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Virus-induced gene silencing in *Nicotiana benthamiana* triggered by heterologous gene sequences from *Viola philippica*

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Abstract

Virus-induced gene silencing (VIGS) is a particularly useful tool for functional genomics. In the present study, we attempted to utilize this technology to infer the function of genes from *Viola philippica* using a tobacco rattle virus (TRV) construct. Firstly, the *phytoene desaturase* gene from *V. philippica* (*VpPDS*) was silenced, and local leaf bleaching was observed but did not exhibit systemic effects, thereby limiting utilization of TRV-mediated gene silencing in the recipient plant. However, we observed systemic gene silencing in *Nicotiana benthamiana* when the *VpPDS* sequence was used as a trigger, thereby suggesting that heterologous gene sequences could elicit gene silencing. We then investigated the role of gene *PISTILLATA* from *V. philippica* (*VpPI*) of the B-class MADS-box gene family, which regulates the identity and development of stamens and petals. Using the coding region of *VpPI* as triggers, we determined the gene silencing efficiency of the corresponding *GLOBOSA* paralogs in *N. benthamiana* (*NbGLO1* and *NbGLO2*), and we observed stamen-to-carpel transformation and distorted corollas in *N. benthamiana* suggesting that *VpPI* functioned as the *NbGLO* gene. However, the 3'-untranslated region (3'UTR) of *VpPI* and each 3'UTR of the *NbGLO* genes downregulated its own corresponding gene, hardly producing floral homeotic alterations. This work provides a better understanding of gene-specific probe design for gene silencing as well as shows that heterologous sequence-triggered VIGS is an efficient way to investigate functional conservation of orthologous genes.

Additional key words: gene-specific probe, *GLOBOSA*, *phytoene desaturase*, *PISTILLATA*.

Introduction

Virus-induced gene silencing (VIGS) is a method that utilizes RNA interference (RNAi) to induce transient gene knockdown in plants (Dinesh-Kumar *et al.* 2003). Virus-induced gene silencing exploits the plant endogenous RNA-mediated defense system that targets viral RNAs for sequence-specific degradation (Robertson 2004, Becker and Lange 2010). A previous study showed that a double-strand RNA (dsRNA) fragment (a silencing inducer) 300 - 500 bp in length with at least one stretch of 21 - 23 nucleotides with 100 % identity to a targeted transgene mRNA is essential to gene silencing in plants (Thomas *et al.* 2001). Virus-induced gene silencing is an effective

technology for the analysis of gene functions because it is easy, rapid, and circumvents the need for genetic transformation of plants. Recently, VIGS has been performed in various dicot plant species including those from *Solanaceae* (*Nicotiana* spp., *Solanum* spp., *Capsicum annuum*, *Lycium* spp., and *Physalis floridana*; Kumagai *et al.* 1995, Ratcliff *et al.* 1997, 2001, Liu *et al.* 2002, Brigneti *et al.* 2004, Chung *et al.* 2004, Senthil-Kumar *et al.* 2007, Liu *et al.* 2014, Zhang *et al.* 2014a,b, Meng *et al.* 2016), *Leguminosae* (*Pisum sativum* and *Glycine max*; Constantin *et al.* 2004, Zhang and Ghabrial 2006,

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Abbreviations: *AP1* - *APETAL1*; cDNA - complementary DNA; *GLO* - *GLOBOSA*; ORF - open reading frame; *PDS* - *phytoene desaturase*; *PI* - *PISTILLATA*; quantitative PCR - qPCR; RNAi - RNA interference; TRV - tobacco rattle virus; VIGS - virus-induced gene silencing; UTR - untranslated region.

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Zhang *et al.* 2010), *Brassicaceae* (*Arabidopsis thaliana*; Burch-Smith *et al.* 2006, Pflieger *et al.* 2008), *Ranunculaceae* (*Aquilegia vulgaris*; Gould and Kramer 2007), *Malvaceae* (*Gossypium hirsutum*; Gao *et al.* 2011, Tuttle *et al.* 2012), *Rosaceae* (*Malus pumila*; Sasaki *et al.* 2011), and some monocot species such as *Hordeum vulgare* (Hein *et al.* 2005, Yuan *et al.* 2011), *Triticum aestivum* (Ma *et al.* 2012), *Zea mays* (Benavente *et al.* 2012), and *Oryza sativa* (Purkayastha *et al.* 2010).

Viola philippica is a common species with the typical chasmogamous-cleistogamous mixed breeding system. *V. philippica* develops chasmogamous flowers under a short photoperiod, whereas an extended photoperiod induces the formation of cleistogamous flowers (Li *et al.*

2016), thereby serving as a good model for investigating the developmental evolution of dimorphic flowers. In the present study, we explored the tobacco rattle virus (TRV)-mediated VIGS of genes belonging to two families in *V. philippica*. Phytoene desaturase (PDS) is an important enzyme in the carotenoid biosynthetic pathway, in which silencing leads to chlorophyll photo-oxidation (Kumagai *et al.* 1995), whereas *PISTILLATA* (*PI*) or *GLOBOSA* (*GLO*) of the B-class of MADS-box gene family regulates the identity and development of stamens and petals (Bowman *et al.* 1989, Hill and Lord 1989). Our aim was to provide new insights into investigating *V. philippica* gene function in *N. benthamiana* based on heterologous sequence-triggered VIGS.

Materials and methods

Sequence cloning: Total RNA was extracted from leaves and flowers of *Nicotiana benthamiana* Domin and leaves of *Viola philippica* Cav. using a TRIzol® reagent (Tiangen, Beijing, China), and first-strand cDNA synthesis was performed using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). The cDNA fragments of phytoene desaturase of *V. philippica* (*VpPDS*) were isolated using degenerate primers (Table 1 Suppl.), which were designed based on the conserved regions of the *PDS* orthologs from various plant species. The full-length cDNA was assembled by 3'- and 5'-rapid amplification of cDNA ends using gene-specific primers (Table 1 Suppl.). Universal 3' and 5' PCR primers were supplied by a SMARTer™ cDNA library construction kit (Clontech, Mountain View, CA, USA). After rapid amplification of cDNA ends, the full-length cDNA was amplified by routine reverse transcription (RT) PCR using gene-specific primers (Table 1 Suppl.). The cDNA of *VpPDS* and *VpPI* of *V. philippica* and the cDNA of *NbGLO1*, *NbGLO2*, and *NbAPI* (*APETALA1*) of *N. benthamiana* were amplified using gene-specific or degenerate primers (Table 1 Suppl.). The *Ex Taq* enzyme (TaKaRa) was used in routine reverse transcription (RT)-PCR. The amplified cDNA fragments were cloned using a vector pMD19-T (TaKaRa) and sequenced by Huada (Beijing, China).

The VIGS plasmid construction: The *pTRV1* and *pTRV2* VIGS vectors (Fig. 1A,B) were used in this study as previously described (Liu *et al.* 2002, Gould and Kramer 2007). The probe of each gene was amplified from the obtained cDNAs of *VpPDS*, *VpPI*, *NbGLO1*, and *NbGLO2* using gene-specific primers (Table 1 Suppl.). The amplified gene fragments were cloned into the *pTRV2* vector using restriction enzymes *NcoI* and *BamHI*. The constructs *TRV2::VpPDS*-ORF, *TRV2::VpPI*-ORF, *TRV2::VpPI*-3'UTR, *TRV2::NbGLO1*-3'UTR, and *TRV2::NbGLO2*-3'UTR (Fig. 1C-G) were then generated. Positive clones of the above-mentioned VIGS constructs were detected using 156 primers (located in the *pTRV2*

vector) and sequenced by Huada (Beijing, China). The plasmid constructs were extracted using a *TIANprep* mini plasmid kit (Tiangen).

Plant growth and agroinfiltration: Plasmids were introduced into *Agrobacterium tumefaciens* L. strain GV3101 by heat shock. The *A. tumefaciens* strain GV3101 containing *pTRV1* and *pTRV2*-derivatives (Fig. 1C-G) were plated on a yeast and beef extract (YEB) solid medium containing kanamycin (50 µg cm⁻³) and rifampicin (100 µg cm⁻³) and cultivated at a temperature of 28 °C in the dark. Two days later, a single colony was selected and inoculated into 5 cm³ of YEB liquid medium, which contained 100 µg cm⁻³ rifampicin and 50 µg cm⁻³ kanamycin and cultured overnight on a shaker (250 rpm). Then, the cultures were transferred into 250 cm³ of YEB liquid medium containing 100 µg cm⁻³ rifampicin, 50 µg cm⁻³ kanamycin, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), and 0.2 mM acetosyringone and grown overnight at 28 °C (250 rpm). *Agrobacterium* cells were harvested by centrifugation at 4 000 g for 15 min, and the pellets were resuspended in an infiltration buffer (10 mM MgCl₂, 10 mM MES, and 200 mM acetosyringone) to reach an absorbance of 2.0 at a wavelength of 600 nm. Infiltration cultures were incubated at room temperature in a triangle flask for 3 h, and the *pTRV1* and *pTRV2* derivative solutions were mixed at 1:1 (v/v) ratios and then infiltrated into the underside of all leaves of four-leaf stage plants of *N. benthamiana* and *V. philippica* using a 1-cm³ needleless syringe. The infiltrated *N. benthamiana* and *V. philippica* plants were cultured in the dark for 2 d and then were kept at a temperature of 22 - 25 °C, a 16-h photoperiod, an irradiance of 120 µmol m⁻² s⁻¹, and an air humidity of 48 % for phenotypic observation.

Real-time quantitative RT-PCR analysis: To quantify transcript abundances of *VpPDS*, *NbPDS*, *NbAPI*, and B-class MADS-box genes in inoculated *V. philippica* and

N. benthamiana plants, real-time quantitative (q) PCR was performed using three independent biological replicates. To reduce errors, the collection of flowers or leaves were processed at the same time and in a shortest time. Total RNA was extracted from the leaves or flowers using a *TRIzol*® reagent (Tiangen). RNA concentrations were measured using an ultramicrospectrophotometer (*NanoDrop2000*, Thermo Scientific, Massachusetts, USA), and the concentrations of all samples were adjusted

to be the same. For qPCR analysis, a *PrimeScript RT* reagent kit (*TaKaRa*), *SYBR Premix EX Taq II* (*TaKaRa*), and gene-specific primers were used (Table 1 Suppl.). The *Vp18sRNA* and *Nbactin* RNA genes were used as internal references for *V. philippica* and *N. benthamiana*, respectively. The amplification conditions were 95 °C for 30 s for one cycle followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Three independent biological samples were used. Expressions were calculated according to Livak and Schmittgen (2001).

Fig. 1. Virus induced gene silencing (VIGS) constructs generated in this study. *A* A scheme of the tobacco rattle virus 1 (*TRV1*) vector. *B* A scheme of the *TRV2* vector. *C* to *G* - Schemes of the *TRV2*-based gene-specific VIGS constructs. The gene-specific primer positions are indicated, and the sequences are presented in Table 1 Suppl. LB - left border; RB - right border; RdRp - RNA-dependent RNA polymerase; MP - movement protein; 16 K - 16 kd protein; Rz - self-cleaving ribozyme; NOST - NOS terminator; CP - coat protein; MCS - multiple cloning site; ORF - open reading frame; 3'UTR - 3'-untranslated regions. The schemes of the *TRV1* and *TRV2* vectors were modified from the previous investigations (Liu *et al.* 2002, Gao *et al.* 2011).

Morphological observations: Flower bud morphology was assessed under a stereomicroscope (*Olympus SZ61*, Tokyo, Japan). Ten flower buds from each plant were observed, and 28 plants for each gene silencing were used for statistical analysis. Representative flower images were taken using a camera linked to the stereo-microscope. Gene silencing frequency was defined as the ratio of the number of plants showing a silencing phenotype (bleaching or abnormal flower buds) and the total number of plants infiltrated (Senthil-Kumar *et al.* 2007).

Sequencing analysis: The nucleotide sequences of the genes were aligned using *CLUSTALW* as implemented in *MegAlign* (<http://www.dnastar.com>), and sequence identities were calculated. The multiple sequence alignments were saved as *GCG pileup* files and exported using *GeneDoc* (<http://genedoc.software.informer.com>) with manual adjustments, and gaps were introduced for proper alignment. Neighbor-joining phylogenetic trees of protein sequences were constructed using *MEGA5* (Tamura *et al.* 2011). Bootstrap values were based on 1 000 replicates.

Statistics: Means $\pm$ standard deviations (SDs) were calculated from three independent biological replicates. Significance differences and the related correlations were analyzed in *SPASS13.0*.

Data availability: The sequences reported in this study

have been deposited in *GenBank* (<http://www.ncbi.nlm.nih.gov>) as accession numbers KU318324 (*VpPI*), MF156690 (*VpPDS*), MF156691 [*NbGLO1*, open reading frame (ORF)], MF156692 (*NbGLO2*, ORF), HQ005417 [*NbGLO1*, 3'-untranslated region (3'UTR)], HQ005418 (*NbGLO2*, 3'UTR), and MG637440 (*NbAPI*).

Results

To apply VIGS in *V. philippica*, we firstly tried to silence *VpPDS*. The leaves that were inoculated with *A. tumefaciens* carrying *TRV1* and *TRV2::VpPDS* exhibited photobleaching, whereas the newly developed leaves were as green as the wild-type leaves (Table 2 Suppl. and Fig. 1 Suppl.). Furthermore, *VpPDS* expression was significantly reduced in the photobleached leaves (N1 - N4) correlating with the visual observations on the area of white patches, whereas no change was observed in the young leaves of *V. philippica* and the non-photobleached ones, which were designated as N0 leaves (Table 2 Suppl. and Fig. 1 Suppl.). These results indicate that TRV-mediated local gene silencing was possible in *Viola*, although no efficient and systemic gene silencing occurred.

In parallel, we used the *V. philippica* sequence as the heterologous gene sequence for VIGS in *N. benthamiana*. *N. benthamiana* was inoculated with *TRV2::VpPDS* and

one month later, 100 % of the plants exhibited photobleaching symptoms (Fig. 2A,B and Table 2 Suppl.). Systemic gene silencing and the consequent phenotypic variations were observed in the newly developed leaves of *N. benthamiana* (Fig. 2B), and no floral homeotic changes were observed. Real-time qPCR analyses indicate a significant reduction in endogenous *PDS* expression in the visually categorized N1 - N4 photobleached leaves of *N. benthamiana* (Fig. 2C). Thus, the heterologous sequence of *V. philippica* could knock down the corresponding ortholog or closely related homologs in *N. benthamiana*.

Based on this finding, we investigated the function of the *VpPI* gene (Li *et al.* 2016). Phylogenetic reconstruction in combination with sequence identity suggested that *VpPI* is the putative ortholog of *NbGLO2* in *N. benthamiana* (Fig. 2 Suppl.).

Fig. 2. Gene silencing in *Nicotiana benthamiana* using *VpPDS* as a trigger sequence. A - Wild-type. B - Silencing endogenous *NbPDS* genes in *N. benthamiana* by inoculating with *Agrobacterium tumefaciens* containing *TRV2::VpPDS*. The red arrows indicate the white patches on the leaves (pt - photobleaching). C - Relative expression of *NbPDS* in the leaves of *N. benthamiana*; WT - wild-type leaves; N0 - WT-like leaves with no photobleaching; leaves showing an increased severity of photobleaching were visually categorized to N1 - N4 according to the area of white patches. Means $\pm$ SDs, $n = 3$. * indicate significant differences at $P \leq 0.01$ relative to the WT. The leaves for quantitative PCR analysis were collected about 35 d post-inoculation.

To silence the *NbGLO* genes, we firstly inoculated the leaves of *N. benthamiana* with *TRV2::VpPI*-ORF (Fig. 1D). Approximately 54 % of the inoculated plants generated flowers with abnormal morphological structures, particularly the stamens and petals (Fig. 3A-J and Table 2 Suppl.). Compared to the wild-type flowers (Fig. 3A), stamen-to-pistil (ovule) transformation was mainly observed in the *TRV2::VpPI*-ORF plants, and the number of transformed organs varied from one to five (Fig. 3B-J). The *TRV2::VpPI*-ORF plants depicted five degrees of variation, which we designated as N0 - N4. The

N0 flowers resembled the wild-type flowers. The N1 flowers harbored stamens with microspores, which were transformed into ovules, and no stamen adnation to the corolla tube was observed, thereby generating free stamens (Fig. 3B,C). The tops of the stamens in the N2 flowers generated stigmas and stamens changed into ovules (Fig. 3D,E). Nonetheless, the petals did not show any significant changes. In the N3 flowers, some stamens were completely transformed into pistils (Fig. 3F,G), whereas the N4 flowers displayed wrinkled and distorted petals (Fig. 3H), and the stamens were completely

transformed into pistils (Fig. 3I,J).

To elucidate the mechanism underlying the observed floral phenotypic variations, we investigated the expressions of B-class MADS-box genes (*NbGLO1*, *NbGLO2*, *NbDEF*, and *NbTM6*) in *N. benthamiana* flowers that displayed different degrees of phenotypic variations. We also assessed the expression of *APETALA1* (*NbAPI*), which belongs to the A-class MADS-box gene family, a close homolog of B-class MADS-box genes. The expressions of all B-class MADS-box genes, particularly *NbGLO2*, which shared the highest identity with *VpPI* (Fig. 2 Suppl.) were significantly reduced in the abnormal flowers of *N. benthamiana* compared to the wild-type flowers (Fig. 3K). However, the variation in *NbAPI* expression was antithetical to that of the B-class MADS-box genes in *N. benthamiana* suggesting the specificity of gene silencing. Upregulation of *NbAPI* by downregulating *NbGLO2* was in line with the observation in *Arabidopsis*, where *API* transcription increase when the expressions of B-class MADS-box genes decrease (Sundström *et al.* 2006). Moreover, we observed systemic silencing all B-class MADS-box genes (Fig. 3K and Table 3 Suppl.). A correlation between the extent of floral variation and the

reduction in the expression of these genes was observed (*NbGLO1*: $r = 0.934$, $P < 0.01$; *NbGLO2*: $r = 0.968$, $P < 0.01$; *NbDEF*: $r = 0.951$, $P < 0.01$; *NbTM6*: $r = 0.805$, $P < 0.01$). The N4 flowers that exhibited wrinkled petals and five stamens that were fully transformed to pistils showed the most significant reductions in gene expression, whereas the N0 flowers with petals and stamens that remained unchanged depicted the smallest decreases in gene expression (Fig. 3K). The systemic silencing effect of all B-class MADS-box genes might be due to the high sequence identity between the trigger sequence *VpPI* and the target sequence *NbGLO2* and its homologs (Table 1 and Table 3 Suppl.).

To exclude this possibility, we inoculated *N. benthamiana* plants with *TRV2::VpPI-3'UTR* (Fig. 1E). In this case, no gene silencing effect was expected because the 3'UTR is relatively gene-specific. However, the results indicate that 21 % of the VIGS plants had flowers with abnormal morphological structures, particularly the petals (Fig. 4 and Table 2 Suppl.). Compared with the wild-type flowers (Fig. 4A), N0 flowers, which resembled the wild-type flowers of the *TRV2::VpPI-3'UTR* plants, were observed although floral variations including unhealed

Fig. 3. Silencing *Globosa* (*GLO*) genes using the open reading frame (ORF) fragment of the *VpPI* gene. A - A wild-type flower. B - A VIGS flower displaying anthers that have undergone dehiscence and ovules. C - Stamens from a wild-type flower (the upper part) and from a virus-induced gene silencing (VIGS) flower (a lower part). D - A VIGS flower without stamen adnation to the corolla tube, thereby resulting in free stamens. E - Five stamens had stigma-like structures, and anthers were transformed into ovules. F - A VIGS flower displaying anthers, which were transformed into pistils. G - The transformed pistils from the stamens in F. H - A VIGS flower showing a crumpled corolla tube. I - A VIGS flower with a crumpled corolla tube in H showing that five stamens were all transformed into pistils. J - The transformed pistils from stamens from the VIGS flower in H and I. ov - ovules; sg - stigma-like structure. K - Relative expressions of B-class genes and *NbAPI* in VIGS flowers. WT - wild-type flower, N0 - VIGS flowers resembling WT, N1 - flowers with one to three anthers that transformed into ovules, N2 - flowers with five stamens that have stigma-like structures and anthers transformed into ovules, N3 - flowers with one to three stamens that transformed into pistils, and N4 - flowers with five stamens that transformed into pistils and wrinkled petals. Means $\pm$ SDs, $n = 3$, * - significant differences at $P \leq 0.01$ relative to the WT. The flowers for quantitative PCR analysis were collected about 60 d post-inoculation.

petal tubes (N1 flowers; Fig. 4B), and distorted and wrinkled petals were observed (N2 and N3, Fig. 4C,D). Besides *NbTM6*, the expressions of three other genes, particularly *NbGLO2*, were significantly reduced in these flowers (Fig. 4E and Table 3 Suppl.). The auto- and cross-regulatory circuit of obligate heterodimers between B class MADS-box genes was required for the specification of petal and stamen identity in angiosperms (Zahn *et al.* 2005, Zhang *et al.* 2014a). Thus, the reciprocal regulations among B-class MADS-box genes could account for the reduction of expressions of *NbGLO1*, *NbDEF*, and *NbTM6* when *NbGLO2* was silenced in *N. benthamiana* by the *VpPI*-3'UTR probe (Geuten and Irish 2010, Zhang *et al.*

2014a). Furthermore, the expression of *NbAPI* increased in the abnormal flowers of *N. benthamiana* compared with the wild-type flowers, whereas the expressions of B-class MADS-box genes in the *TRV2::VpPDS* flowers were similar to the wild-type flowers (Fig. 4E).

These results demonstrate that the heterologous gene sequence of *VpPI*, either the ORF or the 3'UTR, could specifically knock down its putative ortholog *NbGLO2* in *N. benthamiana*, thereby leading to floral alterations. Therefore, *VpPI* in *Viola* played a role similar to that of its ortholog *NbGLO2* in *Nicotiana* in controlling petal and stamen identity and development.

Fig. 4. Silencing *NbGLO* by the 3'UTR sequence of the *VpPI* gene. *A* - A wild-type flower. *B* - A flower of a *TRV2::VpPI*-3'UTR-inoculated plant; an unhealed petal tube. *C* - A flower of a *TRV2::VpPI*-3'UTR-inoculated plant. The petals became slightly wrinkled. *D* - A flower of a *TRV2::VpPI*-3'UTR-inoculated plant. The petals became severely wrinkled. *E* - Relative expressions of B-class genes and *NbAPI* in virus-induced gene silencing (VIGS) flowers. WT - wild-type flowers; *TRV2::VpPDS* - flowers of *TRV2::VpPDS* VIGS plants; N0 to N3 - flowers of *TRV2::VpPI*-3'UTR VIGS plants; N0 - flowers that resembled WT; N1 - flowers with unhealed petal tubes; N2 - flowers with slightly distorted corolla tubes; N3 - flowers with severely crumpled corolla tubes. Means $\pm$ SDs, $n = 3$, * - significant differences at $P \leq 0.01$ relative to WT. The flowers were collected about 60 d post-inoculation.

An apparent weak gene silencing effect was observed when the 3'UTR of *VpPI* was employed as a trigger sequence. To objectively evaluate this effect, we inoculated the leaves of *N. benthamiana* with *TRV2::NbGLO1*-3'UTR (Fig. 1F) and *TRV2::NbGLO2*-3'UTR (Fig. 1G). No mutated flowers were observed in the *TRV2::NbGLO1*-3'UTR plants, whereas 32 % of the *TRV2::NbGLO2*-3'UTR plants produced abnormal flowers (Fig. 5 and Table 2 Suppl.). Unlike the flowers from the wild-type and the *TRV2::NbGLO1*-3'UTR plants (Fig. 5A,B), unclosed petal tubes (Fig. 5C), severely wrinkled petals, and/or stigmas in one stamen were observed in the *TRV2::NbGLO2*-3'UTR flowers (Fig. 5D,E). The qPCR analysis showed that the expressions of the target genes were significantly downregulated in both cases (Fig. 5F,G and Table 3 Suppl.). In these analyses, four categories of flowers (N0 - N3) were selected from

TRV2::NbGLO2-3'UTR (Fig. 5G), and four *TRV2::NbGLO1*-3'UTR flower categories demonstrated different degrees of gene silencing (Fig. 5F) although no phenotypic variations were observed. Meanwhile, the expression of *NbAPI* was also upregulated in the both cases (Fig. 5F,G). These results suggest that gene silencing triggered by the 3'UTR was effective and gene-specific. However, the extent of phenotypic variation when only the 3'UTR was used as a triggered sequence was indeed weaker than previous observations, in which part of the coding sequences were included in the trigger constructs (Geuten and Irish 2010). The insufficient downregulation of another paralog triggered by the 3'UTR fragments might account for the observed weak to negligible phenotypic variation, further indicating that a single *NbGLO1* or *NbGLO2* mutant was unlikely to be capable of inducing pronounced floral variations.

To assess variations in gene silencing using different trigger sequences, we conducted sequence analysis of the target gene sequence and the trigger sequence. The *NbPDS* and the 409-bp trigger *VpPDS* fragment showed 79.7 % nucleotide identity, whereas a 26-nucleotide stretch depicted 100 % identity (Fig. 3 Suppl.). The *VpPI*-ORF trigger sequence was 356 bp long and shared 65.6 and 69.0 % identity at the nucleotide level with the putative target sequences of *NbGLO1* and *NbGLO2*, respectively, and there was a 23-nucleotide stretch in *VpPI* and *NbGLO1* or *NbGLO2* showing 100 % identity, with one base mismatch (Table 1 and Fig. 3 Suppl.). The *VpPI*-3'UTR trigger sequence was 177 bp long and shared 45.6 and 51.8 % sequence identity at the nucleotide level with those

of *NbGLO1*-3'UTR and *NbGLO2*-3'UTR, respectively, and the number of mismatched bases was 8 and 7, respectively, within a 21-nucleotide stretch shared between the *VpPI*-3'UTR sequence and the *NbGLO1*-3'UTR, and *NbGLO2*-3'UTR sequences (Table 1 and Fig. 3 Suppl.). The trigger sequences of both *VpPDS*-ORF and *VpPI*-ORF imparted strong gene silencing effects, which might be due to the high sequence identity between the exogenous genes and the trigger sequence and the longer length of the trigger sequences (Table 1 and Table 2 Suppl.). These analyses indicate that sequence identity was not the only determinant for effective gene silencing. Nonetheless, a pure 3'UTR was effective in inducing specific gene silencing, as expected.

Fig. 5. Silencing *NbGLO* by the 3'UTR sequences of the *NbGLO* genes. A - A wild-type flower. B - A wild-type flower of a *TRV2::NbGLO1*-3'UTR-inoculated plant. C - A flower of a *TRV2::NbGLO2*-3'UTR virus-induced gene silencing (VIGS) plant with unclosed petal tubes. D - A flower of a *TRV2::NbGLO2*-3'UTR VIGS plant with wrinkled petals. E - A flower of a *TRV2::NbGLO2*-3'UTR VIGS plant with curving filaments and a stamen that transformed into a stigma-like structure. F - Relative expressions of B-class MADS-box genes and *NbAPI* in VIGS flowers as mediated by *TRV2::NbGLO1*-3'UTR. WT - wild-type flowers; N0 to N3 - flowers of *TRV2::NbGLO1*-3'UTR-inoculated plants. According to the expression of *NbGLO1*, the four categories from N0 to N3 were selected. G - Relative expressions of B-class genes and *NbAPI* in VIGS flowers mediated by *TRV2::NbGLO2*-3'UTR. WT - wild-type flowers; N0 - WT-like flowers in the *TRV2::NbGLO2*-3'UTR VIGS plants. N1 - flowers with unclosed petal tubes; N2 - flowers with crumpled corolla tubes; N3 - flowers with crumpled corolla tubes and one anther that was transformed into a stigma-like structure. Means $\pm$ SDs, $n = 3$, * -significant differences at $P \leq 0.01$ relative to WT. The flowers were collected about 60 d post-inoculation.

Discussion

Virus-induced gene silencing is a particularly useful tool for plant functional genomics because it permits the knock down of genes of interest, the observation of elicited phenotypes within one month, and does not require the creation of stable plant lines (Burch-Smith *et al.* 2004). Efficient VIGS has been successfully accomplished in a few plant species, and more than 30 VIGS vectors have been developed, with the majority having a narrow host range (Yuan *et al.* 2011). Tobacco rattle virus is a VIGS system that was initially designed for *Solanaceae* plants, with wide applications to other plant species (Gould and Kramer 2007, Tian *et al.* 2015, Bilichak and Kovalchuk

2017). The present study first tried to apply the system to *V. philippica*, an optimal model for investigating the developmental evolution of dimorphic flowers (Li *et al.* 2016). The attempt was not successful because no systemic gene silencing effect was observed in *V. philippica*. This might be due to the limited distribution of TRV transcripts in this *Viola* species, as observed in *Eschscholzia californica* (Wege *et al.* 2007). Nonetheless, we observed the systemic gene silencing effect in *N. benthamiana* when both the *N. benthamiana*-native and *V. philippica*-derived gene probes were used, which indicates that heterologous probes effectively triggered VIGS.

Table 1. Nucleotide sequence identities and mismatches between trigger and target sequences. For details, see Table 3 Suppl.

Trigger sequence	Gene silencing extent <i>NbGLO1</i> [%]	Gene silencing extent <i>NbGLO2</i> [%]	Inserted cDNA length [bp]	Nucleotide identity with <i>NbGLO1</i> [%]	Nucleotide identity with <i>NbGLO2</i> [%]	Maximum stretch of nucleotides having 100 % identity with <i>NbGLO1</i>	Maximum stretch of nucleotides having 100 % identity with <i>NbGLO2</i>	Number of nucleotide mismatch in 21-nucleotide stretch having 10 % identity with <i>NbGLO1</i>	Number of nucleotide mismatch in 21-nucleotide stretch having 100 % identity with <i>NbGLO2</i>
<i>VpPI</i> ORF	64.00	70.74	356	65.6	69.0	13	19	1	1
<i>VpPI</i> 3'UTR	46.70	68.68	177	45.6	51.8	6	5	8	7
<i>NbGLO1</i> 3'UTR	55.86	22.97	161	100.0	39.9	161	3	0	11
<i>NbGLO2</i> 3'UTR	44.61	74.11	181	39.9	100.0	3	181	11	0

This phenomenon may easily occur between closely related species; for example, gene probes from one *Solanaceae* species could silence the corresponding genes in the other *Solanaceae* species (Ryu *et al.* 2004, Senthil-Kumar *et al.* 2007) although a dsRNA fragment (a silencing inducer) of 300 - 500 bp in length with at least one stretch of 23 nucleotides with a 100 % identity to a targeted transgene mRNA is essential (Thomas *et al.* 2001). Our work has added further evidence that gene silencing occurs in *N. benthamiana* using gene probes from distantly related species (Howes and Kumagai 2005, Senthil-Kumar *et al.* 2006). Furthermore, our findings also indicate that a 100 % nucleotide sequence identity between the target gene and the probe is not essential for VIGS. Nevertheless, there is a need to determine the minimal nucleotide identity and number of base mismatches between the trigger sequence and the target sequence that could effectively trigger a knockdown effect on endogenous genes. We took advantage of the probe construct that we made using genes from *V. philippica* to test this in *N. benthamiana*.

The *PDS* gene is a structural gene that is conserved

among plant species and is often used as a reporter for gene silencing (Chen *et al.* 2004, Burch-Smith *et al.* 2006, Yuan *et al.* 2011, Liu *et al.* 2014). The *VpPDS* has a 79.7 % nucleotide identity and a 26-nucleotide stretch that shares 100 % identity with *NbPDS*; therefore, 100 % gene silencing was observed. However, different situations were observed when *VpPI* was used as a probe. The *VpPI* gene encodes a B-class MADS-box regulatory protein in *V. philippica* (Li *et al.* 2016) and is the putative ortholog of *NbGLO2*. We considered only the silencing effect of the orthologous gene pair, and other downregulations are considered to be reciprocal regulations among B-class MADS-box genes in *Nicotiana* (Geuten and Irish 2010). The *VpPI*-ORF fragment that was used as a trigger sequence had a 69 % nucleotide identity with the corresponding target section of *NbGLO2*-ORF in *Nicotiana*. Except for the single-base mismatch, a 21-nucleotide stretch with a 100 % identity between *VpPI*-ORF and *NbGLO2*-ORF was detected. *NbGLO2* was effectively knocked down, which induced visible floral phenotypic variations, wherein the pistils replaced stamens, and the petals shrank. The wrinkled petals

seemed to be the consequence of the strong transformation of petal midveins into sepal midveins, thereby forcing the second whorl floral organ to fold (Geuten and Irish 2010). Therefore, *VpPI* in *Viola* plays the same role as its ortholog *NbGLO2* in *Nicotiana*. In this case, the number of base mismatches coincides with previous findings (Senthil-Kumar *et al.* 2007). However, the 3'UTR sequence of *VpPI* had only a 51.8 % nucleotide identity with the corresponding sequence in *NbGLO2*, and up to 7 base mismatches within a 21-nucleotide stretch compared to the target sequence of *NbGLO2*-3'UTR were observed. *NbGLO2* was also efficiently knocked down in *N. benthamiana*, which in turn induced floral phenotypic variations. Nonetheless, no *NbGLO1* and *NbGLO2* were silenced in the *TRV2::VpPDS* inoculated flowers, no *NbAPI* was silenced in the flowers inoculated with *TRV2::VpPI*-3'UTR constructs, and no mutated flowers were observed in plants inoculated with the *TRV2::NbGLO1*-3'UTR constructs. These observations further support that the silencing phenotypes in flowers with *TRV2::VpPI*-3'UTR constructs are specific for the *VpPI*-3'UTR construct in *N. benthamiana* instead of the side effects of silencing process. Thus, a 21-nucleotide stretch of identity with 1 - 7 base mismatches was sufficient for silencing target genes in this particular case. Moreover, the native trigger sequences *NbGLO2*-3'UTR and *NbGLO1*-3'UTR demonstrated different degrees of gene silencing. However, the specificity and mechanism of gene silencing triggered by the lower homology sequence need investigations. We currently support that the length and position of the triggers in the cDNA may affect the efficiency of silencing target genes (Liu and Page 2008), and the structure of RNA might also play a putative role (Senthil-Kumar *et al.* 2007). In addition, using the 3'UTR as a trigger sequence for effective gene silencing did not lead to the same phenotypic deviations as those observed using ORFs (Geuten and Irish 2010) and heterologous *VpPI*-ORF fragments (this study), where both paralogs were significantly downregulated, thereby indicating that

the two paralogs *NbGLO1* and *NbGLO2* might play similar roles in organ identity determination during stamens and corolla development.

Homologous genes, including orthologous and paralogous genes, generally share similar functions or functions in closely related pathways (Vandenbussche *et al.* 2003, Geuten and Irish 2010, Roque *et al.* 2013, Zhang *et al.* 2015). TRV-mediated gene silencing technology is currently not applicable to *V. philippica*; however, the findings of the present study provide a platform for inferring the function of specific plant genes where a native functional inference is impossible. For example, in *V. philippica*, *VpPI* could knock *NbGLO2* down and induce phenotypic variations similar to the *NbGLO2* mutant (Geuten and Irish 2010), thereby suggesting that *VpPI* functions as a B-class MADS-box gene, which determines petal and stamen organ identities in *V. philippica*. The direct downregulation of *NbGLO1*, the paralog of *NbGLO2*, as induced by *VpPI*-derived triggers, is not excluded although the reciprocal regulation among B-class MADS-box genes has been observed (Geuten and Irish 2010, Zhang *et al.* 2014b, Zhang *et al.* 2015). However, the phenotype of the mutated flowers with the *VpPI*-3'UTR as a trigger sequence was similar to that using *NbGLO2*-3'UTR as a trigger sequence. *VpPI*-3'UTR should be a specific probe, which is similar to *NbGLO2*-3'UTR. Therefore, designing gene-specific probes for gene silencing or knockdowns should be performed with caution. If trigger sequences are not specific, then all paralogous genes, instead of a single gene, could be knocked down, and the observed phenotypic variations might be the collective effect of silencing all paralogous genes instead of a single paralogous gene, thereby resulting in unreliable functional inferences. Therefore, when no relative gene-specific coding region encompassing the variable region of the gene of interest is available, the 3'UTR may be included in the selection of trigger sequences.

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