# Construction and optimization of TRV-mediated VIGS silencing system of *Helianthus annuus*

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**Abstract:** To analyze gene function in plants lacking a stable genetic transformation system, virus-induced gene silencing (VIGS) is essential. Sunflower (*Helianthus annuus*), the world's fourth most important oil crop and a significant cash crop in China, was selected as the experimental material. Three specific fragments of the *H. annuus* phytoene desaturase gene (*HaPDS*) were amplified and cloned into the pTRV vector to construct the recombinant vector pTRV-*HaPDS* for VIGS. Confectionery sunflower cultivars Huiyuan119, LD5009, and 3936, as well as oilseed sunflower cultivars R5 and GK2002, were used to optimize silencing conditions. *Agrobacterium tumefaciens*-mediated infiltration was performed, and *HaPDS* gene silencing was evaluated by considering factors such as the position of the silencing fragment within the gene, vacuum treatment, *A. tumefaciens* co-cultivation time, *A. tumefaciens* concentration ($OD_{600}$), and plant cultivation temperature. Silencing efficiency was statistically analyzed, and *HaPDS* expression levels were quantified by qPCR. After various trials, it was determined that seed vacuum infiltration followed by 6 h of co-cultivation produced the most effective VIGS results. The optimal conditions involved silencing the *HaPDS3* fragment near the 3′ end, an $OD_{600}$ value of 1.0, and a plant ambient temperature of 22°C, resulting in relatively high silencing efficiency in Huiyuan119 and GK 2002. This study aims to develop an efficient and stable gene silencing system for sunflower, establishing a foundation for the subsequent validation of gene functions in this species.

**Keywords:** *H. annuus*, *HaPDS* gene, VIGS system, gene silencing
**DOI:** 10.25165/j.ijabe.20261902.10338

**Citation:** Liu D Q, Kong X J, Lu Y, Guo S, Jing L. Construction and optimization of TRV-mediated VIGS silencing system of *Helianthus annuus*. Int J Agric & Biol Eng, 2026; 19(2): 78–87.

## 1 Introduction

Sunflower (*Helianthus annuus*) is an annual herbaceous dicotyledonous plant belonging to the Asteraceae family. According to the Food and Agriculture Organization of the United Nations (FAO) in 2024, the global sunflower planting area exceeds $2.95\times10^7$ $hm^2$. China ranks ninth worldwide in sunflower planting area and fourth in production output[1]. Despite this, sunflower currently lacks efficient methods for gene function validation, which hinders progress in molecular breeding. To tackle this issue, this study reviewed successful applications of the TRV-VIGS system in validating gene functions in crops such as tomato (*Solanum lycopersicum*)[2-4], tobacco (*Nicotiana benthamiana*)[5], cotton (*Gossypium hirsutum*)[6], and cassava (*Manihot esculenta*)[7]. Drawing from these achievements, this study proposed that TRV-mediated VIGS could overcome the challenges of gene function validation in sunflower, offering a novel validation approach.

Kumagai et al. used a recombinant virus to silence the phytoene desaturase (PDS) gene in *N. benthamiana* in 1995, achieving post-transcriptional gene silencing (PTGS)[8]. This experiment laid the foundation for the development of virus-induced gene silencing (VIGS) technology and marked the first demonstration of PTGS via viral vectors. Furthermore, the VIGS process, which serves as a defense mechanism against viral invasion, typically occurs in the cytoplasm and is considered as a form of PTGS in plants. In contrast, RNA interference (RNAi), a similar mechanism, is observed in animals[9,10]. The most widely used VIGS inoculation method for practical applications is *A. tumefaciens*-mediated delivery, in which the VIGS vector is carried by the T-DNA of *A. tumefaciens* and subsequently integrated into the host genome.

In 2001, Ratcliff et al. developed a VIGS vector based on Tobacco Rattle Virus (TRV), which has become the most widely used VIGS vector due to several advantages. These include mild viral symptoms in host plants, high silencing efficiency, prolonged silencing duration, absence of phenotype masking, and the ability to induce gene silencing across various tissues[11,12]. The transmission and replication of TRV involve two viral RNAs, TRV1 (RNA1) and TRV2 (RNA2). In TRV2, multiple cloning sites replace two non-structural protein-coding genes, allowing the insertion of target gene fragments for silencing. Constructs derived from TRV1 and TRV2 are cloned into a binary vector for infiltration mediated by *A. tumefaciens*[13].

Phytoene desaturase (PDS) is a critical rate-limiting enzyme in the carotenoid biosynthesis pathway[14]. Silencing the *PDS* gene disrupts normal carotenoid biosynthesis, resulting in photobleaching in plants. Due to the easily observable nature of photobleaching phenotypes, the *PDS* gene serves as a control in VIGS experiments. The ratio of photobleached plants to the total number of treated plants can indirectly reflect the silencing efficiency of VIGS. This indicator gene has been utilized in VIGS systems across various species, including *Arabidopsis thaliana*[3], *N. benthamiana*[8], *S. lycopersicum*[15], eggplant (*Solanum melongena*)[16], *T. aestivum*[17,18],

**Received date:** 2025-11-22 **Accepted date:** 2026-04-01
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saffron (*Crocus sativus*)[19], *H. annuus*[20], barley (*Hordeum vulgare*), and foxtail millet (*Setaria italica*)[18].

Numerous factors influence the silencing efficiency of VIGS, including environmental conditions, the gene fragments inserted, and the inoculation methods used. In *S. lycopersicum*, the silencing efficiency of the *SlPDS* gene is enhanced under low-temperature (15°C) and low-humidity (30%) conditions[21]. A reduction in cultivation temperature from 25°C to 22°C results in a 33% increase in the silencing rate of the *SlPDS* gene[22]. In *N. benthamiana*, gene silencing is restricted to the infiltrated leaf tissues when exposed to high light intensity (≥450 $\mu$E/m$^2$/s) and high temperature (≥30°C), with no systemic spread observed[23]. Both the homology and length of the inserted fragment, as well as the characteristics of the target gene, affect VIGS silencing efficiency. Among solanaceous plants, heterologous gene sequences from distantly related species can effectively silence their respective orthologous genes through TRV-mediated VIGS in *N. benthamiana*[24]. The specific region of *NbPDS* cDNA used for silencing has a limited impact on efficiency; insertions at the 5′ and 3′ ends of the target gene are less effective than those positioned in the middle[25]. The practical limit for inserting gene sequences into pTRV2 for silencing is approximately 1.5 kb, with typical fragment sizes ranging from 300 to 500 bp. To ensure effective silencing of endogenous genes, multiple fragments with homology exceeding 23 nucleotides are employed[26]. Inserting target gene fragments in the forward orientation into the VIGS vector results in lower silencing efficiency compared to reverse insertion[27]. Additionally, the inoculation method influences gene silencing efficiency. *A. tumefaciens*-mediated inoculation is currently the most widely used method due to its convenience and high infection efficiency[28]. Depending on the characteristics of different plant species, alternative methods such as rubbing inoculation, particle bombardment, injection, root irrigation, high-pressure spray, seed vacuum infiltration, and cotyledon infiltration are also available[27].

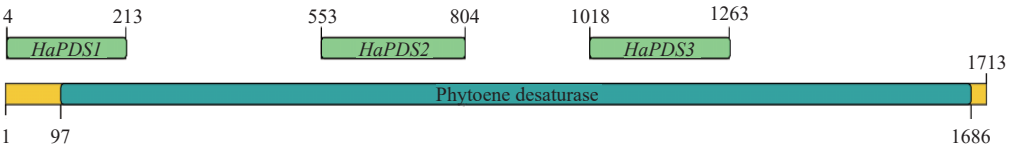
Variations in gene silencing efficiency have been observed among different cultivars of the same species when using the TRV-VIGS system. For example, in soybean (*Glycine max*), effective silencing of the *GmPDS* gene occurs in cultivars such as NanNong 47 and AnDou 203, but not in HeDou 12 and ZhongHuang 311[28]. The silencing efficiency of the *CaPDS* gene in chili pepper (*Capsicum annuum*) ranks as Early Calwonder>PBC137>CM34[29]. Although gene silencing has been successfully achieved using the sunflower TRV-VIGS system[20], a fully optimized TRV-VIGS protocol specifically tailored for sunflower cultivars has yet to be developed. To achieve optimal gene silencing in sunflower, it is essential to develop a customized silencing system tailored to specific cultivars.

This study utilizes TRV as a viral vector and *HaPDS* as a reporter gene to develop a VIGS system in *H. annuus*. The establishment of this system is significant not only for elucidating the gene functions of *H. annuus* and the *Helianthus* genus but also for providing a theoretical foundation for developing VIGS systems in plants where genetic transformation systems are difficult to establish.

## 2 Materials and methods

### 2.1 TRV-VIGS vector construction and transformation

Three gene fragments, each 200 bp-350 bp in length, were randomly selected from the coding sequence (CDS) of *HaPDS* (NCBI accession number: XM_022157947.2) in the forward direction and designated *HaPDS1* (4-213 bp), *HaPDS2* (553-804 bp), and *HaPDS3* (1018-1263 bp) (Figure 1). The plasmids pTRV1 and pTRV2 were provided by Professor Hada (Inner Mongolia University, China). Specific primers targeting CDS region of the gene were designed using SnapGene software (Table 1). Sunflower genomic cDNA were used as the PCR template. The PCR procedure followed the protocol supplied with the Phanta Max Super-Fidelity DNA Polymerase kit (Vazyme Biotech Co., Ltd., Nanjing, China).




Note: The full-length region denotes the CDS of the *HaPDS* gene (1 bp-1713 bp); the blue-green region signifies the conserved domain of *PDS* (97 bp-1686 bp); the light green region indicates the gene fragments of *HaPDS*.

Figure 1 Diagram of the *PDS* fragments

The linear pTRV2 vector was generated by digesting the pTRV2 plasmid with the *Bam*HI restriction endonuclease (New England Biolabs, Ltd., Beijing, China) at 37°C for 15 min. The digested products were then analyzed by 1% agarose gel electrophoresis; the correct band was excised, purified, and recovered using the HiPure Gel Pure DNA Mini Kit (Magen Biotechnology Co., Ltd., Guangzhou, China). Gene fragments were inserted into the pTRV2-empty vector in reverse orientation, with the 5′ homology arm of the vector template strand ligated to the 3′ end of the gene fragment, and the 3′ homology arm of the vector template strand ligated to the 5′ end of the gene fragment. The PCR product of *HaPDS* was subsequently ligated to the linear pTRV2 vector using the ClonExpress® II One Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China). Following ligation at 37°C for 30 min, the reaction mixture was cooled to 4°C and placed on ice. Transformation of the recombinant vector into competent *Escherichia coli* cells was performed using the DH5α Chemically Competent Cell kit (Weidi Biotechnology Co., Ltd., Shanghai, China). Single colonies were selected and cultured in 1 mL of LB liquid medium supplemented with 50 $\mu$g/mL kanamycin, incubated at 37°C and 200 r/min for 12 h. PCR identification of bacterial cultures was conducted using specific primers (Table 1). Bacterial cultures from positive clones were sent to Shanghai Sangon Biotech Co., Ltd. for sequencing. The pTRV2-*HaPDS* recombinant plasmid was extracted using the TIANprep Mini Plasmid Kit (TIANGEN Biotech Co., Ltd., Beijing, China). All TRV constructs (pTRV1, pTRV2-empty, pTRV2-*HaPDS1*, pTRV2-*HaPDS2*, and pTRV2-*HaPDS3*) were transformed into *A. tumefaciens* using the GV3101 Chemically Competent Cell kit (Weidi Biotechnology Co., Ltd., Shanghai, China).

A starter culture of *A. tumefaciens* was prepared by inoculating a PCR-validated colony into 5 mL of LB liquid medium supplemented with 50 $\mu$g/mL kanamycin and 20 $\mu$g/mL rifampicin. The culture was incubated for 1.5 d at 28°C and 180 r/min.

Subsequently, 200 $\mu$L of each culture was transferred into 50 mL portions of LB medium containing the same antibiotic concentrations and grown at 28°C and 180 r/min until reaching optical densities ($OD_{600}$) of 0.5, 1.0, and 1.5. The *A. tumefaciens* cells were harvested by centrifugation at 4000 r/min for 5 min, and the resulting pellet was resuspended in infiltration buffer composed of 10 mmol/L 2-morpholinoethanesulfonic acid (MES), 200 $\mu$mol/L acetosyringone (AS), 10 mmol/L $MgCl_2$, and 5% (w/v) sucrose. This washing process was repeated twice, and the final suspension was incubated at room temperature for 4 h before VIGS infection. An agroinfiltration solution of 20 mL was prepared by combining *A. tumefaciens* carrying pTRV1 with *A. tumefaciens* carrying either the pTRV2 empty vector or various pTRV2-derived vectors (pTRV2-*HaPDS1*, pTRV2-*HaPDS2*, and pTRV2-*HaPDS3*) in a 1:1 ratio. Sterilized sunflower seeds were soaked in 20 mL of the agroinfiltration solution in Petri dishes.

**Table 1 Primers sequence**

| Gene name | Primer (5′-3′) | Tm/ °C | Amplicon size/bp |
|---|---|---|---|
| TRV-*HaPDS1*-F | **AGAAGGCCTCCATGG**GGATCCTCTT GGATAGTCCACGCAGACC | 55 | 210 |
| TRV-*HaPDS1*-R | **CGTGAGCTCGGTACC**GGATCCTCTCT GCTTGGAAATGTATCCG | | |
| TRV-*HaPDS2*-F | **AGAAGGCCTCCATGG**GGATCCTCAG TGGTAACTCGATCCGGT | 55 | 245 |
| TRV-*HaPDS2*-R | **CGTGAGCTCGGTACC**GGATCCGCAA TGCCAAACAAGCCTGG | | |
| TRV-*HaPDS3*-F | **AGAAGGCCTCCATGG**GGATCCGCTC CTGCTGAAAAGTAGGTGA | 54 | 246 |
| TRV-*HaPDS3*-R | **CGTGAGCTCGGTACC**GGATCCATCG AGTTAAACAAGGATGGAACT | | |
| TRV2-F | GTTCAGGCGGTTCTTGTGTGTC | 56 | - |
| TRV2-R | GTCGAGAATGTCAATCTCGTAGGT | | |
| qPCR-*HaPDS*-F | TTGGAATTGGTTTTTGCACCTGC | 56 | 121 |
| qPCR-*HaPDS*-R | TGCTCCCATCTTCTGCAATTT | | |
| qPCR-*HaActin*-F | GAAACATCGTGCTTAGTGGCG | 60 | 178 |
| qPCR-*HaActin*-R | TGCTGGAAGGCTGAGTGA | | |

Note: The parts in bold are the vector homology arms; the underlined part is the *Bam*H I recognition site.

### 2.2 Plant material and growing conditions

The confectionery sunflower cultivars Huiyuan119, LD5009, and 3936, along with the oilseed sunflower line R5 and cultivar GK2002, all fully mature and dry, were obtained from the Plant Pathology Laboratory of Inner Mongolia Agricultural University. After sequentially removing the outer and inner seed coats, the seeds were surface-sterilized with a 3% hydrogen peroxide solution for 30 min, followed by three rinses with sterile water. Subsequently, the peeled seeds were gently and evenly scraped with a sterile inoculating needle. The seeds were then immersed in the agroinfiltration suspension in a petri dish at room temperature. Vacuum infiltration was performed using a vacuum pump at a pressure of 0.80 MPa, involving three cycles of 3 min of vacuum followed by 3 min of pause each. The Petri dishes were sealed with Parafilm and placed on a shaker at 28°C and 50 r/min for several hours. The infected seeds were cultivated in a growth chamber in square (6 cm×6 cm) and round (14 cm diameter) plastic pots filled with a growth medium consisting of a 1:1 ratio of nutrient soil to vermiculite. The growth chamber environment was maintained at a controlled temperature, with an 18 h light and 6 h dark cycle, approximately 55% relative humidity, and a light intensity of 6000 lx.

### 2.3 Optimization of the silencing system

#### 2.3.1 Exploring high-efficiency inserted PDS fragments

*A. tumefaciens* containing either the pTRV1 and pTRV2 empty vectors or the pTRV1 and pTRV2-*HaPDS1*, *HaPDS2*, and *HaPDS3* recombinant vectors were adjusted to an $OD_{600}$ of 1.0. The seeds were then immersed in the *A. tumefaciens* suspension, subjected to vacuum infiltration, and subsequently incubated in the dark on a shaker at 28°C and 50 r/min for 6 h. Following treatment, the seeds were planted in pots and cultivated in a growth chamber set at 22°C. The infection efficiency of pTRV-*PDS* was evaluated by observing photobleaching in 28-day-old seedlings. Nine seedlings were counted from each of the five cultivars to determine silencing efficiency. The qPCR test samples consisted of the third and fourth true leaves of plants with photo-bleaching or TRV virus phenotypes, flash-frozen in liquid nitrogen, and stored at –80°C.

#### 2.3.2 Exploring high-efficiency vacuum infiltration

*A. tumefaciens* harboring pTRV1 and the most efficient pTRV2-*HaPDS* construct, as determined above, was standardized to an $OD_{600}$ of 1.0. The seeds were soaked in the *A. tumefaciens* suspention and subjected to either vacuum or non-vacuum infiltration. After a 6 h incubation in darkness on a shaker at 28°C and 50 r/min, all seeds were transferred to a growth chamber set at 22°C. Infection efficiency was evaluated following the procedure described in Section 2.3.1.

#### 2.3.3 Exploring high-efficiency co-cultivation time

*A. tumefaciens* harboring pTRV1 and the most efficient pTRV2-*HaPDS* construct was standardized to an $OD_{600}$ of 1.0. Seeds were submerged in the *A. tumefaciens* suspension and subsequently vacuum-infiltrated. This was followed by dark incubation on a shaker at 28°C and 50 r/min for 2, 6, and 18 h. All seeds were then grown in a growth chamber maintained at 22°C. Infection efficiency was evaluated following the procedure described in Section 2.3.1.

#### 2.3.4 Exploring high-efficiency agroinfiltration concentration

*A. tumefaciens* harboring pTRV1 and the most efficient pTRV2-*HaPDS* construct were prepared at varying optical densities ($OD_{600}$= 0.5, 1.0, and 1.5). Seeds were submerged in the *A. tumefaciens* suspension, vacuum infiltrated, and then incubated on a shaker in the dark at 28°C and 50 r/min for 6 h. Subsequently, the seeds were cultivated in a growth chamber maintained at 22°C. Infection efficiency was evaluated following the procedure described in Section 2.3.1.

#### 2.3.5 Exploring high-efficiency cultivation temperature

*A. tumefaciens* harboring pTRV1 and the most efficient pTRV2-*HaPDS* construct were adjusted to an $OD_{600}$ of 1.0. Seeds were submerged in the *A. tumefaciens* suspension and then vacuum infiltrated, followed by a 6 h dark incubation on a shaker at 28°C and 50 r/min. The seeds were subsequently in growth chambers set to either 22°C or 25°C. Infection efficiency was evaluated following the procedure described in Section 2.3.1.

### 2.4 Total RNA extraction and expression analyses

A 100 mg leaf sample was pulverized using a mortar. Total RNA was extracted from the sample using the RNAsimple Total RNA Kit (TIANGEN Biotech Co., Ltd., Beijing, China). The concentration and integrity of the RNA were assessed using micro-UV spectrophotometry. Subsequently, 4 $\mu$L of the RNA sample was mixed with 2 $\mu$L of 6× DNA loading buffer, and electrophoresis was performed on a 1% agarose gel at 140 V for 25 min. The RNA was reverse-transcribed to generate cDNA using the PrimeScript™ RT Master Mix reagent kit (TaKaRa Biotechnology Co., Ltd., Beijing, China). After completion, the cDNA was promptly stored at –20°C. PCR reactions were conducted according to the

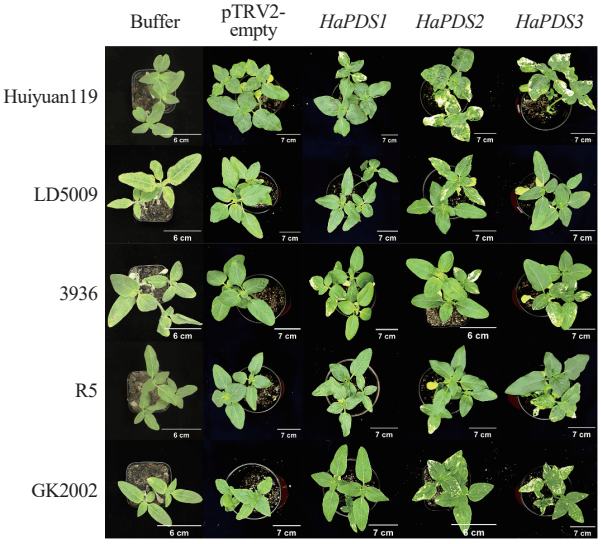
instructions provided with the Phanta Max Super-Fidelity DNA Polymerase kit (Vazyme Biotech Co., Ltd., Nanjing, China). The reaction volume was 50 μL, and the thermal cycling program consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 15 s, and extension at 72°C for 1 min, concluding with a final extension at 72°C for 5 min. Each reaction was conducted in triplicate with three independent biological replicates. The primers used for detection were TRV2-F and TRV2-R (Table 1). For gel electrophoresis, 4 μL of the PCR product was combined with 2 μL of 6× DNA loading buffer, and the mixture was loaded onto a 2% agarose gel. Electrophoresis was carried out at 140 V for 30 min and the results were visualized using a UV gel imager.

Primers were designed based on the coding sequence of the *HaPDS* gene using SnapGene software (Table 1). The internal reference gene selected was *HaActin* (NCBI accession number: XM_022142191.2). For qRT-PCR quantification, a 20 μL reaction mixture was prepared using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit from TaKaRa Biotechnology Co., Ltd., Beijing, China. The thermal cycling program included pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 60°C for 1 min, repeated for 40 cycles. Each reaction was performed in triplicate. Subsequently, melting and amplification curves were analyzed. The relative expression levels of the target gene under various treatment conditions were calculated using the $2^{-\Delta\Delta Ct}$ method. Data analysis was conducted using Excel 2019, while GraphPad Prism 9.5 was employed for plotting and correlation analysis.

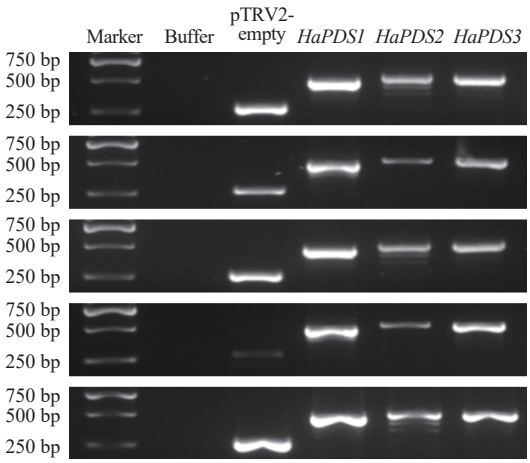
## 3 Results

### 3.1 Effect of different PDS fragments on VIGS efficiency in agroinoculated plants

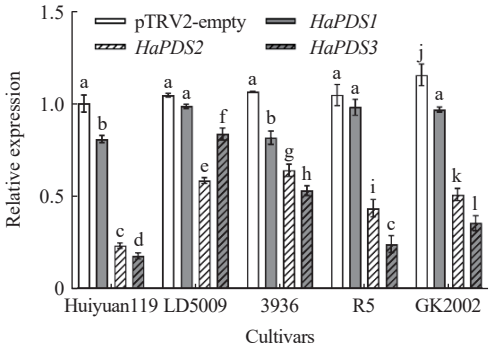
Silencing *HaPDS1* induced leaf photo-bleaching in two cultivars: Huiyuan119 and 3936. Silencing *HaPDS2* induced leaf photo-bleaching in five cultivars: Huiyuan119, LD5009, 3936, R5, and GK2002. Silencing *HaPDS3* induced leaf photo-bleaching in four cultivars: Huiyuan119, 3936, R5, and GK2002 (Figure 2a). The highest silencing efficiency of the *HaPDS3* fragment was observed in Huiyuan119 (88.89%) and GK2002 (88.89%). The highest silencing efficiency of the *HaPDS2* fragment was observed in




a. Symptoms of plants inoculated with *A. tumefaciens* harboring different TRV2-*HaPDS* constructs




b. RT-PCR results of PDS fragments with primers designed on the regions of TRV2 RNA flanking the insertion site




c. qRT-PCR analysis of *HaPDS* transcript levels in leaves inoculated with *A. tumefaciens* harboring different TRV2-*HaPDS* constructs

Note: The procedure involved vacuum infiltration with *A. tumefaciens* co-cultivation for 6 h, $OD_{600}$ of 1.0, and a plant ambient temperature of 22°C; Buffer: Infiltration buffer consisted of 10 mmol/L 2-morpholinoethanesulfonic acid (MES), 200 μmol/L acetosyringone (AS), 10 mmol/L $MgCl_2$, and 5% (w/v) sucrose; Letters indicate significant differences using the Fisher LSD test at $p \leq 0.05$.

Figure 2 Effect of different PDS fragments on silencing efficiency

Huiyuan119 (55.56%). The highest silencing efficiency of the *HaPDS1* fragment was observed in Huiyuan119 (11.11%) and 3936 (11.11%) (Table 2). PCR analysis confirmed the successful introduction of the pTRV2 vector, along with the recombinant vectors pTRV2-*HaPDS1*, pTRV2-*HaPDS2*, and pTRV2-*HaPDS3*, into the plants (Figure 2b). Silencing of *HaPDS1* did not cause a significant decrease in PDS gene expression levels in two cultivars: LD5009 and R5. In contrast, silencing of *HaPDS2* and *HaPDS3* resulted in a significant decrease in PDS gene expression levels across all five cultivars (Figure 2c). Based on the comparison provided, it is evident that *HaPDS3* demonstrates superior silencing effects and enhanced silencing efficiency in Huiyuan119, 3936, R5, and GK2002 (Table 2).

### 3.2 Effect of vacuum-induced agroinfiltration on VIGS efficiency in plants

Within the vacuum treatment group, leaf photo-bleaching was observed in all cultivars except LD5009. Within the non-vacuum treatment group, leaf photo-bleaching was observed in the Huiyuan119 and GK2002 (Figure 3a). The silencing efficiencies in the vacuum group were as follows: Huiyuan119 (88.89%), 3936 (55.56%), R5 (44.44%), GK2002 (88.89%), and LD5009 (0.00%). Within the non-vacuum treatment group, the efficiencies were Huiyuan119 (33.33%), 3936 (0.00%), R5 (0.00%), GK2002 (66.67%), and LD5009 (0.00%). Compared to the non-vacuum treatment, vacuum treatment significantly enhanced the silencing efficiency of the VIGS system (Table 3). PCR analysis confirmed the successful introduction of the pTRV2-*HaPDS3* recombinant vector into the plants (Figure 3b). The results of *HaPDS* expression levels showed that compared with non-vacuum treatment, vacuum treatment significantly decreased the transcript levels of the *HaPDS* gene in all five cultivars (Figure 3c).

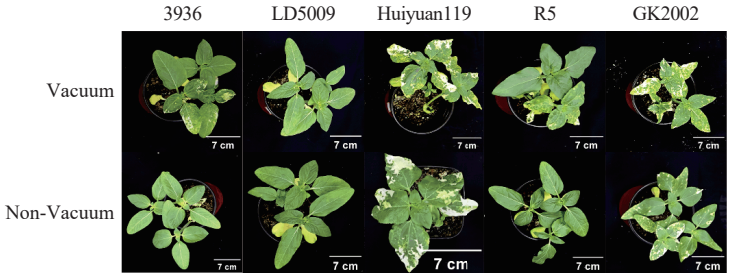
**Table 2 Number of *H. annuus* with photo-bleaching leaves after silencing different PDS fragments**

| Cultivars | Treatments | Number of photo-bleaching plants | Total number of plants | Silence efficiency/% |
|---|---|---|---|---|
| Huiyuan119 | *HaPDS1* | 1 | 9 | 11.11 |
| | *HaPDS2* | 5 | 9 | 55.56 |
| | *HaPDS3* | 8 | 9 | 88.89 |
| LD5009 | *HaPDS1* | 0 | 9 | 0.00 |
| | *HaPDS2* | 4 | 9 | 44.44 |
| | *HaPDS3* | 0 | 9 | 0.00 |
| 3936 | *HaPDS1* | 1 | 9 | 11.11 |
| | *HaPDS2* | 4 | 9 | 44.44 |
| | *HaPDS3* | 5 | 9 | 55.56 |
| R5 | *HaPDS1* | 0 | 9 | 0.00 |
| | *HaPDS2* | 3 | 9 | 33.33 |
| | *HaPDS3* | 4 | 9 | 44.44 |
| GK2002 | *HaPDS1* | 0 | 9 | 0.00 |
| | *HaPDS2* | 4 | 9 | 44.44 |
| | *HaPDS3* | 8 | 9 | 88.89 |

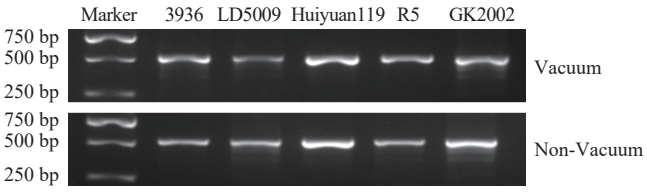
Note: The silence efficiency is calculated as:

$$\text{Silence efficiency} = \frac{\text{Number of plants with leaf photo-bleaching}}{\text{Total number of plants}}$$

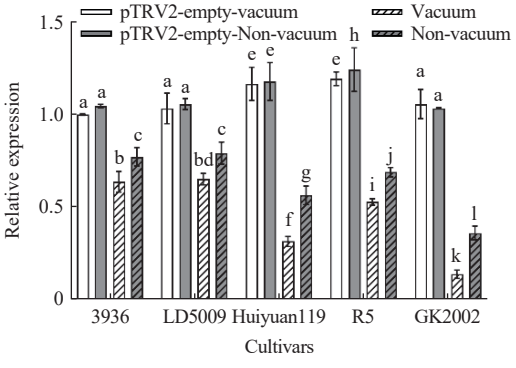
Same as below.




a. Symptoms of plants inoculated following vacuum-induced agroinfiltration




b. RT-PCR results of vacuum treatment with primers designed on the regions of TRV2 RNA flanking the insertion site




c. qRT-PCR analysis of *HaPDS* transcript levels in leaves following vacuum-induced agroinfiltration

Note: The procedure involved using a fragment of the *HaPDS3* gene for silencing, with *A. tumefaciens* co-cultivation of 6 h; $OD_{600}$ of 1.0, and a plant ambient temperature of 22°C; Letters indicate significant differences using the Fisher LSD test at $p \leq 0.05$.

Figure 3 Effect of vacuum-induced agroinfiltration on VIGS efficiency in *H. annuus*

### 3.3 Effect of *A. tumefaciens* co-cultivation durations on VIGS efficiency in plants

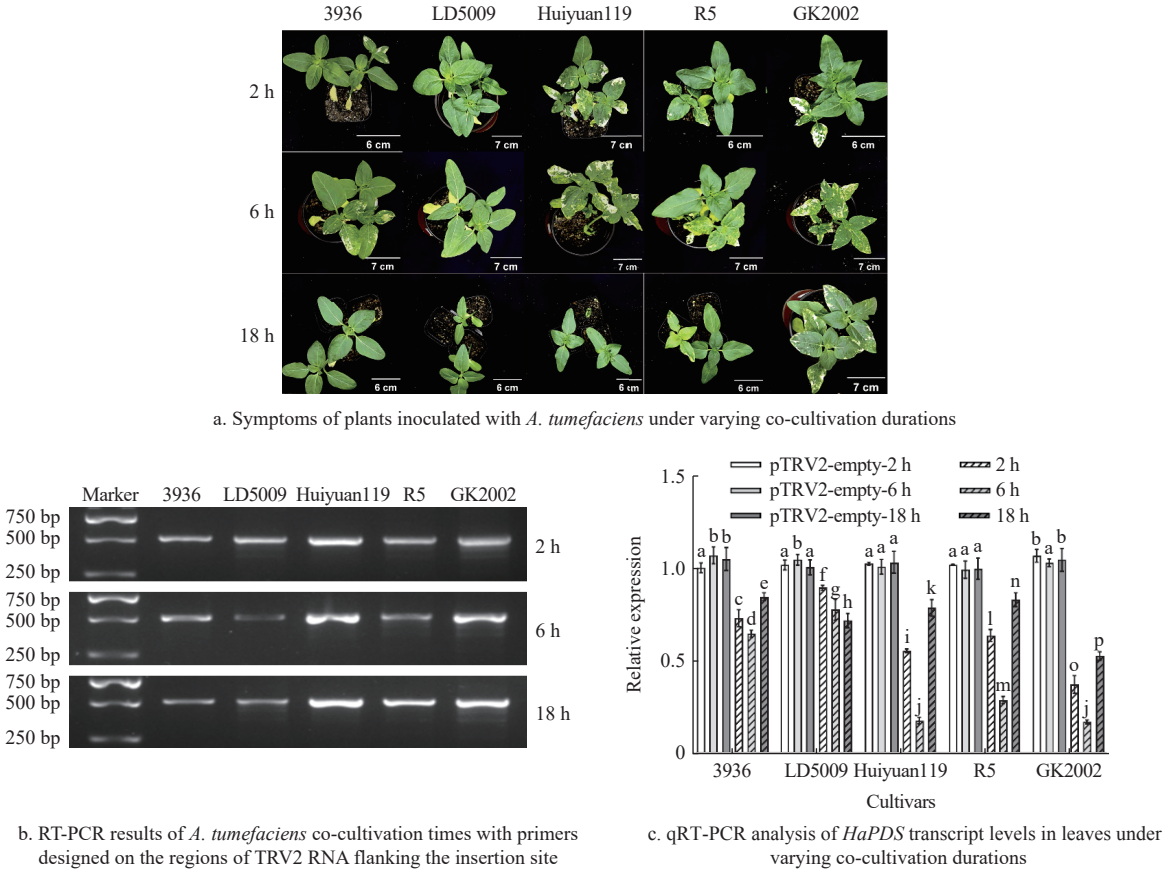
The duration of co-cultivation with *A. tumefaciens* significantly influenced gene silencing efficiency, showing variability across sunflower cultivars. Leaf photo-bleaching was observed in the cultivars 3936, R5, Huiyuan119, and GK2002 after 2 h and 6 h of co-cultivation. After 18 h of co-cultivation, leaf photo-bleaching was observed only in R5 and GK2002 (Figure 4a). Gene silencing efficiencies following 2 h of co-cultivation were Huiyuan 119 (33.33%), 3936 (11.11%), R5 (44.44%), GK2002 (22.22%), and

LD5009 (0.00%). After 6 h of co-cultivation, efficiencies increased to Huiyuan 119 (88.89%), 3936 (55.56%), R5 (44.44%), and GK2002 (88.89%) (Table 4). The VIGS system demonstrated significantly enhanced silencing efficiency after 6 h compared to 2 h of co-cultivation. An 18 h co-cultivation period resulted in the lowest overall efficiency and caused stunted growth in all five cultivars.

PCR analysis confirmed the successful integration of the pTRV2-*HaPDS3* recombinant vector into the plants (Figure 4b). The results of *HaPDS* expression levels showed co-cultivation with *A. tumefaciens* for 6 h significantly decreased the transcript levels of the *HaPDS* gene in the VIGS system compared to 2 h and 18 h in four cultivars: 3936, Huiyuan119, R5, and GK2002 (Figure 3c).

**Table 3 Number of *H. annuus* with photo-bleaching leaves following vacuum-induced agroinfiltration**

| Cultivars | Treatments | Number of photo-bleaching plants | Total number of plants | Silence efficiency/% |
|---|---|---|---|---|
| Huiyuan119 | Vacuum | 8 | 9 | 88.89 |
| | Non-vacuum | 3 | 9 | 33.33 |
| LD5009 | Vacuum | 0 | 9 | 0.00 |
| | Non-vacuum | 0 | 9 | 0.00 |
| 3936 | Vacuum | 5 | 9 | 55.56 |
| | Non-vacuum | 0 | 9 | 0.00 |
| R5 | Vacuum | 4 | 9 | 44.44 |
| | Non-vacuum | 0 | 9 | 0.00 |
| GK2002 | Vacuum | 8 | 9 | 88.89 |
| | Non-vacuum | 6 | 9 | 66.67 |




a. Symptoms of plants inoculated with *A. tumefaciens* under varying co-cultivation durations

b. RT-PCR results of *A. tumefaciens* co-cultivation times with primers designed on the regions of TRV2 RNA flanking the insertion site

c. qRT-PCR analysis of *HaPDS* transcript levels in leaves under varying co-cultivation durations

Note: The procedure involved using a fragment of the *HaPDS3* gene for silencing with vacuum infiltration, $OD_{600}$ of 1.0, and a plant ambient temperature of 22°C; Letters indicate significant differences using the Fisher LSD test at $p \leq 0.05$.

Figure 4 Effect of *A. tumefaciens* co-cultivation durations on VIGS efficiency in *H. annuus*

### 3.4 Effect of different concentrations of agroinoculum on VIGS efficiency in plants

The concentration of *A. tumefaciens* as measured by $OD_{600}$ influenced silencing efficiency across different cultivars (Figure 5a). At an $OD_{600}$ of 1.0, leaf photo-bleaching occurred in 3936, R5, Huiyuan 119, and GK2002, resulting in highest silencing efficiencies of 55.56%, 44.44%, 88.89%, and 88.89%, respectively. In cultivar LD5009, highest silencing efficiency of 22.22% was observed at an $OD_{600}$ of 1.5 (Table 5).

PCR analysis confirmed the successful introduction of the pTRV2-*HaPDS3* recombinant vector into the plants (Figure 5b). The results of the relative expression levels of the *HaPDS* gene showed that in four cultivars (3936, Huiyuan119, R5, and GK2002), agroinoculum treatment at an $OD_{600}$ of 1.0 significantly decreased the transcript levels of the *HaPDS* gene in the VIGS system compared to treatments at $OD_{600}$ of 0.5 and 1.5. In LD5009, treatment with *A. tumefaciens* at an $OD_{600}$ of 1.5 significantly decreased the transcript levels of the *HaPDS* gene in the VIGS system, achieving the highest silencing efficiency (Figure 5c).

### 3.5 Effect of different ambient temperatures on VIGS efficiency in agroinoculated plants

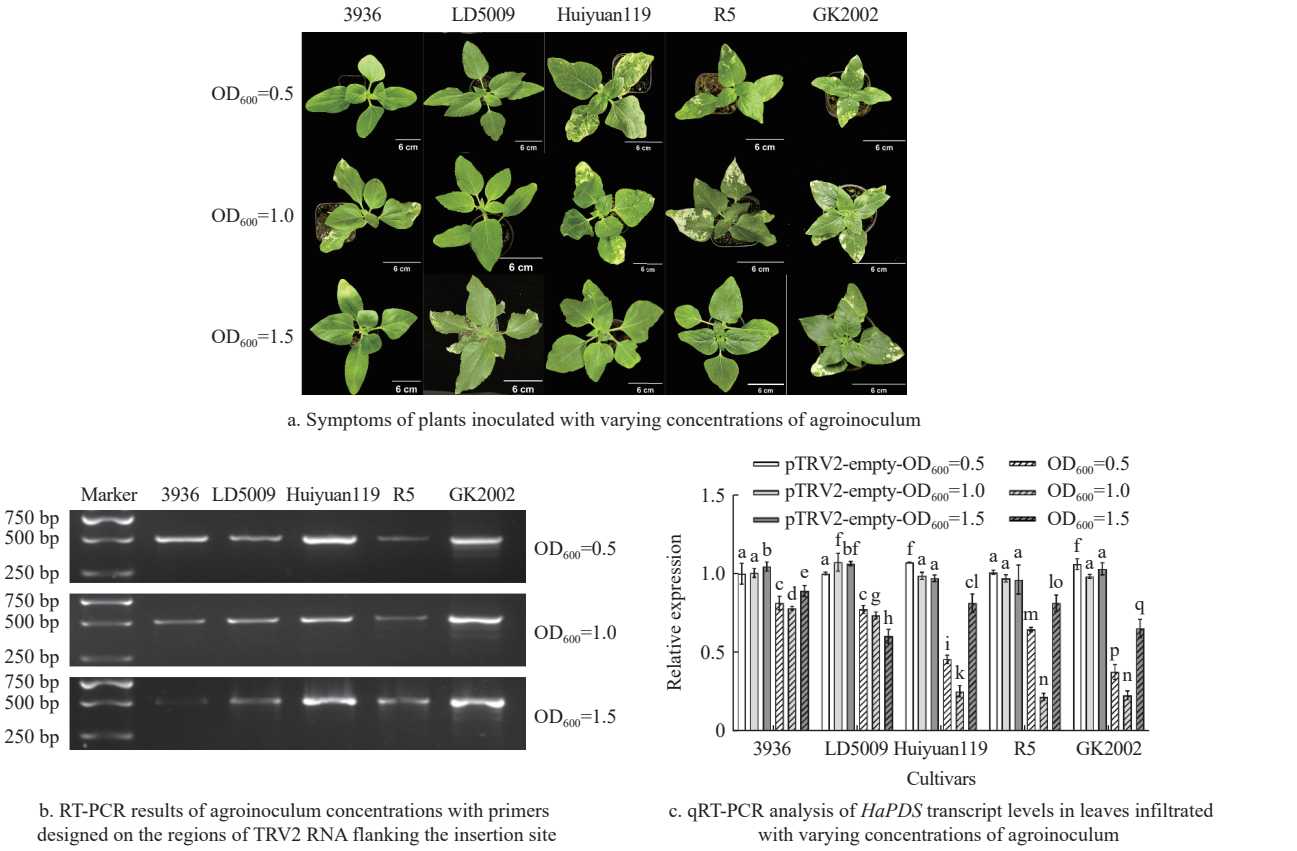
The ambient temperature of plants influenced silencing efficiency across different cultivars except LD5009 (Figure 6a). Leaf photo-bleaching was observed in the cultivars 3936, R5, Huiyuan119, and GK2002 at ambient temperatures of 22°C and 25°C. At an ambient temperature of 22°C, the silencing efficiencies were 3936 (55.56%), R5 (44.44%), GK2002 (88.89%), and Huiyuan119 (88.89%). When the temperature increased to 25°C, the silencing efficiencies decreased to 3936 (44.44%), R5 (33.33%), GK2002 (66.67%), and Huiyuan119 (77.78%) (Table 6). PCR

**Table 4 Number of *H. annuus* with photo-bleaching leaves under varying *A. tumefaciens* co-cultivation durations**

| Cultivars | Treatments | Number of photo-bleaching plants | Total number of plants | Silence efficiency/% |
|---|---|---|---|---|
| Huiyuan119 | 2 h | 3 | 9 | 33.33 |
| | 6 h | 8 | 9 | 88.89 |
| | 18 h | 0 | 9 | 0.00 |
| LD5009 | 2 h | 0 | 9 | 0.00 |
| | 6 h | 0 | 9 | 0.00 |
| | 18 h | 0 | 9 | 0.00 |
| 3936 | 2 h | 1 | 9 | 11.11 |
| | 6 h | 5 | 9 | 55.56 |
| | 18 h | 0 | 9 | 0.00 |
| R5 | 2 h | 4 | 9 | 44.44 |
| | 6 h | 4 | 9 | 44.44 |
| | 18 h | 1 | 9 | 11.11 |
| GK2002 | 2 h | 2 | 9 | 22.22 |
| | 6 h | 8 | 9 | 88.89 |
| | 18 h | 6 | 9 | 66.67 |

**Table 5 Number of *H. annuus* with photo-bleaching leaves at varying concentrations of agroinoculum**

| Cultivars | Treatments | Number of photo-bleaching plants | Total number of plants | Silence efficiency/% |
|---|---|---|---|---|
| Huiyuan119 | $OD_{600}$ of 0.5 | 6 | 9 | 66.67 |
| | $OD_{600}$ of 1.0 | 8 | 9 | 88.89 |
| | $OD_{600}$ of 1.5 | 4 | 9 | 44.44 |
| LD5009 | $OD_{600}$ of 0.5 | 0 | 9 | 0.00 |
| | $OD_{600}$ of 1.0 | 0 | 9 | 0.00 |
| | $OD_{600}$ of 1.5 | 2 | 9 | 22.22 |
| 3936 | $OD_{600}$ of 0.5 | 0 | 9 | 0.00 |
| | $OD_{600}$ of 1.0 | 5 | 9 | 55.56 |
| | $OD_{600}$ of 1.5 | 0 | 9 | 0.00 |
| R5 | $OD_{600}$ of 0.5 | 2 | 9 | 22.22 |
| | $OD_{600}$ of 1.0 | 4 | 9 | 44.44 |
| | $OD_{600}$ of 1.5 | 0 | 9 | 0.00 |
| GK2002 | $OD_{600}$ of 0.5 | 7 | 9 | 77.78 |
| | $OD_{600}$ of 1.0 | 8 | 9 | 88.89 |
| | $OD_{600}$ of 1.5 | 5 | 9 | 55.56 |




a. Symptoms of plants inoculated with varying concentrations of agroinoculum

b. RT-PCR results of agroinoculum concentrations with primers designed on the regions of TRV2 RNA flanking the insertion site

c. qRT-PCR analysis of *HaPDS* transcript levels in leaves infiltrated with varying concentrations of agroinoculum

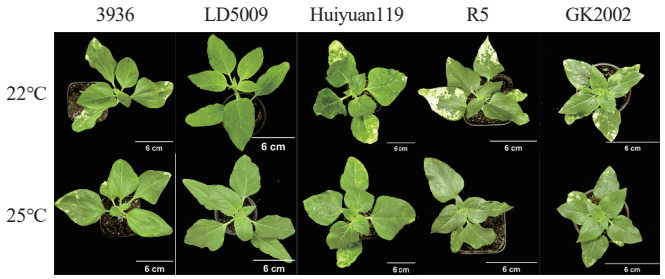
Note: The procedure involved using a fragment of the *HaPDS3* gene for silencing with vacuum infiltration, *A. tumefaciens* co-cultivation of 6 h, and a plant ambient temperature of 22°C; Letters indicate significant differences using the Fisher LSD test at $p \leq 0.05$.

Figure 5 Effect of concentrations of agroinoculum on VIGS efficiency in *H. annuus*

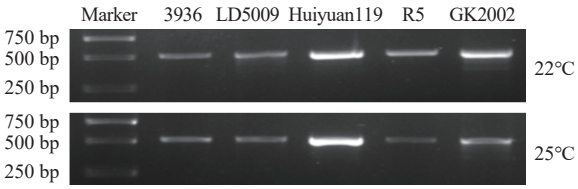
analysis confirmed the successful introduction of the pTRV2-*HaPDS3* recombinant vector into the plants (Figure 6b). Among all five cultivars, the results of *HaPDS* expression levels indicated that an ambient temperature of 22°C significantly decreased the transcript levels of the *HaPDS* gene in the VIGS system compared to an ambient temperature of 25°C (Figure 6c).

## 4 Discussion

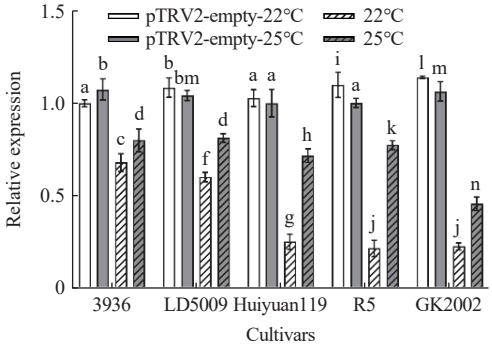
Sunflowers often rely on heterologous transformation systems, such as *A. thaliana* or *N. benthamiana*, to indirectly assess gene function due to the absence of a stable genetic transformation system. The VIGS system offers a novel approach for validating gene function by directly silencing target genes within the crop, thereby enabling reverse validation of their roles. The effectiveness of gene silencing via VIGS critically impacts gene function validation and depends on the virus's ability to efficiently infect plants and replicate at high levels within target tissues[30]. Four main factors influence this efficiency: 1) the choice of viral vectors; among various options, the TRV vector is commonly used in VIGS studies of both dicot and monocot plants due to its advantages, including mild viral symptoms and the capacity to invade meristematic tissues[31]; 2) the characteristics of the inserted




a. Symptoms of plants inoculated at varying ambient temperatures




a. RT-PCR results of ambient temperatures with primers designed on the regions of TRV2 RNA flanking the insertion site




b. qRT-PCR analysis of *HaPDS* transcript levels in leaves at varying ambient temperatures

Note: The procedure involved using a fragment of the *HaPDS3* gene for silencing with vacuum infiltration, *A. tumefaciens* co-cultivation of 6 h, and $OD_{600}$ of 1.0; Letters indicate significant differences using the Fisher LSD test at $p \leq 0.05$.

Figure 6 Effect of ambient temperatures on VIGS efficiency in *H. annuus*

**Table 6 Number of *H. annuus* with photo-bleaching leaves at varying ambient temperatures**

| Cultivars | Treatments | Number of photo-bleaching plants | Total number of plants | Silence efficiency/% |
|---|---|---|---|---|
| Huiyuan119 | 22°C | 8 | 9 | 88.89 |
| | 25°C | 7 | 9 | 77.78 |
| LD5009 | 22°C | 0 | 9 | 0.00 |
| | 25°C | 0 | 9 | 0.00 |
| 3936 | 22°C | 5 | 9 | 55.56 |
| | 25°C | 4 | 9 | 44.44 |
| R5 | 22°C | 4 | 9 | 44.44 |
| | 25°C | 3 | 9 | 33.33 |
| GK2002 | 22°C | 8 | 9 | 88.89 |
| | 25°C | 6 | 9 | 66.67 |

fragments, including length and sequence homology[22]; 3) operational techniques, such as optimizing *A. tumefaciens* suspension concentration, vacuum infiltration parameters, and co-cultivation duration[32]; and 4) intrinsic plant factors, including cultivars background[28], physiological age[33], and tissue type[34]. Together, these factors determine the actual silencing efficiency of the VIGS system in sunflowers.

In this experiment, the *HaPDS3* gene fragment demonstrated the highest silencing efficiency among the cultivars Huiyuan119, R5, GK2002, and 3936. Conversely, the *HaPDS2* gene fragment exhibited the greatest silencing efficiency in the LD5009, while the *HaPDS1* gene fragment was relatively ineffective across all five cultivars. In soybean, gene fragments containing conserved domains showed a 20% higher silencing efficiency in roots and leaves compared to those lacking conserved domains[35]. As shown in Figure 1, the *HaPDS1* gene fragment partially contains the *PDS* conserved domain sequence. Therefore, the *HaPDS1* fragment exhibits lower silencing efficiency for the *PDS* gene compared to *HaPDS2* and *HaPDS3*. In the LD5009 variety, the *HaPDS2* fragment showed greater silencing efficiency than *HaPDS3*, likely because siRNAs targeting regions closer to the start codon are generally more effective than those targeting regions further downstream[30]. When employing the TRV viral vector to induce gene silencing in *N. benthamiana*, silencing fragments can activate silencing across nearly any region of the *NbPDS* gene cDNA. In contrast, the Potato Virus X (PVX) vector allows gene fragments inserted into the middle and 5′ end of the *NbPDS* gene cDNA to effectively silence the PDS gene, while insertions at the 5′ and 3′ intron regions fail to induce silencing. Several studies have identified VIGS gene silencing fragments situated within the middle of the coding sequence (CDS) of the target gene[25]. To enhance the specificity of the silenced gene fragment, targeting the 5′ or 3′ ends can more accurately silence specific family members of the gene.

Vacuum-assisted infiltration of *A. tumefaciens* suspension enhances the efficiency of genetic transformation and VIGS infection, as seen in wheat, maize (*Zea mays*)[32], *N. benthamiana*[36], and *A. thaliana*[37]. In this experiment, vacuum treatment enhanced silencing efficiency in all cultivars except LD5009, in which silencing of the *HaPDS3* gene fragment was ineffective.

In wheat and maize, appropriately extending the co-cultivation time of seeds with *A. tumefaciens* can increase the number of infected plant cells, thereby enhancing silencing efficiency. However, exceeding the optimal co-cultivation duration reduces VIGS efficiency, likely due to plant mortality caused by *A. tumefaciens* infection[32]. In this experiment, photo-bleaching symptoms appeared after 2 h of co-cultivation. As the co-cultivation time increased, silencing efficiency in Huiyuan119, R5, GK2002, and 3936 improved, reaching a peak at 6 h. However, silencing efficiency declined at 18 h, with all five cultivars exhibiting plant death or stunting due to prolonged *A. tumefaciens* infiltration. LD5009 did not display leaf photo-bleaching, and varying the co-cultivation time did not enhance silencing of the *HaPDS3* gene fragment. Although no leaf photo-bleaching was observed in

LD5009, the expression level of the *PDS* gene decreased; a similar pattern was also observed in other cultivars.

The concentration of the *A. tumefaciens* culture significantly influences the efficiency of VIGS silencing. Lower concentrations lead to diminished efficiency, whereas higher concentrations may negatively impact plant survival rates[32]. The optimal concentration of *A. tumefaciens* culture for VIGS varies among different crops. For instance, the highest silencing efficiency in *A. thaliana* occurs at an $OD_{600}$ of 2.0[37], in tomato at an $OD_{600}$ of 1.5[24], and in chili pepper at an $OD_{600}$ of 1.0[29]. Excessively high concentrations of *A. tumefaciens* culture can induce necrosis; specifically, $OD_{600}$ values exceeding 1.0 result in necrosis in *N. benthamiana*[3]. Soaking naked cotton seeds in an *A. tumefaciens* culture with an $OD_{600}$ of 1.5 for 90 min produces the most effective seed-soak agroinoculation (SSA)-VIGS silencing[38]. For wheat and maize, optimal conditions for VIGS necessitate the use of *A. tumefaciens* culture with an optical $OD_{600}$ of 0.3, followed by a vacuum treatment lasting 30 to 60 s. Subsequently, co-cultivation occurs with cultures at the same concentration for 15 h[32]. These results indicate that different cultivars require certain concentrations of *A. tumefaciens* culture; thus, the concentration should be tailored for each TRV-VIGS experiment. In the cases of Huiyuan119 and GK2002, the *HaPDS* gene can be effectively silenced using the VIGS system at an $OD_{600}$ of 0.5, with the highest silencing efficiency observed at an $OD_{600}$ of 1.0 as the concentration of *A. tumefaciens* increases. The rate of change in silencing efficiency is greater for 3936 and R5 than for Huiyuan119 and GK2002; however, at an $OD_{600}$ of 0.5, silencing efficiency remains low or ineffective. LD5009 exhibits photo-bleaching at an $OD_{600}$ of 1.5. It is hypothesized that as the concentration of *A. tumefaciens* increases, the probability of generating effective silencing small interfering RNA (siRNA) also rises, thereby enhancing silencing efficiency; however, this increase is accompanied by a higher risk of plant mortality.

Temperature has a significant influence on TRV replication and propagation in plants, with low temperatures potentially increasing the VIGS efficiency[3]. While most plant viruses thrive at 20°C-25°C[22], the optimal temperature for maximal viral silencing effectiveness varies across different crop VIGS systems. For instance, in *N. benthamiana*, the highest VIGS silencing efficiency is achieved at 25°C[26], whereas in *C. annuum*[29] and *A. thaliana*[37], 22°C is deemed optimal. Similarly, in *S. lycopersicum*, temperatures of 21°C or lower result in high silencing efficiency[38]. Notably, compared to a temperature of 25°C, the VIGS silencing efficiency of Huiyuan119, R5, GK2002, and 3936 is enhanced at 22°C, with Huiyuan119 and GK2002 showing more pronounced improvements than R5 and 3936.

Genetic variation among cultivars of the same species can result in differing sensitivities to TRV virus infection, thereby affecting silencing efficiency[29,39]. The replication and transport efficiency of the TRV virus within crops significantly influence the silencing effectiveness of VIGS[24,40]. Varietal differences in sensitivity have been documented in crops such as *Cannabis sativa*[41], *Gerbera hybrida*[42], and *Rhododendron* species[43]. In this study, Huiyuan119 and GK2002 exhibited more pronounced leaf photo-bleaching and higher statistical silencing efficiency compared to other tested varieties, likely due to their increased susceptibility to TRV infection and more efficient viral replication and transmission. LD5009 is resistant to *Orobanche coerulescens*, whereas R5 and 3936 are believed to possess broad-spectrum resistance. These cultivars may also exhibit some resistance to the TRV virus, resulting in comparatively reduced silencing efficacy.

## 5 Conclusions

In light of the lack of suitable functional genetic tools for sunflowers, this study established and optimized a TRV-VIGS system using existing sunflower cultivars. By silencing the *HaPDS* gene, the resulting photo-bleaching phenotype served as a visual marker to indirectly assess silencing efficiency, thereby providing a practical tool for functional studies of sunflower genes. Experimental results showed that among three fragments targeting different regions of the *HaPDS* cDNA, *HaPDS3*, located near the 3′conserved domain, exhibited the highest silencing efficiency. The silencing efficiency of the TRV-VIGS system in sunflower was significantly enhanced by optimizing several key factors: vacuum treatment, a 6 h co-cultivation with *A. tumefaciens*, an agroinoculum concentration of 1.0 ($OD_{600}$), and a post-infiltration growth temperature of 22°C. Among the cultivars tested, GK2002 and Huiyuan119 showed superior silencing efficiency under these optimized conditions. Although this study preliminarily identified influential factors and established a high-efficiency silencing protocol, the underlying causes of silencing variation require further investigation.

## Acknowledgements

This research was supported by the Basic Research Funds of Inner Mongolia Agricultural University (Grant No. BR22-13-0922-13) and Natural Science Foundation of Inner Mongolia (Grant No. 2024LHMS03031), and the Open Project Program (Grant No. SKLCSRHPKF16) of State Key Laboratory for Crop Stress Resistance and High-Efficiency Production, Northwest A&F University.

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