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Protocol status: Working
We use this protocol and it's working

Simple Seed-vacuum Protocol for Agrobacterium-mediated Virus Induced Gene Silencing (VIGS) in Sunflower Helianthus annuus L.

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ABSTRACT

Virus-Induced Gene Silencing (VIGS) stands out as one of the most powerful tools for functional genomic research. The underlying principle of the VIGS system is based on harnessing the innate defense mechanism of the studied species to induce post-transcriptional gene silencing. In brief, the process involves introducing a modified viral constructs carrying a fragment of the host targeted gene (300-500 bp). The introduced virus will prompt a group of enzymes within the plant's defense system, setting off the degradation of viral RNA products together with any similar sequences in the cell, including the host's own mRNA. As a result, the expression level of the targeted gene is altered or, in optimal scenarios, completely silenced.

The introduction of the viral constructs is typically achieved through agrobacterium-mediated transformation. Numerous agrobacterium-VIGS systems have been firmly established for a number of crops and model plant species (Zhang Y. et al., 2019; Ramegowda V. et al., 2014). However, it is important to note that the infiltration procedure and viral construct often exhibit strong species-specificity. And as research advances, customizing new protocols becomes imperative for successful implementation across diverse plant species. Here, we represent a simple and reproducible agrobacterium-mediated VIGS protocol for sunflower (Helianthus annuus L.). Our methodology is seed vacuum-based without the necessity for pre-germination, MS medium recovery, or even seeds sterilization. Within this protocol, we provide comprehensive instructions that ensure the consistent replication of results. For demonstration we used PHYTOENE DESATURASE gene (PDS) of *Helianthus annuus* as a target for VIGS. The viral constructs are derived from Tobacco Rattle Virus (TRV) (Liu Y. et al., 2002). Information on vectors assembly and post-experimental validation are also provided. The protocol's reproducibility has been rigorously tested on a breeding line and various commercial sunflower cultivars.

BEFORE START INSTRUCTIONS

The VIGS constructs should be prepared beforehand and introduced into agrobacterium cells using the standard transformation method employed in your laboratory.

The constructs utilized for demonstration in this protocol have been developed based on TRV vectors. The primary target gene for VIGS is the PDS of sunflower (*Helianthus annuus* L.). This gene serves as a common positive control in VIGS experiments due to its easily detectable phenotypic characteristics. Silencing effects manifest as color-bleaching of the leaves.

The PDS fragment for VIGS (siRNA) was designed utilizing the open-access web server https://www.zhaolab.org/pssRNAit/. The PDS fragment used within these VIGS constructs was amplified from sunflower genomic DNA using these primers: 5'-taattctagaATGGCATTTTTAGATGGCAGCCC-3' and 5'-taatggatccTGGAGTAGCAAATACATAAGCATCCCC-3'. Subsequently, the resulting

amplicon was ligated into the pYL156 TRV RNA2 vector following standard procedure. The resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101 using a standard electroporation procedure.

TRV2 constructs used in this protocol:

addgene-plasmid-148969-TRV2.dna

TRV2 Ha PDS.dna

PDS gene in sunflower and the location of primers:

PDS Helianthus anuus.dna

link for pYL192 (TRV1): https://www.addgene.org/148968/
link for pYL156 (TRV RNA2): https://www.addgene.org/148969/

Primers information for cultures validation and results analysis:

	A	B	C	D	E	F
	forword primer	sequence	reverse primer	sequence	PCR template	comments
	pYL156_TRV2_F_flank	GTTCAGGCGGTTCTTGTGTGTC	TRV2-tRNA_flank_R	TTGAACCTAAAACTTCAGACACGGATCTAC	pYL156 (TRV RNA2)	agrobacterium colony check
					TRV2 Ha PDS	agrobacterium colony check
					cDNA from VIGS-treated plant	validation of succefull viral infection
	TRV1_F	AGACAACTTAATAACACATTGCGGACG	TRV1_R	CTTTGACGTTGGAGTCCACGTTC	pYL192 (TRV1)	agrobacterium colony check
	Ha_PDS_VIGS_insertion_F	taattctagaATGGCATTTTTAGATGGCAGCCC	Ha_PDS_VIGS_insertion_R	taatggatccTGGAGTAGCAAATACATAAGCATCCCC	genomic DNA of sunflower	amplyfing PDS insertion for VIGS construct assembly

Infiltration suspension preparation

1 Streak previously prepared glycerol stocks of agrobacterium carrying the required VIGS constructs (TRV1 and TRV2) on agar-LB plates. For each plate: 20 mL LB medium + 1% agar + 50 µg/mL kanamycin + 10 µg/mL gentamycin + 100 µg/mL rifampicin.

Note

This step should be executed at least 3 days before the designated date of the primary seed vacuum infiltration. Nevertheless, agrobacterium LB plates can be stored for up to 3 months at 4°C, allowing for advance preparation and PCR validation of these plates for multiple forthcoming VIGS experiments during this timeframe.

2 Incubate at 28°C. Individual, well-separated colonies will start to emerge within 24-38 hours.

Expected result

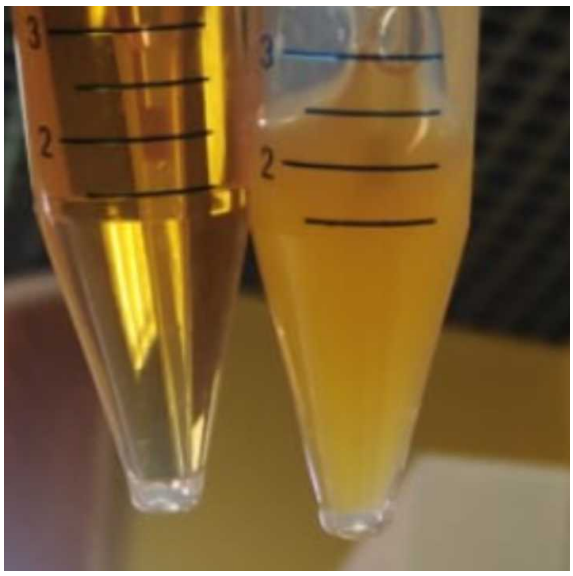
Streak from agrobacterium glycerol stock after 35 hours incubation at 28°C

Note

Before proceeding to the next step, a PCR check is recommended for the selected colonies. However, this step is not necessary if the PCR check has already been conducted prior to the preparation of the glycerol stocks. (Information on primers and PCR conditions can be found in the "BEFORE START" section)

3 To prepare the starting culture, inoculate 5 mL of liquid LB containing antibiotics with single colony and incubated at 28°C/180rpm for another 24-38 hours.

Expected result



Starting culture after 40 hours growth. Left tube: negative control; Right tube: agrobacterium transformed with TRV2-empty plasmid.

4 To prepare the infiltration culture, transfer the starting culture to a sterile flask containing 50mL of LB (10 times the volume of the starting culture) suplemented with antibiotics, MES (10 mM), and acetosyringone (200 µM) .

5 Incubate at 28°C/180rpm and monitor OD_{600} every 1-1.5 hour. Remove from the incubator when OD reaches 1.5.

Note

If not all cultures have reached an OD of 1.5 simultaneously, transfer the ready ones to the refrigerator (at 4 °C) to halt their growth, while allowing the other cultures to continue growing.

6 Transfer the infiltration culture to a 50-mL tube and collect the agrobacterium cells by centrifuging at 3000 rcf for 10 minute.

7 Remove the supernatant and wash agrobacterium cells in distilled water for a couple of times.

8 Re-suspend the cells in the infiltration buffer to achieve a volume that sustains an OD of 1.5. The infiltration buffer is a water-based solution comprising MES (10 mM), acetosyringone (200 µM), and $MgCl_2$ (10mM). After resuspending, allow the infiltration suspension to sit at room temperature for 2-3 hours.

Performing seed-vacuum agrobacterium infiltration

9 After removing the outer coat of sunflower seeds immerse them in tap water for approximately 2 hours. This will facilitate the removal of the inner membrane.

10 Transfer the seeds to a sterile Petri dish. Add a 1:1 ratio of TRV1:TRV2 infiltration suspension. Each Petri dish should contain a total volume of 20-25 mL of the infiltration suspension (TRV1 + TRV2).

In our example, three different Petri dishes for different VIGS variants:
1. TRV1 + TRV2-empty
2. TRV1 + TRV2-PDS
3. Infiltration buffer (negative control)

11 In each Petri dish, introduce a 2-3 g of autoclave-sterile silica sand. In order to produce wounds in the surface of the seeds, gently rub using index and middle fingers (wearing gloves). The rubbing should be executed carefully in a circular motion, taking extra care not to fracture the seeds.

Note

- Change gloves between different Petri dishes with various TRV2 constructs to prevent cross-contamination.
- Following the immersion of seeds in water for 2 hours during the peeling step, they can sometimes become fragile for mechanical manipulation.

12 Initiate the vacuum process as follows: Apply vacuum for 3 minutes, followed by a 3-minute pause, and then another 3 minutes of vacuum.

13 Seal the Petri dishes and incubate at 28°C/50rpm for 6 hours.

Note

- Retain the sand within the Petri dish. Allowing the sand to remain during the incubation period at low rpm will sustain the ongoing seed wounding process. This, in turn, has the potential to enhance the efficiency of agrobacterium transformation.
- Our experience revealed that, in comparison to a 2-hour incubation, a 6-hour incubation period produced optimal PDS silencing results, evident in the increased occurrence of color-bleached leaves closer to the epical meristem. However, an 18-hour incubation demonstrated adverse effects, with less pronounced color-bleaching symptoms.

14 Plant the treated seeds in clean, humid soil. Subsequently, cover the pots with plastic wrap and transfer them to a dark location for a period of 2 days.

15 After 2 days, remove the plastic wrap and transfer the pots to a greenhouse environment. Maintain a light/dark cycle of 16 hours of light and 8 hours of darkness for optimal growth conditions.
The PDS silencing symptoms (color-bleaching of leaves) will start to be visible within 11-13 days.

Expected result