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Establishment of Virus-Induced Gene Silencing (VIGS) System in Perennial *Rosa* Plants under Field Conditions

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ABSTRACT

Virus-induced gene silencing (VIGS) technique, which is developed in recent years, is a rapid identification of plant gene function from reverse genetics. It is a manifestation of post-transcriptional gene silencing mechanism. Compared with the traditional transgenic technology, VIGS is a transient expression system, which can achieve good results in a short time. At present, it is widely used to study the function of plant genes, but most of them are model plants, and the experiments are carried out always in the indoor environment with controlled light and temperature conditions. In this study, we creatively provided a method to establish VIGS system using perennial *Rosa* plants as experimental materials under field conditions. The recombinant virus vector was constructed with *RrGT1* gene as reporter gene and modified TRV-GFP virus as vector, and the perennial *R. rugosa* "Zizhi" and *R. davurica* were used as experimental verification materials. According to the growth conditions of *Rosa* plants, the natural environment in the field and the optimal conditions for the occurrence of VIGS, the technical problems such as the confirmation of the inoculation period, the preparation of the infective fluid, the inoculation technology of the virus vector and the light and temperature conditions of plant materials cultured after inoculation were solved one by one. When the *RrGT1* gene was silenced, the *Rosa* plants showed a pale petal color phenotype. By detection, it was found that the expression of endogenous *RrGT1* gene was significantly down-regulated, and the content of all anthocyanins also decreased significantly. Therefore, we believed that the attempt to establish VIGS system in perennial *Rosa* plants under field conditions was very successful.

1. INTRODUCTION

Many plants in the genus *Rosa*, such as *Rosa rugosa*, *Rosa chinensis* and *Rosa davidiana*, have great

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scientific research value. However, traditional hybrid breeding is still the main method in the study of many important characters (flower color, flower fragrance, etc.). With the development of functional genomics, more and more attention has been paid to the study of key genes that control traits. However, the method of gene function verification is very simple, the most widely used is transgenic technology [1-3]. For some plants that have not solved the problem of tissue culture, the implementation of transgenic technology is usually based on model plants, such as *Arabidopsis thaliana* and tobacco, as experimental materials. But for the key genes controlling flower color, flower fragrance, and other ornamental characters in the plants that were the subject of the study, even if the change of characters is observed in transgenic model plants, it cannot be equated with the characters of the plants themselves as the object of study. This is also a major restriction on the use of transgenic technology to verify gene function.

In addition to using transgenic technology to study the function of genes, virus-induced gene silencing (VIGS) is also an efficient genetic tool for functional genomics in plants. Usually, VIGS needs to construct a recombinant virus carrying a fragment of the target gene to silence a specific endogenous gene [4]. The host defense machinery, such as post-transcriptional gene silencing (PTGS), will be activated when the virus invades the host plant. PTGS will target double-stranded RNA (dsRNA), which is commonly used for replication of most plant viruses, and lead to cleavage of dsRNA and sequential generation of small interfering RNA (siRNA). The siRNAs are loaded into the RNAi silencing complex (RISC) to degrade any endogenous transcript which shows sequence homology to the siRNA [5-7]. In general, VIGS has become a simple and fast method to study gene function because of its simplicity and efficiency.

However, VIGS technology also has its limitations. Due to the limitation of host range, different host plants may need to select corresponding virus vectors, and different virus vectors may have different requirements for the length of inserted exogenous gene fragments [8]. In addition, the sensitivity of plant materials to virus vectors also determines the method of introducing virus vectors suitable for use in plant materials. And the optimal plant culture temperature, the circadian growth rhythm and the optimum propagation temperature of the virus carrier determine the light and temperature conditions of the whole experimental system [9]. These uncertainties directly lead to the possibility that different genera and even different species of the same genus may need an independent VIGS operating system, but cannot use the same set of universal VIGS system simply to achieve the purpose of efficient silencing function gene.

For the selection of viral vectors, the most widely used vector is TRV virus. It is applicable for many plants because the virus has a broad host range [10]. TRV virus is a double-stranded RNA virus [11]. The modified TRV-GFP vectors are also widely used because of its small molecular weight, wide host range, high infection rate, low pathogenicity and green fluorescence visualization after infection.

At present, there are many inoculation methods for VIGS, and the following are commonly used: vacuum infiltration method, high pressure spray gun method (leaf surface direct spraying method), root absorption method (root irrigation method), back leaf injection method, etc. Generally speaking, the vacuum infiltration method has the highest silencing efficiency, but it has a high demand for utensils and cannot be used under the condition of the field. Although the high pressure spray gun method can be operated in a large area, because the leaves of *Rosa* are smaller and fluffier in the inoculation period, the contact area between the liquid and the blade is small and cannot penetrate the leaf surface.

The root absorption method is simple, but the species limitation is higher, so it is not suitable for *Rosa*. In addition, high pressure spray gun method and root absorption method need to consume a large amount of infection liquid, which is also not suitable for operation under field conditions. Although the operation of leaf back injection is relatively simple, it cannot be injected with syringe because of the shallow leaves of *Rosa*.

In order to solve the technical problems mentioned above, in this study, we provided a method for establishing VIGS system based on perennial *Rosa* plants as experimental objects under field conditions. According to the growth conditions of *Rosa* plants, the natural environment in the field and the optimal conditions for the occurrence of VIGS, the technical problems were solved one by one. And the successful establishment of VIGS system was confirmed by determining some biochemical indexes.

2. MATERIALS AND METHODS

2.1. Plant Materials

For *Rosa*, *R. rugosa* "Zizhi" and *R. davurica* cultivated in Rose germplasm nursery of Shandong Agricultural University was used as the test material. We collected the samples (leaves and petals) in the forenoon on sunny days from 20 April to 10 May 2017. After quick freezing of liquid nitrogen, all samples collected with three replicates were put into −80˚C refrigerator for storage.

2.2. Extraction of Total RNA and Synthesis of the First-Strand cDNA

The extraction of total RNA is based on the specification of EASY spin plant RNA rapid extraction kit (Aidlab Biotech, Beijing, China). The integrity of RNA was detected by gel electrophoresis with 1.0% non-denatured agarose, the purity and concentration of RNA were detected by Nanodrop2000C ultramicro spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA), and the qualified RNA was preserved at −80˚C. The first-strand cDNA was synthesized by referring to the steps of 5×All-In-One RT MasterMix reverse transcription kit (ABM Company,Vancouver, Canada) and synthesized according to the requirements of RT-PCR and qRT-PCR.

2.3. VIGS in *R. rugosa*

Based on the modified TRV-GFP vector, the recombinant viral vector TRV-GFP-*RrGT1* was constructed. pTRV1 and pTRV2-GFP are two RNA strands of TRV-GFP virus vector respectively, and the multiple clone site are mainly on pTRV2-GFP. In order to specifically silence the *RrGT1* gene in *R. rugosa*, the relative conserved region of the *RrGT1* gene was removed, and a sequence of the length of 543bp was finally selected as the specific fragment, by analyzing the conserved domain of the *RrGT1* gene. Two restriction endonuclease sites (*XbaI* and *XhoI*) were designed at the two ends of the fragment. After double enzyme digestion, the digestion products were ligated with DNA ligase and transformed into *Agrobacterium tumefaciens*. The recombinant viral vector was confirmed by PCR and sequencing.

The plasmids of pTRV1, pTRV2-GFP and pTRV2-GFP-*RrGT1* were transformed into *A. tumefaciens*, and cultured in YEB medium containing kanamycin, rifampicin and AS at 28˚C for 14-16 h to reach OD_{600} = 1.5. Before the infection, the *A. tumefaciens* infective liquid with pTRV2-GFP and pTRV2-GFP-*RrGT1* was mixed with the *A. tumefaciens* infective liquid carrying pTRV1 in equal volume respectively to form the complete TRV-GFP and TRV-GFP-*RrGT1* virus carrier. Then the mixed bacteria solution was kept at room temperature for 4 h with darkness.

The experimental materials for gene silencing induced by virus were the perennial *Rosa* plants grown naturally in the field, and the experimental treatment time was about one month before *Rosa* flowering. Because the leaves and twigs are difficult to be injected with syringes and vacuum infiltration cannot be used in the field, we have adopted the method of infecting leaves and twigs with *A. tumefaciens* after scratching them. The specific operation was to completely immerse the scratched leaves and branchlets in the 5 ml centrifuge tube containing the infective fluid for infection. In order to improve the infection efficiency of *A. tumefaciens*, 0.01% Silwet L-77 was added to the infective fluid and the plants was treated with black plastic bags for 24 h after the infection for 10 min.

2.4. qRT-PCR Detection

We analyzed the gene expression by qRT-PCR on a Bio-Rad CFX96™ Real-Time PCR instrument (Bio-Rad, Inc., USA). The qRT-PCR mixture (20 μL total volume) contained 10 μL of SYBR® Premix Ex Taq™ (TaKaRa, Inc., Japan), 8.2 μL of ddH_2O, 0.4 μL of each primer and 1 μL of cDNA. The PCR program was carried out with an initial step of 95˚C for 30 s; 40 cycles of 95˚C for 5 s, 60˚C for 30 s; and then, 95˚C for 10 s, 65˚C for 5 s and 95˚C for 5 s for the dissociation stage. Each gene was assessed with three biological replications. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta Ct}$ method [12].

2.5. Total Anthocyanin Extractions and HPLC Analysis

All samples (0.1 g fresh weight) were homogenized in liquid nitrogen, after which they were extracted with 5 mL of an acidic methanol solution (70:0.1:29.9, v/v/v; CH_3OH:HCl:H_2O) at 4˚C in darkness for 24 h and then sonicated for 30 min. After centrifugation, each extract was passed through a membrane filter (0.22 mm). The aqueous phase was used to determine the absorbance at 530 nm and 657 nm. The total anthocyanin contents were quantified via the following equation: $Q_{Anthocyanins} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$, where $Q_{Anthocyanins}$ is the amount of anthocyanins, A_{530} and A_{657} are the absorptions at the indicated wavelengths, and M is the weight of the plant material in grams used for extraction.

Qualitative and quantitative analyses of anthocyanins were performed via HPLC. Three independent biological replicates were measured for each sample. The specific conditions of the anthocyanin analysis were the same as those in the report of Yang et al. with some modifications [13]. The chromatographic analysis was conducted using an Agilent 1100 series HPLC system (TC-C18 column, 5 μm, 4.6 mm × 250 mm) with the detection wavelength at 530 nm and the column was maintained at 30˚C. The eluent was aqueous solution A (0.1% formic acid in water) and organic solvent B (acetonitrile). The gradient elution program was modified as follows: 0 min, 10% B; 15min, 17% B; 20min, 23% B; 25 min, 23% B; and 30 min, 10% B; the eluent flow rate was 1.0 ml/min with 10 μl injection volume. Cy3G, Cy3G5G, Pg3G, Pg3G5G, Pn3G, Pn3G5G, Dp3G and Dp3G5G were used as references for the anthocyanin analysis.

3 RESULTS

3.1. Construction and Verification of Recombinant Virus Vector pTRV2-GFP-*RrGT1*

The virus vector pTRV2-GFP and the specific fragment of the *RrGT1* gene were respectively digested (**Figure 1(a)**) and then were combined together. Then the PCR (**Figure 1(b)**) was verified by specific primers (**Table 1**), and the base mutation and deletion were verified by double enzyme digestion (**Figure 1(c)**) and sequencing. All of the above results indicated that the recombinant virus vector pTRV2-GFP-*RrGT1* was successfully constructed.

3.2. VIGS of *RrGT1* Gene Reduced the Transcription Abundance of Endogenous *RrGT1* Gene

At 14 days after infection, the leaves of control plants and the infected plants (TRV-GFP and TRV-GFP-*RrGT1*) were collected for semiquantitative RT-PCR detection, and the *RrGAPDH* gene was used as internal control to confirm the efficiency of VIGS. The results (**Figure 2(a)**) showed that *TRV* and *GFP* genes could be detected in leaves treated with VIGS (TRV-GFP and TRV-GFP-*RrGT1*), but the abundance of the *RrGT1* gene was significantly decreased only in leaves treated with TRV-GFP-*RrGT1*, and the expression of the *RrGT1* gene was normal in the leaves treated with TRV-GFP. While in the control group, the *TRV* and *GFP* genes could not be detected and the *RrGT1* gene was normally expressed.

At 30 - 40 days after infection, *in vitro* observation, semiquantitative RT-PCR and qRT-PCR were performed on the blooming petals of control plants and the infected plants (TRV-GFP and TRV-GFP-*RrGT1*) of two *Rosa* species. The results were as expected: we can clearly observe that the control group and TRV-GFP group had no obvious change of flower color, but the TRV-GFP-*RrGT1* group had obviously shallower petals (**Figure 3(a)**). The presences of *TRV* virus and *GFP* gene could be detected in the petals treated with VIGS (TRV-GFP and TRV-GFP-*RrGT1*). In the petals treated with TRV-GFP-*RrGT1*, the expression of *RrGT1* gene was almost not detected, but was normal in the petals treated with TRV-GFP; in contrast, *TRV* and *GFP* genes were not detected in the control group, and the *RrGT1* gene was expressed normally (**Figure 2(b)**). The results of the qRT-PCR detection (**Figure 3(b)**) were consistent with the results above. In the two *Rosa* species, the trend of relative expression of *RrGT1* gene is also basically consistent: the relative expression level of *RrGT1* gene in the petals treated with TRV-GFP was basically the same as that in control group. However, the transcription abundance of endogenous *RrGT1* gene in petals treated with TRV-GFP-*RrGT1* was significantly downregulated.

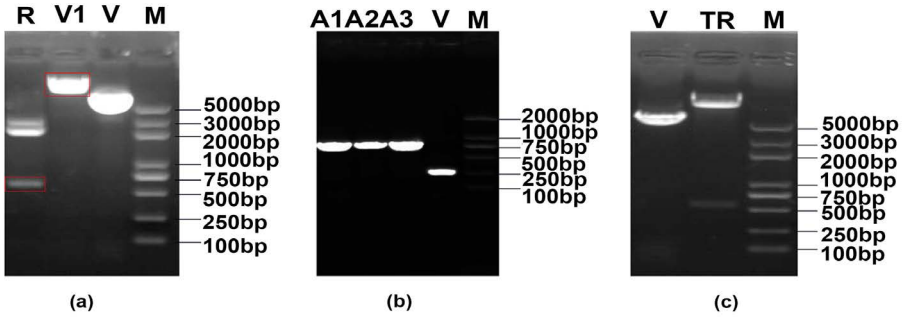

Figure 1. Construction and validation of the recombinant virus vector TRV-GFP-*RrGT1*. (a) The virus vector and target gene fragment were digested separately. M: Marker; V: The plasmid of the empty virus vector was used as the control; V1: The empty virus vector was digested by double enzymes; R: The plasmid carrying the target gene fragment was digested by two enzymes. The red box indicates the portion that needs to be retained for subsequent operations. (b) The result of PCR verification. Three repeated PCR assays were performed on the recombinant virus vector and the results showed positive. M: Marker; V: The empty virus vector was used as the control; A1, A2, A3 was the three repetitions which were larger than the empty virus vector due to the inserted *RrGT1* fragment. (c) Double enzyme digestion was used to verify the plasmid of the recombinant vector. M: Marker; V: The plasmid of the empty virus vector was used as the control; TR: The plasmid of the recombinant vector was digested into two correct parts.

Table 1. Primers used in the present study.

Primer Name	Sequence(5'-3')	Description
TGR-F	GCTCTAGAATGTCAGGAAATCCACTGGATGC	Generation for *pTRV2-GFP-RrGT1*
TGR-R	CCCTCGAGCTGCAGTGGTAATGAGAGGGAG	
TRV1-F	TTACAGGTTATTTGGGCTAG	RT-PCR for *TRV*1
TRV1-R	CCGGGTTCAATTCCTTATC	
TRV2-F	TGGGAGATGATACGCTGTT	Confirmation for recombinant silencing vector and RT-PCR for *TRV*2 RT-PCR for *GFP*
TRV2-R	CCTAAAACTTCAGACACG	
GFP-F	ATGGTGAGCAAGGGCGAGGA	
GFP-R	CTTGTACAGCTCGTCCATGCC	
RrGAPDH-F	TTCTGCCTGCTCTCAATG	RT-PCR and qRT-PCR for *RrGAPDH*
RrGAPDH-R	TGCCTTCTTCTCAAGTCTG	
q*RrGT1*-F	GTATTTGCCAACACACTGAGTAA	qRT-PCR for *RrGT1*
q*RrGT1*-R	CTGCAGTGGTAATGAGAGGGAG	

*Restriction enzyme site are underlined.

3.3. Silencing Efficiency of VIGS

Total RNA was extracted from all petals of VIGS treated samples and the first strand cDNA was synthesized. The results of PCR and agarose gel electrophoresis showed that: The silencing efficiency of VIGS was 75% in *R. rugosa* "Zizhi" and 85% in *R. davurica* (Table 2).

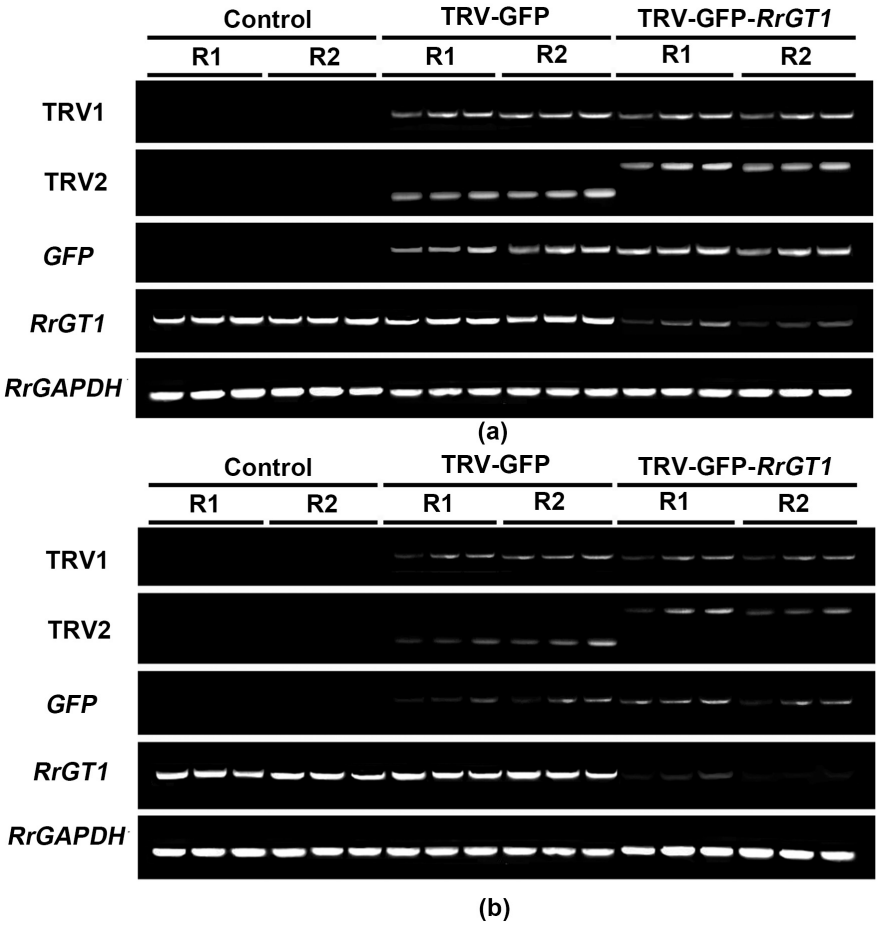

Figure 2. Semiquantitative RT-PCR in leaves (a) and petals (b). Semiquantitative RT-PCR of *TRV1*, *TRV2*, *GFP*, and *RrGT1* in control group and VIGS-treated group. R1, *R. rugosa* "Zizhi"; R2, *R. davurica*. *RrGAPDH* was used as an internal control. The *TRV2* fragment was larger in plants infected by TRV-GFP-*RrGT1* due to the inserted *RrGT1* fragment.

3.4. HPLC Analysis

The anthocyanin HPLC chromatograms for *R. rugosa* "Zizhi" (Figure 3(c)) and *R. davurica* (Figure 3(d)) show that the components were well separated. Comparisons with standards allow the contents of different substances to be calculated by their peak area (Table 3). For "Zizhi", six kinds of anthocyanins were detected: Cy3G5G, Pg3G5G, Cy3G, Pn3G5G, Pg3G, and Pn3G. Pn3G5G had the highest content, while Cy3G5G had the second highest; the contents of the other four anthocyanins were relatively low. In response to VIGS treatment, reductions in the contents of several anthocyanins compared with those in the control group and TRV-GFP group were obvious. Pn3G5G exhibited the greatest drop in content, followed by Cy3G5G; the content in Pg3G was no longer detectable. For *R. davurica*, the six anthocyanins listed above were also detected. However, Cy3G5G had the highest content, and Cy3G had the second highest content; the contents of the other four anthocyanins were relatively low. In response to VIGS treatment, reductions in the contents of the six anthocyanins compared with those in the control group and TRV-GFP group were obvious. Cy3G5G exhibited the greatest drop in content, followed by Cy3G; no detection of Pn3G was observed.

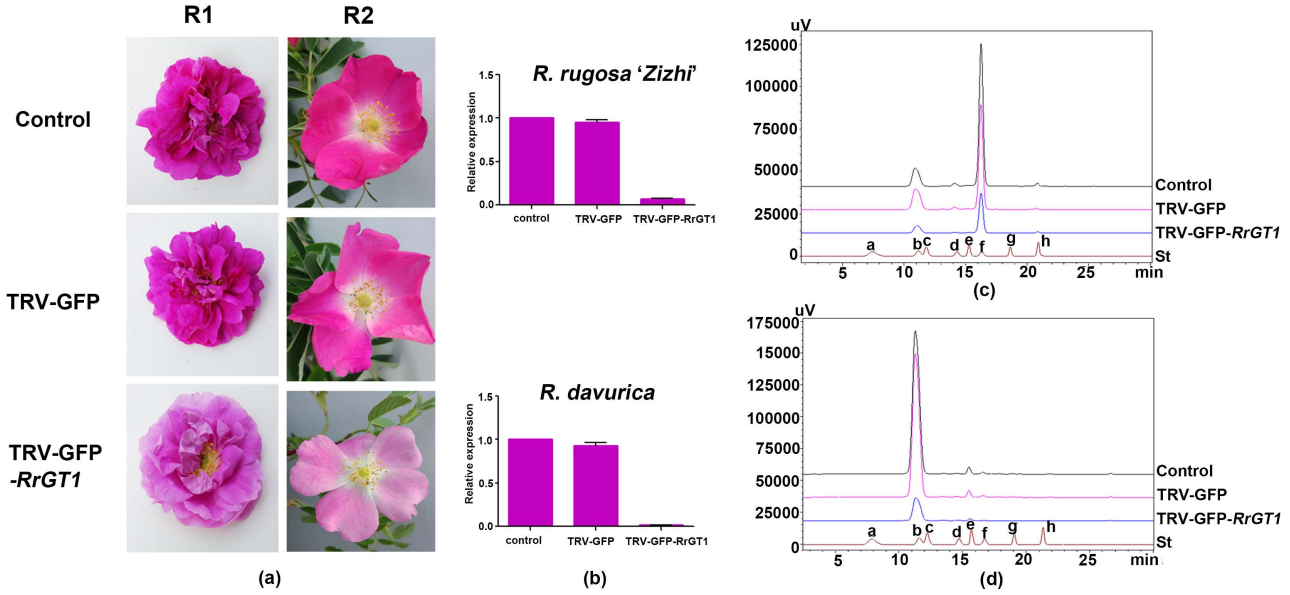

Figure 3. Validation of VIGS in flowers of *R. rugosa* "Zizhi" and *R. davurica*. (a) Contrast of flower color phenotypes. R1, *R. rugosa* "Zizhi"; R2, *R. davurica*. (b) The results of the qRT-PCR detection. The error bars represent the SDs of triplicate reactions. The experiment was repeated three times, and each yielded similar results. HPLC chromatograms in *R. rugosa* "Zizhi" (c) and *R. davurica* (d). Eight kinds of anthocyanin standards (St) were used for detection: (a) Dp3G5G; (b) Cy3G5G; (c) Dp3G; (d) Pg3G5G; (e) Cy3G; (f) Pn3G5G; (g) Pg3G; and (h) Pn3G.

Table 2. Silencing efficiency of VIGS in *R. rugosa* "Zizhi" and *R. davurica*.

Plant		TRV-GFP	TRV-GFP-*RrGT1*
R. rugosa "Zizhi"	Total	20	20
	GFP positive	17	15
	RrGT1 silencing	_	15
	GSEI	_	75%
R. davurica	Total	20	20
	GFP positive	18	17
	RrGT1 silencing	_	17
	GSEI	_	85%

GSEI, gene silencing efficiency of all infected plants.

4. DISCUSSION

In the past few years, molecular biology has made great progress in model plants and crops. However, the molecular biology of *Rosa* lags far behind. One reason is that the *Rosa* genera are usually perennial woody plants, usually taking a long time to achieve results, and another reason is that the transgenic operation of the *Rosa* genus is difficult.

As a simple and rapid tool in functional genomics, VIGS has been widely used to analyze gene function in many plant species, as well as in multiple development processes and in response to extrinsic stimuli. Therefore, in the past decade, tremendous improvements VIGS have been reported, such as

Table 3. Contents of anthocyanins in the flowers of *R. rugosa* "Zizhi" and *R. davurica* subjected to different VIGS treatments (μg·g^{-1} FW).

Name	treatments	Cy3G	Cy3G5G	Pn3G	Pn3G5G	Pg3G	Pg3G5G	Dp3G	Dp3G5G
R. rugosa "Zizhi"	Control	13.05 ± 0.04A	298.38 ± 3.85A	17.63 ± 0.12A	1773.93 ± 4.31A	5.04 ± 0.09A	52.88 ± 0.45A	-	-
	TRV-GFP	11.86 ± 0.70A	301.14 ± 1.57A	17.33 ± 0.07A	1660.26 ± 13.07B	4.82 ± 0.13A	45.32 ± 1.23B	-	-
	TRV-GFP-*RrGT1*	3.22 ± 0.12B	96.17 ± 2.22B	5.16 ± 0.06B	472.31± 1.28C	-	10.84 ± 0.39C	-	-
R. davurica	Control	56.72± 0.20A	2590.18 ± 2.45A	2.55 ± 0.03A	40.28± 0.09A	7.20 ± 0.01A	25.7 ± 0.36B	-	-
	TRV-GFP	55.59 ± 0.26B	2565.85 ± 3.26B	2.50 ± 0.01A	40.36± 0.11A	7.19 ± 0.01A	28.13 ± 0.27A	-	-
	TRV-GFP-*RrGT1*	15.33 ± 0.17C	443.17 ± 4.04C	-	1.95± 0.02B	0.74 ± 0.03B	6.99 ± 0.06C	-	-

*Data are the mean values ± SE of three independent replicates. Different upper case letters represent significant difference which is calculated using LSD analysis at the level of $P < 0.01$. "-" means that no corresponding anthocyanin was detected.

developing new vectors and improving the vector delivery methods [14, 15]. At present, few reports exist about the use of the VIGS system in plant floral organs, and most of the tested species belong to the Solanaceae family. For example, VIGS technology was used to study the genes controlling floral fragrance in *Petunia hybrida* [16], and the roles of the *SlMADSI*, *NbMADS*4-1 and *NbMADS*4-2 genes in tobacco flowers were also identified via the VIGS [17]. Furthermore, the TRV recombinant virus vector successfully induced the silencing of the *CHS* gene and *GLO*1 gene in *Gerbera jamesonii* [18]. In this study, we developed a VIGS system for use with perennial *Rosa* plants grown naturally in the field as experimental materials for the first time, and we used the system to study key genes of *Rosa* color and obtained results.

At present, the main factors affecting gene silencing effect are summarized as follows: firstly, the matching degree between virus vector and target plant; Secondly, the position of the target gene fragment inserted into the virus vector and the length of the target gene fragment in the whole length of the gene; thirdly, the determination of the inoculation period, the preparation of the infective fluid, the inoculation technology of the virus vector and the light and temperature conditions of plant materials cultured after inoculation after the construction of the recombinant virus vector with the target gene [19, 20].

In this study, according to the optimum conditions of VIGS in field, we select the most suitable virus vector, the best growth stage of *Rosa* plants inoculation, the best method of preparation of infective liquid, the most suitable inoculation method and the most reasonable culture management method after inoculation in order to achieve the most efficient gene silencing effect. Because of the influence of natural environment under field conditions and the ability of resistance to infection of perennial plant materials, we increased the concentration of A. tumefaciens carrying virus vector in the preparation of infective fluid. In order to ensure the sufficient survival base of A. tumefaciens carrying virus vector in the inoculation area under natural conditions, so as to improve the infection efficiency of the whole experiment. The use of low concentration Silwet L-77 in the formulation of infective liquid also achieved a good effect, which not only increased the hydrophilicity of plant material surface, but also increased the infection efficiency of the infective liquid. Moreover, low concentration of components will not produce toxic effect on plant materials, and its pollution rate is low, which is beneficial to environmental protection. The intensive scratching of the treatment site and the prolongation of the time of infiltration and infection can not only increase the

area of contact between the wound and the infective fluid, but also improve the infection efficiency. Moreover, due to the strong growth of the treatment site, its normal growth will not be greatly affected by the condition that it is not broken and incomplete. The bagging dark treatment after inoculation can not only achieve the effect of heat preservation and moisture preservation, but also resist the bad weather to a certain extent.

In this study, we developed a VIGS system for use with perennial *Rosa* plants grown naturally in the field as experimental materials for the first time, and we used the system to study key genes of *Rosa* color and obtained results. Under conditions of the established optimal VIGS system, the petal color of both *Rosa* species was clearly lighter, which was consistent with the significantly downregulated transcript abundance of the endogenous *RrGT1* gene and the decrease in the contents of all the different kinds of anthocyanins after HPLC analysis. This undoubtedly proved that our attempt to establish VIGS system using perennial *Rosa* plants as experimental materials under field conditions was successful.

5. CONCLUSION

In conclusion, the construction of this VIGS system not only saved the cost but also improved the efficiency of gene silencing. The efficiency of gene silencing in the treatment of samples was more than 75%, which will greatly promote the research in the field of genetic engineering in *Rosa* plants. It will also promote the transition from research objects to experimental verification materials and greatly reduce the time of gene function verification.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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