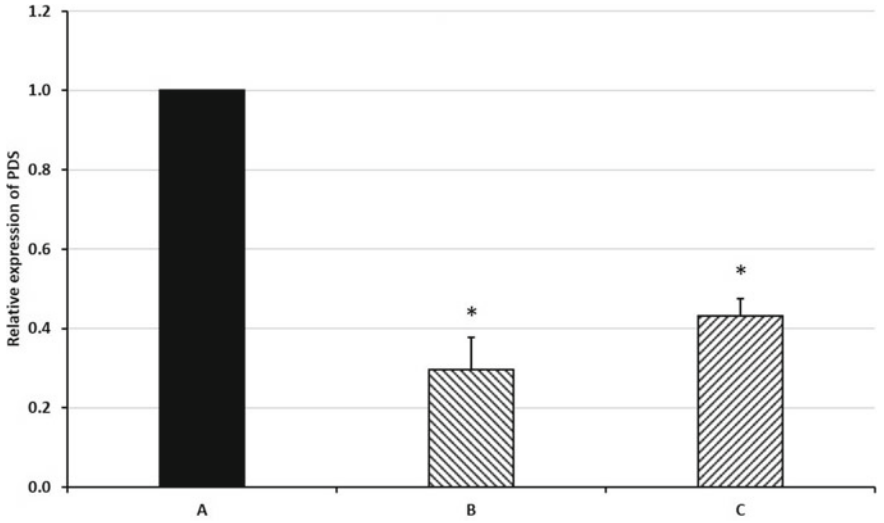

Fig. 8.4 RT-PCR analysis of expression of the *PDS* gene in silenced zoysiagrass plants (Data shown here are relative expression in zoysiagrass empty vector control [A], and two individually silenced plants [B and C]. The asterisk represents significant difference between control and VIGS plants, at the *P* level of 0.05)

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Notes

1. In order to get ready for zoysiagrass inoculation, sow *N. benthamiana* seeds four weeks before zoysiagrass inoculation day, and zoysiagrass seeds three weeks before zoysiagrass inoculation day.
2. Use 3' end of gene sequence in order to obtain more gene-specific fragments, however, avoid regions with high GC content or repetitive elements.
3. *Critical point*: be careful to remove the supernatant, since the DNA pellet is nearly invisible! It is recommended to use a pipettor to carefully aspirate it out. Same precaution applies to the rest of the purification steps.
4. The final step of incubation time at 16 °C could be shortened to 1 h, however, this may lead to decreased yield of ligation product.
5. It is recommended to choose the first four true leaves of *N. benthamiana* to infiltrate, since those leaves are thicker and juicier. Two-week old *N. benthamiana* plants usually have four true leaves, which are ideal for agro-infiltration.

6. *Critical point*: leaf age is of vital importance for successful VIGS assay. To ensure maximum VIGS efficiency, use "Zenith" seedlings that have three or less leaf blades. VIGS efficiency significantly decreases with older seedlings.

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