A multifunctional protein encoded by *Turkey herpesvirus* suppresses RNA silencing in *Nicotiana benthamiana*

Running head: silencing suppression by an avian viral protein

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ABSTRACT

Many of plant and animal viruses counteract the RNA silencing-mediated defense by encoding diverse RNA silencing suppressors (RSSs). Here, we characterized HVT063, a multifunctional protein encoded by Turkey herpesvirus (HVT), as a silencing suppressor in co-infiltration assays with GFP transgenic Nicotiana benthamiana line 16c. Our results indicated that HVT063 could strongly suppress both local and systemic RNA silencing induced by either sense RNA or double-stranded RNA. HVT063 could reverse local silencing, but not systemic silencing in newly emerging leaves. The local silencing suppression activity of HVT063 was also verified using the heterologous vector PVX. Further, single alanine substitution of arginine or lysine residues of HVT063 protein showed that each selected single amino acid contributed to suppression activity of HVT063 and the region1 (138-141) was more important, because three out of four single amino acid mutations in this region could abolish the silencing suppressor activity of HVT063. Moreover, HVT063 seemed induce a cell death phenotype in infiltrated leaf region, and the HVT063 dilutions could decrease silencing suppressor activity and alleviate cell death phenotype. Collectively, these results suggest that HVT063 function as a viral suppressor of RNA silencing that targets a downstream step of the dsRNA formation in RNA silencing process. The positively charged amino acids in HVT063, such as arginine and lysine, might contribute to the suppressor activity by boosting the interaction between HVT063 and RNA, as HVT063 was demonstrated as an RNA
INTRODUCTION

Small RNA-guided gene silencing serves as a key component of host defense strategy against viruses in plants, invertebrates, and fungi as well as higher animals (2, 5, 9, 20). RNA silencing refers to the suppression of gene expression through homology-dependent mRNA degradation and it is thought to be initiated by double stranded RNA (dsRNA) molecules (21). The dsRNA is recognized and processed by Dicer or Dicer-like (DCL) proteins into small RNA duplexes of 21-24nt (5), and one strand of the duplex is subsequently incorporated into a multisubunit endonuclease called the RNA-induced silencing complex (RISC) that initiates the sequence-specific degradation of target RNAs (48).

To counteract host antiviral defenses, viruses have evolved sophisticated mechanisms including encoding proteins that are capable of suppressing the RNA silencing process (52). The first silencing suppressor was discovered in plant virus, and then the list for different suppressor proteins were identified in many plant viruses as well as animal viruses (3). The discovery of RNA silencing suppressor (RSS) functions in animal viruses provided evidence of conserved RNA silencing pathway in the plant and animal kingdoms (45, 59, 61). Up to now, more than 70 RSSs have been found; however, less than 15 animal proteins have been identified as RSSs. The RSSs are extremely diverse within and across kingdoms, with no obvious sequence homology, appearing to have evolved independently to overcome silencing-mediated defense. The various RSSs target at distinct silencing stages during the RNA silencing
process, such as viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification (7, 28, 29, 36). For example, P14 protein of *Pothos latent aureusvirus* (PoLV), P38 protein of *Turnip crinkle virus* (TCV), 2b protein of *Tomato aspermy cucumovirus* (TAV) and B2 protein of the insect–infecting *Flock house virus* (FHV) have been shown to bind dsRNA in a size-independent way and inhibit the processing of dsRNA to siRNAs (11, 12, 37, 38); p19 protein of *Tombusviruses* (TBSV) and *Influenza A virus* NS1 protein prevent RNA silencing by siRNA sequestration through binding siRNA in a size-specific manner (17, 47); 2b protein of *Cucumber Mosaic virus* (CMV) and P0 protein of *Potato leaf roll virus* (PLRV) target the AGO protein to prevent RISC assembly (9); P38 protein of *Turnip crinkle virus* (TCV) and P1 protein of *Sweet potato mild mottle ipomovirus* (SPMMV) interact with AGO proteins by the glycine/tryptophan (GW/WG) residues (4, 22); *Human immunodeficiency virus type 1* (HIV-1) Tat protein and the core protein of *Hepatitis C virus* (HCV) interact with Dicer to ablate the effect of RNAi (6, 42, 60). So, the continuing studies on the functions of various viral suppressors should contribute significantly to our understanding of the specific steps of RNA silencing.

RNA silencing is non-cell autonomous, which initially was induced at the single-cell level then transmitted to remote cells or tissues to cause systemic RNA silencing. In plants, the silencing signal moves from cell to cell through plasmodesmata and over long distance via the vascular system (24, 40, 53, 54, 57). Recent studies suggest that the short distances spread of RNA silencing is mediated by 21-nt siRNAs, however, the role of small RNAs in long distance signaling remains
Roth et al. reported that several suppressors, such as CMV 2b, TBSV p19, TSWV NSs and PVX P25 could prevent gene silencing in systemic tissue (44). Among many of the known mammalian viral suppressors, B2 protein of *Flock house virus* (30) and *influenza A virus* NS1 protein (8, 18) first exhibit RNA silencing suppressor activity in plant cells. For this reason, RNA silencing represented as an important antiviral defence mechanism in plants most likely also plays an antiviral role in animal cells (31).

The identification of novel viral suppressors and elucidation of their mode of action are important to understand RNA silencing mechanisms as well as virus-host interactions. Here, we present HVT063 encoded by *Turkey herpesvirus* (HVT) is a strong RSS. HVT is a double-stranded DNA avian virus, and it is classified as the third serotype of the *Marek’s disease virus* (MDV) group (1). The complete genome of HVT is 159,160 bp, which encodes an estimated 99 putative proteins (1). HVT063, one of these proteins, located at the ends of the unique long (UL) region of HVT (1), a multifunctional expression regulator, holds some features that make it a good RSS candidate such as binding RNA and shuttling between nucleus and cytoplasm (refer to its sequence information from NCBI). The results showed that HVT063 suppressed both local and systemic RNA silencing induced by sense RNA or dsRNA. And HVT063 protein could reverse local silencing with a preexisting silenced transgene, and its RSS activities were dosage-dependent. Moreover, the single alanine substitutions of arginine or lysine residues indicated that the RSS activity of HVT063 was likely relational to RNA or siRNA binding ability.
MATERIALS AND METHODS

Plant materials and plasmid constructs. GFP transgenic *N. benthamiana* line 16c plants were grown at 24 ± 2°C under a 16h illumination (34W fluorescent bulbs) and 8h dark regimen.

The HVT063 gene (NP_073349) used in this experiment was PCR amplified from *Turkey herpesvirus* genome using specific primers HVT063-F/HVT063-R (Table S1). Furthermore, pp24 gene (ABF72222), pp38 gene (ABF72309) and MEQ gene (ABF72204) were PCR amplified from *Marek’s Disease Virus* genome with specific primers pp24-F/pp24-R, pp38-F/pp38-R, MEQ-F/MEQ-R and N gene (AC037573) was amplified by reverse transcription-PCR (RT-PCR) from total RNA extracted from *Avian infectious bronchitis virus* with specific primers N-F/N-R (Table S1). The resulting PCR products were cloned into PMD-18T vector (TaKaRa, DaLian, China) to produce PMD-HVT063, PMD-pp24, PMD-MEQ and PMD-N. After that, the above constructs were digested with specific enzymes and inserted into the binary vector pBI121 between the 35S promoter and the Nos terminator to yield constructs 35S-HVT063, 35S-pp24, 35S-pp38, 35S-MEQ and 35S-N. All single alanine point mutations of the HVT063 protein were produced by reverse PCR from the entire plasmid PMD-HVT063 using specific primers (i.e. HVT063K138A-F/HVT063K138A-R, HVT063R139A-F/HVT063R139A-R, HVT063R140A-F/HVT063R140A-R, HVT063R141A-F/HVT063R141A-R, respectively, Table S1). The PCR products were circularized by blunt end-ligation and
digested with DpnI to remove any residual parental plasmid before transformation into *E. coli*. mHVT063, an untranslatable mutant of HVT063 protein, was made by replacing the initiating codon ATG with GTG using PCR and primer mHVT063-F (Table S1). These constructs were digested with *Xba*I and *BamHI* restriction enzymes and cloned into the pBI121 vector.

The GFP gene (792 nucleotides) was amplified from total DNA of *N. benthamiana* line 16c using the GFP primes GFP-F/GFP-R (Table S1) and incorporated into pBI121 vector to construct 35S-GFP and the p19 gene of *Tomato bushy stuntvirus* (TBSV) was amplified from pBIN61-p19 plasmid with primes p19-F/p19-R (Table S1) and incorporated into pBI121 vector to generate 35S-p19 as a positive control in various experiments. An inverted repeat sequence of GFP amplified from the plasmid 35S-GFP with primers GFP-Xba-F/GFP-Bam-R, and GFP-Sac-F/GFP-Kpn-R (Table S1) was introduced into the binary vector pBI121 to generate 35S-dsGFP. To produce PVX-HVT063, the HVT063 sequence was amplified from plasmid PMD-HVT063 with primes PVX-HVT063-F/PVX-HVT063-R (Table S1), and then inserted into the ClaI/SalI site in the PVX vector pGR106.

All constructs generated by PCR were confirmed correct by nucleotide sequencing, and all plasmids described above were verified by restriction site analysis. Each of the constructs was transformed into *A. tumefaciens* strain GV3101 containing the helper plasmid pJIC SA_Rep by the freeze-thaw method (27).

**Co-infiltration and GFP imaging.** The GFP expressing *Nicotiana benthamiana* 16c plants with four to five leaves were infiltrated with the *A. tumefaciens* GV3101
strain carrying the above constructs according to Voinnet et al. (57). Prior to co-infiltration, each culture OD$_{600}$ was adjusted to 1.0. Co-expression of GFP with different viral proteins, the corresponding bacterial cultures were mixed in 1:1 (v:v) ratio before co-infiltration which was achieved by pressure infiltration. The cells of *A. tumefaciens* cultures were incubated at room temperature for at least 3h before infiltration. Local and systemic RNA silencing were determined through observing the GFP fluorescence in both infiltrated and the newly emerging leaves under long-wavelength UV (365nm) light (Spectroline model SB-100P/A, UV products, New York, USA), plants were photographed with a Coolpix 5400 Nikon digital camera. Laser-scanning microscope (LSM) photographs were taken using a Zeiss LSM510 microscope.

**Total RNA and siRNA isolation for Northern blot analysis.** Approximately 1g of frozen fresh samples of leaf infiltrated zones or systemic silencing leaves were ground into powder in liquid nitrogen, then transferred to 15mL tubes containing 2 to 3mL hot phenol buffer (Extraction buffer: 0.1M LiCl, 100mM Tris-Cl pH8.0, 10mM EDTA and 1% SDS. Phenol buffer: extraction buffer was mixed with an equal volume of phenol water, preheated at 80°C for 5min). 1/4 volume of chloroform was added and vortexed for 20s. The tubes were centrifuged at 10,000g for 10min at room temperature and the aqueous phase was transferred to new tubes, then an equal volume of 4M LiCl was added. The contents were gently mixed and left at –20°C overnight. The total RNA pellets were obtained by centrifugation at 10,000g for 15min at 4°C (keep the supernatant for siRNAs isolation). The pellets were
resuspended in 300μL DEPC treated TE buffer (10mM Tris-Cl, 1mM EDTA pH8.0),
about 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol absolute
were added, incubated for at least 3h at −20°C. The RNA was precipitated by
centrifugation at 10, 000g for 15min at 4°C, washed with 70% ethanol and
resuspended in 50 to 100μL DEPC treated H2O.

Low-molecular-weight RNAs were enriched from isolation of mRNA. Firstly, add
1/10 volume of 3M sodium acetate (pH5.2) and 2 volumes of ethanol to the
supernatant, incubated for at least 3h at −20°C. The RNA pellets were precipitated by
centrifugation at 10, 000g for 15min at 4°C, then washed with 70% ethanol and
resuspended in 100μL DEPC treated H2O. Add 900 μL of Trizol to the supernatant
followed by vortexing for 20s, then add 200μL chloroform and vortex for 20s. Then,
tubes were centrifuged at 12,000g for 15min at 4°C, transferred the aqueous phase into
a fresh eppendorf tube and added 500μL isopropanol and vortex for 15s. The RNA
was precipitated by centrifugation at 12, 000g for 10min at 4°C and washed with
75 % ethanol twice. The pellets were air dried and dissolved in 50-60μL DEPC
treated H2O.

**Total RNA Northern blot analysis.** For northern blot analysis of
high-molecular-weight RNA, 10μg of total RNA was separated on a 1%
agarose/MOPS/formaldehyde gel in 1×MOPS buffer, transferred to Hybond-N+
membranes (Amersham) for Northern blot analysis as previously described (43).
Thereafter, the membrane UV crosslinked and treated at 80°C for at least 2h, then
stored at 4°C until used. The membrane was briefly washed in 6×SSC for 10min at
room temperature. Then, the wash solution was discarded and enough
pre-hybridization solution was added to prehybridize the membrane at 42°C for 6–12h.
The membrane was hybridized with [α-32P] dCTP-labelled full-length GFP probe or
PVX MP probe, which synthesized using the primer-a-gene labelling kit (Promega).
The probe was added to the membrane and then incubated for 12-24h at 42°C. The
membranes were washed three times as follows: 2×SSC+0.2% SDS at 42°C for
20min, 0.2×SSC+0.2% SDS at 42°C for 10min twice. The membrane was covered
with a filter paper and excess wash buffer removed before exposure to X-ray film.
The membranes were reprobed once after stripping them in 1% SDS at 85°C for
30min.

**siRNA Northern hybridizations.** For small RNAs detection, 20µg siRNA was
separated on 15% polyacrylamide -7M urea gels in 0.5×TBE buffer, RNAs were
visualized by staining in ethidium bromide (0.5µg/mL in 0.5×TBE buffer) and
photographed. The siRNA was transferred into Hybond N⁺ membranes (Amersham)
by electrobloting in 0.5×TBE at 2mA/cm² for 1.5h. After that, we started with a
novel method, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)–mediated,
chemical cross-linking step that enhances detection of small RNA (41). Simply, first
prepared EDC fresh, wetted 3mm filter paper with EDC, then placed membrane with
RNA side face-up on wet 3mm filter paper, finally wrapped EDC saturated 3mm filter
paper and membrane in Saran and incubate at 60°C for 1h. The transferred membrane
was UV cross-linked and treated at 80°C for 2h, stored at 4°C until used. The
following steps were consistent with the hybridization of total RNA.
RESULTS

HVT063 blocks local RNA silencing triggered by both sense RNA and dsRNA.

To screen potential RNA silencing suppressors encoded by avian viruses, HVT063 protein of *Turkey herpesvirus* (HVT); pp24, pp38 and MEQ protein of *Marek's disease virus* and N protein of *Avian infectious bronchitis virus* were tested through *Agrobacterium* co-infiltration assay. Transgenic *N. benthamiana* (16c) plants infiltrated with cultures harboring 35S-GFP and cultures harboring either an empty vector, 35S-mHVT063 (which initiating codon ATG of HVT063 ORF was replaced to GTG) or a vector containing 35S-p19 were used as negative and positive controls respectively. When leaves were infiltrated with a mixture of cultures carrying a sense GFP construct plus the empty vector (data not shown) or 35S-mHVT063, GFP fluorescence reached the highest level in the infiltrated zone 2 to 3 days post-infiltration (dpi), and then declined to almost disappear at 6dpi as a consequence of RNA silencing activation (Fig. 1A). The same results were observed in leaves co-infiltrated with sense GFP and any avian viral protein constructs (data not shown) except for the HVT063 construct. Leaves co-infiltrated with cultures carrying the GFP reporter gene plus that expressing HVT063 showed a marked increase in green fluorescence in infiltrated area at 3dpi, and remained at a high level until 6dpi, observed under a hand-held UV light or under a confocal microscope (Fig. 1A). The GFP fluorescence in regions co-infiltrated with 35S-GFP and 35S-HVT063 was slightly higher than that in areas co-infiltrated with the established plant viral silencing suppressor p19 of TBSV at 3dpi (Fig. 1A). However, at 6dpi, GFP intensity
in leaves co-infiltrated with 35S-GFP and 35S-HVT063 was substantially lower than that in co-infiltrations with 35S-GFP and 35S-p19 (Fig. 1A).

RNA silencing induced by sense GFP triggers degradation of target GFP mRNA and thereby reduces the amount of transcripts available for translation into the fluorescent proteins. We carried out Northern blot hybridization to examine the accumulation of GFP mRNA of various mixtures. The results showed that the levels of GFP mRNA extracted from sectors co-infiltrated 35S-GFP with 35S-HVT063 or 35S-p19 accumulated to a markedly higher abundance than that of 35S-GFP and 35S-mHVT063 co-infiltrations, both at 3dpi and 6dpi (Fig. 1B). Moreover, the level of GFP mRNA accumulation from regions co-infiltrated with 35S-GFP and 35S-HVT063 was higher than that from leaves co-infiltrated with 35S-GFP and 35S-p19 at 3dpi, whereas GFP mRNA levels of HVT063 co-infiltrations were significantly lower than that of p19 at 6dpi (Fig. 1B), which was consistent with the visual observations. The formation of GFP-derived siRNAs is a hallmark of RNA silencing in the silenced tissue. Further siRNA analysis showed that the GFP silencing in tissues co-infiltrated with 35S-GFP and 35S-mHVT063 was correlated with the accumulation of GFP-specific 21 and 24nt siRNAs, which were especially abundant at 6dpi (Fig. 1B). However, this accumulation was drastically reduced to an undetected level in tissues infiltrated with 35S-GFP and 35S-HVT063 or 35S-GFP and 35S-p19, either at 3dpi or 6dpi (Fig. 1B).

The inverted repeats are considered as the strong inducers of RNA silencing since they produce high level of functional siRNAs. To test whether HVT063 could
interfere with GFP silencing initiated by a dsGFP (inverted repeat generating GFP)
dsRNA) inducer, leaves of 16c were co-infiltrated with the *Agrobacterium* harboring
35S-GFP and 35S-dsGFP, and the third *Agrobacterium* carrying either an
35S-mHVT063, 35S-HVT063 or 35S-p19. GFP fluorescence practically disappeared
in tissues co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-mHVT063 at 3dpi (Fig.
2A). In contrast, the green fluorescence intensity remained strong in patches
infiltrated with a mixture of 35S-GFP, 35S-dsGFP and 35S-HVT063 or 35S-p19,
either at 3 or 6dpi (Fig. 2A).

These results were confirmed by Northern blot analysis, which showed that the
steady-state levels of GFP mRNA were significantly higher in leaves co-infiltrated
with 35S-GFP, 35S-dsGFP and 35S-HVT063 or 35S-p19 than that co-infiltrated with
35S-GFP, 35S-dsGFP and 35S-mHVT063 at both 3 and 6dpi (Fig. 2B). Further
analysis of siRNA indicated that by 3dpi, GFP siRNAs had accumulated to the high
levels in leaves co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-mHVT063. They
reached much higher in these leaves by 6dpi (Fig. 2B). However, the GFP-specific
siRNAs levels were remarkably low in leaves co-infiltrated with 35S-GFP,
35S-dsGFP and 35S-HVT063 or 35S-p19, at either 3 or 6dpi (Fig. 2B).

Taken together, these results indicate HVT063 protein encoded by HVT is a strong
RNA silencing suppressor, which can much efficiently suppress local silencing
induced by sense GFP RNA or GFP dsRNA in plant cells.

**Verification of HVT063 suppressor activity using PVX vector.** In order to
validate the silencing suppression activity of HVT063, 16c leaves were in
co-infiltration with 35S-GFP and PVX-mHVT063 or PVX-HVT063, respectively. A weak or absence of GFP fluorescence was observed in tissues co-inoculated with 35S-GFP and PVX-mHVT063 at either 3 or 6dpi, whereas the fluorescence maintained in inoculated patches when HVT063 expressed from the PVX vector till 6dpi (Fig. 3A). The observed phenotypes were confirmed by Northern blot analysis of GFP mRNA (Fig. 3B). Leaves infiltrated with 35S-GFP and PVX-HVT063 revealed high accumulation of GFP mRNAs, in contrast to barely detectable levels observed in leaves co-infiltrated with PVX-mHVT063 (Fig. 3B). Furthermore, the level of PVX RNA was significantly higher in leaves co-infiltrated with PVX-HVT063 than that of leaves co-infiltrated with PVX-mHVT063 at 6 dpi (Fig. 3B). More genomic PVX RNA in the presence of PVX-HVT063 indicated that the HVT063 ORF cloned was retained in the viral progeny and played the role of RSS.

Taken together, these results again indicat that HVT063 presents efficient RSS activity in plant cells. Interestingly, co-infiltration of 35S-GFP and PVX-HVT063 induced the cell death phenotype as early as 7dpi and completely dry necrosis at 10dpi (Fig. 3C).

**HVT063 can reverse established local RNA silencing of GFP.** PVX vector is the most commonly used virus vector for its obvious inability to reverse RNA silencing and very low suppression activity in co-infiltration assays. Previous researches indicated that numerous suppressors had the ability to reverse established silencing. To determine whether HVT063 has the ability to reverse the GFP silencing, we infiltrated 16c with silencing inducer single sense GFP firstly; and then a PVX vector
(pGR106) carrying HVT063 or mHVT063 was inoculated on the upper leaves at 18dpi when silencing occurred throughout the entire plant. The leaves infiltrated with PVX carrying HVT063 recovered green fluorescence under UV light at 7dpi, while the leaves infiltrated with PVX-mHVT063 showed bright red color instead of green fluorescence (Fig. 4A). Consistent with these observations, Northern blot analysis revealed that the very high level of GFP mRNA accumulation was evident in leaves infiltrated with PVX carrying HVT063, and those infiltrated with PVX-mHVT063 did not show any GFP mRNA signal (Fig. 4B). Moreover, the analysis of steady-state levels of PVX RNA indicated that genomic PVX RNA accumulated to a markedly higher level in leaves infiltrated with PVX-HVT063 than that in leaves infiltrated with PVX-mHVT063 (Fig. 4B). Consequently, PVX-HVT063 leading to more genomic PVX RNA than PVX-mHVT063 indeed resulted from HVT063 suppression of virus-induced gene silencing. Nevertheless, the newly emerging leaves of GFP-silenced plants inoculated with PVX-HVT063 or PVX-mHVT063 did not appear obvious GFP fluorescence and presented bright red color all the time (Fig. 4C).

These findings were also demonstrated using the pBI121 vector expressing HVT063. By 7dpi, intense green fluorescence could be observed in leaves of GFP-silenced plants co-infiltrated with pBI121 carrying HVT063 or p19. By contrast, in tissues infiltrated with the pBI121-mHVT063, green fluorescence was undetected (Fig. 4D). This was confirmed by Northern blot analysis and the accumulation of GFP mRNA was consistent with the observed phenotypes (Fig. 4E).

Therefore, these results demonstrated that HVT063 could reverse local silencing.
but not systemic silencing in newly emerging leaves, suggesting that it inhibit a
function required for maintenance of the silenced state.

**HVT063 inhibits systemic RNA silencing triggered by sense RNA or dsRNA.** If
the silencing signal delivered from the co-infiltrated area, the cell-to-cell movement of
the signal should cause shutting down of GFP expression. The above results showed
that HVT063 could suppress local silencing triggered by either sense RNA or dsRNA.
To test whether it interferes with systemic RNA silencing induced by sense GFP in
plant cells, 16c were co-infiltrated in parallel with 35S-GFP plus either
35S-mHVT063, 35S-HVT063 or 35S-p19. The infiltrated plants were monitored
under UV light for the initiation of systemic silencing in the newly emerging leaves.
At 13dpi, plants infiltrated with 35S-GFP and 35S-mHVT063 showed the
characteristic vein proximal GFP silencing in the new leaves (Fig. 5A). The limited
systemic silencing observed in plants infiltrated with 35S-GFP and either
35S-HVT063 or 35S-p19 (Fig. 5A). Furthermore, about 75% of the plants infiltrated
with 35S-GFP and 35S-HVT063 sustainedly showed strong green GFP fluorescence
in the newly emerging leaves even at 35dpi. By that time, GFP expression had been
absolutely lost in plants co-infiltrated with 35S-p19 (data not shown). Systemic
silencing was further explored by determining GFP mRNA levels in the newly
emerging leaves of the infiltrated plants at 13dpi (Fig. 5B). The plants co-infiltrated
with 35S-GFP and 35S-mHVT063 failed to suppress systemic silencing, which
showed a reduced accumulation of GFP mRNA compared to those infiltrated with
35S-GFP and either 35S-HVT063 or 35S-p19 (Fig. 5B).
Moreover, strong GFP expression was only seen in leaves co-infiltrated with 35S-GFP and 35S-p19 at 13dpi, while the fluorescence in leaves co-infiltrated with 35S-GFP plus either 35S-mHVT063 or 35S-HVT063 declined significantly and became almost undetected by 13dpi (Fig. 5A). Our visual observations were confirmed by Northern blot analysis of GFP mRNA isolated from infiltrated leaves of 16c. The levels of GFP mRNA remained high in leaves co-infiltrated with 35S-GFP and 35S-p19 at 13dpi (Fig. 5B). In contrast, the GFP mRNA levels were remarkably low in leaves co-infiltrated with 35S-GFP and either 35S-mHVT063 or 35S-HVT063 (Fig. 5B). Interestingly, the leaves infiltrated with 35S-GFP and 35S-HVT063 also became chlorotic as early as 7dpi, similarly to the observation of PVX-HVT063 assay, but this phenomenon was not found in other treatment groups. The area of the plants infiltrated with 35S-GFP and 35S-HVT063 had been completely dry necrosis at 13dpi (Fig. 5A). The low level of GFP mRNA in plants infiltrated with 35S-GFP and 35S-HVT063 probably reflects the effect of cell death.

To further demonstrate whether HVT063 interferes with systemic RNA silencing triggered by dsGFP, we co-infiltrated 16c with 35S-GFP and 35S-dsGFP plus either 35S-mHVT063, 35S-HVT063 or 35S-p19. Our results showed that systemic GFP silencing occurred in all of the plants co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-mHVT063, showing prominent vein proximal GFP silencing in the newly emerging leaves at 13dpi (Fig. 6A). In contrast, there was strong GFP fluorescence in the newly emerging leaves of the plants whose primary leaves were co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-HVT063 or 35S-p19 (Fig. 6A). The cell death
phenotype in leaves co-infiltrated with 35S-GFP, 35S-dsGFP and 35S-HVT063 was the same to that in leaves co-infiltrated with 35S-GFP and 35S-HVT063. The leaves infiltrated with 35S-GFP, 35S-dsGFP and 35S-HVT063 had been completely dry necrosis and not shown any fluorescence at 13dpi (Fig. 6A).

The GFP fluorescence in local and systemic silencing leaves at 13dpi was correlated with GFP mRNA levels by Northern blot analysis. The GFP mRNA in systemic silencing leaf tissues was very low in 35S-GFP, 35S-dsGFP and 35S-mHVT063 infiltrated plants, while the newly emerging leaves from plants infiltrated with 35S-GFP, 35S-dsGFP and 35S-HVT063 or 35S-p19 had much higher levels of mRNA (Fig. 6B). Leaves infiltrated with 35S-GFP, 35S-dsGFP and 35S-p19, which showed persistently strong fluorescence, had high levels of GFP mRNA at 13dpi (Fig. 6B). On the contrary, GFP mRNA from plants infiltrated with 35S-GFP, 35S-dsGFP and 35S-mHVT063 or 35S-HVT063 was barely detectable which corresponded to the weak fluorescence (Fig. 6B). The low level of GFP mRNA in leaves infiltrated with 35S-GFP, 35S-dsGFP and 35S-HVT063 may be due to the completely dry necrosis area in plants, but not lacking of silencing suppression capability.

These results suggest that HVT063 is able to block the short or long-distance spread of the RNA silencing signal triggered by sense GFP RNA or GFP dsRNA. **HVT063 loses a good part of its suppressor activity at low concentration and the death phenotype induced by HVT063 is dosage dependent.** 16c plants were co-infiltrated with 35S-GFP and 35S-HVT063 or diluted 35S-HVT063 (1:2-50). Our
results showed that by 3dpi, green fluorescence was much brighter in tissues infiltrated with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2-50) than that infiltrated with 35S-GFP and 35S-mHVT063 (data not show). Moreover, at 7dpi, the leaves infiltrated with 35S-GFP and 35S-mHVT063 showed red fluorescence under UV light, indicating the induction of local GFP silencing. The plants co-infiltrated with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2-20) dramatically increased GFP expression, however, the green fluorescence intensity in the patches infiltrated with 35S-GFP and 35S-HVT063 (1:50) declined (Fig. 7A). And in the next two weeks, infiltration of the leaves with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2-50) caused different degrees of the cell death phenotype (Table 1) and the green fluorescence was hardly detectable (data not show).

The suppression activity of HVT063 and HVT063 (1:2-50) was further confirmed by Northern blot with a GFP probe (Fig. 7B). At 7dpi, leaves co-infiltrated with 35S-GFP and 35S-mHVT063 had a drastically decrease in the level of GFP mRNA. There was enhanced accumulation of GFP mRNA in the leaves infiltrated with HVT063 or HVT063 (1:2-20), while a very small amount of GFP mRNA accumulation was observed in plants inoculated with 35S-GFP and 35S-HVT063 (1:50). Interestingly, the accumulation of GFP mRNA in leaves infiltrated with HVT063 (1:5) was more visibly detected than HVT063 (1:1-2) (Fig. 7B). This phenomenon possibly owed to the less cell death caused by HVT063 (1:5) compared to HVT063 (1:1-2) and the still strong RSS activity of HVT063 (1:5). Furthermore, the HVT063 dilutions induced weaker cell death phenotype than that of undiluted
To further compare the RNA silencing suppression capacity between the HVT063 dilutions and HVT063 or p19, we also analyzed the accumulation levels of GFP siRNAs in all infiltrated leaves as described in Fig. 7A (Fig. 7B). The GFP siRNAs showed high levels of accumulation in leaves infiltrated with 35S-GFP and 35S-mHVT063 at 7dpi. However, this accumulation was remarkably reduced in samples infiltrated with 35S-GFP and 35S-p19, 35S-HVT063 or 35S-HVT063 (1:2-5). In contrast, a clearly increased siRNAs was observed in patches co-infiltrated with 35S-GFP and 35S-HVT063 (1:10-50). Consequently, HVT063 dilutions revealed notable effect on GFP siRNAs which was reappearance with increasing dilution of HVT063.

Co-infiltration of 35S-GFP with either 35S-mHVT063 or 35S-p19 did not induce the cell death phenotype, while infiltrations of 35S-GFP with HVT063 or diluted (1:2-50) HVT063 induced different degrees of cell death phenotype (Table 1). To further explore the cell death phenotype, we checked for DNA laddering (a marker for programmed cell death). There was no DNA laddering phenomenon in co-infiltrated zone, indicating that necrosis is likely the type of cell death rather than PCD (programmed cell death) (Fig. S1). At 11dpi, 100% of the plants infiltrated with 35S-GFP and 35S-HVT063 evolved to chlorotic and necrotic mottling. By that time, 20-80% of plants co-infiltrated with 35S-GFP and 35S-HVT063 (1:2-20) resulted in the cell death phenotype and less than 10% of the plants co-infiltrated with 35S-GFP and 35S-HVT063 (1:50) progressed to chlorotic phenotype (Table 1).
This indicated that HVT063 of all above concentrations could cause cell necrosis and high level HVT063 triggered the cell death phenotype more rapidly. These results indicate that the death phenotype induced by HVT063 is dosage dependent.

In order to further explore whether low concentration HVT063 lost its suppressor activity against systemic silencing, GFP expression levels were monitored in the newly emerging leaves of the plants infiltrated with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2-50) (Table 2). By 10dpi, systemic GFP silencing occurred in 100% of the plants infiltrated with 35S-GFP and 35S-mHVT063, and 26% of the plants co-infiltrated with 35S-GFP and 35S-HVT063 (1: 50) (Table 2). In contrast, no plants co-infiltrated with 35S-GFP and 35S-HVT063 or 35S-p19 was systemically silenced even at 20dpi. The plants infiltrated with 35S-GFP and 35S-HVT063 (1:2-50) progressively lost systemic RNA silencing suppressing activity, 33-100% of plants showed systemic silencing at 24dpi (Table 2).

We also monitored GFP expression or silencing in upper noninfiltrated leaves to compare the effects of p19, HVT063 and HVT063 dilutions on systemic RNA silencing at 28dpi (Fig.7C and D). In most of the plants infiltrated with 35S-GFP and 35S-mHVT063, the whole plant lost GFP fluorescence, indicating that the systemic silencing spread completely in plants. In contrast, over 90% of noninfiltrated leaves in plants co-infiltrated with 35S-GFP plus 35S-HVT063 or 35S-p19 retained green fluorescence. The HVT063 dilutions progressively lost systemic RNA silencing suppressing activity, about 20%-80% of noninfiltrated leaves showed systemic silencing (Fig.7C). The steady-state levels of GFP mRNA were much higher in upper
leaves of the plants infiltrated with 35S-GFP and 35S-p19, 35S-HVT063 or 35S-HVT063 (1:2) than that co-infiltrated with 35S-GFP and 35S-mHVT063 at 28dpi. Moreover, the GFP mRNA was hardly detectable in those of plants infiltrated with 35S-GFP and 35S-HVT063 (1:20-50), just like in those co-infiltrated with 35S-mHVT063 (Fig. 7D). Leaves co-infiltrated with 35S-HVT063 (1:5-10) showed slightly higher GFP mRNA level compared to 35S-mHVT063 (Fig. 7D). Therefore, the HVT063 dilutions protect GFP mRNA in a dosage dependent manner at 28dpi.

Taken together, these results suggest that the HVT063 dilutions could suppress local silencing and induce different degrees of the cell death phenotype while they are not efficient to block systemic silencing.

Mutational analysis of HVT063 protein. Numerous studies indicate that the RNA binding activity is an important biochemical characteristic of the silencing suppressor. The conserved, surface-exposed and positively charged amino acids (K, R or H) have been found to be crucial for the activity of dsRNA-binding. So two positively charged amino acid rich regions of the HVT063 protein were selected to test which residues mainly contributes to the RNA silencing activity of HVT063. Region 1 (residues 138–141) and region 2 (residues 185–195) are both located near N terminal of the protein (Fig. 8A). These regions were analyzed by single alanine substitutions. So, four single amino acid substitutions were made in region 1 (K138A, R139A, R140A and R141A) and six in region 2 (R185A, R188A, K189A, R190A, R191A, R193A and R195A). The substitutions were co-infiltrated with 35S-GFP into 16c leaves, along with the negative control 35S-GFP and 35S-mHVT063 (Fig. 8A).
By 3dpi, the leaves infiltrated with 35S-GFP plus the empty vector or 35S-mHVT063, green fluorescence decreased as a consequence of RNA silencing activation. By contrast, in tissues co-infiltrated with 35S-GFP and 35S-p19 or 35S-HVT063, intense green fluorescence was observed. HVT063 mutations showed different RNA silencing suppressor activities (Fig. 8B). GFP intensity was very low in tissues co-infiltrated with 35S-GFP and K138A, R139A, R140A, R191A or R193A constructs and leaves infiltrated with 35S-GFP and R141A, R185A, R188A, K189A, R190A or R195A constructs exhibited an intermediate level of GFP fluorescence (Fig. 8B). Interestingly, all of HVT063 mutations failed to induce the characteristic cell death phenotype even at 13dpi (data not shown).

Northern blot analysis of GFP mRNA was consistent with the above observations. At 3dpi, levels of GFP mRNA was the lowest in leaves infiltrated with 35S-GFP and the empty vector or 35S-mHVT063 while a slightly higher level of GFP mRNA accumulation was observed in leaves infiltrated with 35S-GFP and K138A, R139A, R140A, R191A or R193A constructs (Fig. 8C). GFP mRNA levels of R141A, R185A, R188A, K189A, R190A or R195A constructs co-infiltrations were higher than that of K138A, R139A, R140A, R191A or R193A constructs, but significantly lower than that of p19 and HVT063 (Fig. 8C).

We further carried out Northern blot hybridization to examine the accumulation of GFP specific siRNAs (Fig. 8C). The results clearly revealed that GFP siRNAs had accumulated to high levels in leaves co-infiltrated with 35S-GFP and the empty vector or 35S-mHVT063. Similar results were also found in leaves co-infiltrated with...
35S-GFP and K138A, R139A, R140A, R191A or R193A constructs (Fig. 8C). In contrast, the GFP specific siRNAs levels were not easily detected in tissues co-infiltrated with 35S-GFP and 35S-HVT063 or 35S-p19 (Fig. 8C). An intermediate level of GFP siRNA was found in tissues co-infiltrated with 35S-GFP and R141A, R185A, R188A, K189A, R190A or R195A constructs (Fig. 8C).

To investigate whether HVT063 mutants are active in blocking systemic silencing, GFP expression was monitored in the newly emerging leaves of the plants infiltrated with 35S-GFP and the respective test or control constructs. It was found that no plants that were infiltrated with 35S-GFP and 35S-HVT063 developed GFP silencing at 13dpi, on the other hand, all plants infiltrated with 35S-GFP and single amino acid substitutions of HVT063 developed the characteristic vein proximal GFP silencing in the new leaves (data not shown).

Taken together, these results suggest that the 138K, 139R, 140R, 191R or 193R residues, but not the other residues (141R, 185R, 188R, 189K, 190R or 195 R), are more critical for the silencing ability of HVT063, and the region1 (138-141) might be an important site of silencing suppressor activity of HVT063. Nevertheless, all HVT063 mutations did not induce the cell death phenotype and failed to suppress systemic silencing.

**DISCUSSION**

RNA silencing is an important natural antiviral mechanism in plants and animals, which is a multistep process, including silencing initiation, spread and maintenance (55, 57). To counteract the RNA silencing, viruses encode specific proteins varying
from the sequences to structures, which have the ability to target different stages of RNA silencing pathway (14, 57). In this study, we screened five avian viral proteins using the classical *A. tumefaciens* co-infiltration assay. Our findings indicated that HVT063 protein encoded by *Turkey herpesvirus* (HVT) was a novel RNA silencing suppressor and its suppression activity is obviously dominated by protein rather than mRNA, since an untranslatable mutant of HVT063 could not suppress RNA silencing in co-infiltration assays. In addition, the HVT063 protein could suppress local silencing triggered by sense RNA or dsRNA in the co-infiltrated 16c. This indicated that HVT063 probably interfere with RNA silencing at the downstream of dsRNA formation, either by sequestering siRNAs or by interacting with components of RISC. If suppression only occur before dsRNA formation, HVT063 will no longer suppress RNA silencing once dsRNA form. That is consistent with some reported RSSs, such as ToCV p22, ScYLV P0 and TBSV p19 (10, 34, 47).

The silencing suppression function of HVT063 was also confirmed using heterologous PVX vector. This characteristic is distinct from that of several previously reported suppressors such as ToCV p22 and TCV CP, which were shown to be efficient local silencing suppressors while expressed from transient constructs but not from the heterologous PVX vector (10, 43, 49). An explanation for this phenomenon could be that this kind of proteins effectively inhibit silencing at early stage of RNA silencing process, as the expression of these proteins from the transient constructs might be earlier than that from the recombinant virus. Consequently, HVT063 protein may interfere with the spread and maintenance of RNA silencing.
considering it possesses the capacity to suppress RNA silencing in co-infiltration and
PVX expression system.

Our results showed that the suppression activity of HVT063 was possibly
equivalent to or stronger than p19, a well-studied and widely-used suppressor (47),
especially in the early stage of co-infiltration. The silencing suppression ability of
HVT063 protein was obviously stronger than p19 at 3dpi, subsequently declined
significantly, even weaker than p19 at 6dpi, estimated by the intensity of GFP
fluorescence or the GFP mRNA levels in co-infiltrated regions. This phenomenon
may be related to the death phenotype triggered by HVT063. During the
Agrobacterium co-infiltration assays, we found that the area infiltrated with HVT063
was always exhibiting evident fade or dry phenotypes, which progressed very rapidly
in the subsequent period and turned into completely dry necrosis at about 13dpi. At
the early phase of co-infiltration assays (especially 3dpi), HVT063 protein exerted
strong suppressor activity in the infiltrated area, while GFP expression decreased with
the amount of death cells gradually increasing, despite its strong inhibition capacity.
Indeed, a partial cell death caused by HVT063 protein was likely to affect the
expression of GFP.

The dilution experiments of HVT063 showed that its suppression activity and the
cell death phenotype were both crippled by the HVT063 decreasing in the
co-infiltrated region. Intriguingly, the RNA silencing suppression activities of
HVT063 (1:5) is weaker than that of HVT063 (1:1-2) judged by GFP siRNA level in
the infiltrated area, but inversely by GFP mRNA accumulation. A plausible
explanation could be that the GFP expression level in the co-infiltrated patches depended on the balance between the silencing suppression and the cell death, which is that the diluted HVT063 (1:5) was sufficient to increase the GFP mRNA accumulation by weakening cell death comparing with HVT063 (1:1-2), although meanwhile its suppression activity decreased. However, the enhanced GFP expression was not observed in HVT063 (1:10-50) co-infiltrated tissues, as the decline of RNA silencing suppression caused by diluted HVT063 was more significant than that of cell death. This kind of cell death phenotype caused by RSS had also been reported previously, such as P0 protein expressed by *Sugarcane yellow leaf* virus (ScYLV), which progressed so rapidly and severely that the infected areas appeared to be chlorotic and necrosis at as early as 1dpi (34). Unlike the scenario described for HVT063, P0 dilutions did not change the local silencing suppression activity, but failed to induce the cell death phenotype. We proposed that the cell death phenotype was due to those RSSs, which have certain cytotoxic or could interact with host cellular components of RNA silencing pathway. The specific mechanism of cell death caused by RSSs remains to be further studied.

Furthermore, our results also revealed that HVT063 protein was competent to inhibit systemic silencing induced by sense RNA or dsRNA in 16c plants, similarly to p19 of TBSV (47). There are two possible mechanisms for HVT063 protein to interfere with the signaling pathway of RNA silencing. Firstly, the mobile signal of RNA silencing was inadequate or weak for its strong local silencing suppression activity, which is not enough to trigger systemic silencing in whole plant. On the other
hand, HVT063 could directly interact with RNA silencing signal molecules and limited them in the injected area. Therefore, whether RSSs affect systemic silencing or not might depend on RSSs suppression activity and the mode of action. Although the presence of HVT063 protein can block intercellular RNA silencing in a period of time, the mobile signals are not completely eliminated. Ultimately, the systemic GFP silencing could also be observed in upper noninfiltrated leaves at 40dpi (data not shown). In addition, we showed that HVT063 could reverse GFP silencing of infiltrated patches, but not suppressed systemic gene silencing in newly emerging leaves. Similarly, B3 protein of *Banana bunchy top virus* (BBTV) also only recovered GFP fluorescence again in the inoculated leaves (39), while PVY HC-Pro could restore GFP fluorescence of silenced transgenic plants in both old and new leaves and CMV 2b rescues GFP expression only in new emerging leaves (32). A possible clue account for this result is that HVT063 may directly interact with the essential components of RNA silencing pathway, which is dosage dependent, or this kind of suppression could not be transferred to remote tissues.

Many RNA silencing suppressors were originally identified as determinants of pathogenicity in plants, such as the HC-Pro of *potyviruses* and βC1 of *tomato yellow leaf curl virus* (7, 15, 16, 46). Our study showed that the expression of HVT063 from the heterologous virus enhanced the accumulation of genomic PVX RNA, which is consistent with a role for this protein as an RNA silencing suppressor. However, HVT063 didn’t cause severe symptoms of PVX in infected plants even at 20 dpi, when compared with PVX-mHVT063 or the PVX empty vector (data not
shown). It is possible that HVT063 could only enhance the accumulation of genomic PVX RNA in the early stage of co-infiltration, while the PVX-HVT063 induced the cell death phenotype at as early as 7dpi and completely dry necrosis at 10dpi, which might decrease the accumulation of PVX and prevent PVX spread to other parts of the plant.

The siRNAs and/or long dsRNAs binding are the most common strategy of RSSs for RNA silencing suppression, which prevents the assembly of the RISC effector. Many suppressors, such as p19, 2b, B2, E3L, σ3, NS1 and so on, which contains a dsRNA binding domain, prevent RNA silencing through sequestrating double-stranded siRNAs and/or long dsRNAs (31, 63, 64). Therefore, the dsRNA binding domain of many RSSs is essential for their suppressor activity. According to previous reports, the clustered polar and positively charged amino acids have represented a key part in dsRNA binding region (8, 13, 25, 51, 58). For instance, three alanine substitutions of lysine residues at positions 173–175 of NS3 protein lost both its siRNA binding capacity and RNA silencing suppression activity (26); the inhibitory effect of B2 was almost abolished when replacing lysine by alanine at key position 51 of dsRNA binding domain (33). HVT063 protein was reported to possess dsRNA-binding ability (refer to its sequence information from NCBI), so we speculated that its RNA silencing suppressor activity might be connected to its dsRNA-binding ability. The mutation analysis of two positively charged amino acids rich regions—region1 (138-141) and region2 (185-195) of HVT063 demonstrated that all HVT063 mutations did affect its RNA silencing suppression activities. In
particular, three out of four single alanine mutagenesis in region 1 destroyed HVT063 suppression activities suggesting region 1, which is likely to be RNA binding sites, played an essential role in HVT063 silencing suppression activity. However, further studies on the precise functional motif and the suppression mechanism of HVT063 should be made. Moreover, some RNA binding RSSs also prevents RNA silencing through interacting with RNA silencing components, for example, 2b and p38 inhibits RNA silencing by binding siRNAs or interacting with AGO1(9); Tat has been found to sequester dsRNA or inhibit the activity of Dicer. Whether HVT063 protein has similar characteristics remains to be tested.

Our observations unequivocally demonstrated HVT063 possess strong silencing suppression activity, and it is prospectively to be developed as a novel tool in gene expression researches like TBSV P19. However, the ability of HVT063 to suppress silencing in its natural host cells have remained unknown. Recently, Herpes simplex virus type 1 (HSV-1), which is the prototypic member of the alphaherpesvirus subfamily, was demonstrated that it could suppress silencing in mammalian cells (62). HSV-1 infection suppressed EGFP-specific silencing as demonstrated by increased EGFP mRNA levels and an increase in the EGFP mRNA half-life (62). Remarkably, HVT is very similar at the amino acid level to HSV-1 and 62% of its genome is colinear with genes of HSV-1 (1, 50). It may be assumed that, HVT, which also belongs to alphaherpesvirus subfamily, likely could suppress silencing in mammalian cells. Given that many animal viruses are known to prevent or inhibit RNA silencing by encoding RNA silencing suppressor proteins, it is possible that HVT063...
contributes to RNA silencing suppression in its natural host cells. Doubtlessly, it will be of great importance to investigate the effects of HVT063 on RNA silencing in its natural host cells using the established experimental protocols (23, 35).

ACKNOWLEDGMENTS

We are grateful to David Baulcombe for providing plasmids pGR106, PBIN61-P19 and N. benthamiana 16c seeds. Thanks to Zhizhong Cui and Shuhong Sun for providing avian virus strains.

This work was supported by grants 200802008 (Postdoctoral Innovation Foundation of Shandong Province), 30871620 (National Natural Science Foundation of China, NSFC) and 201003065 (Special Fund for Agro-scientific Research in the Public Interest).

REFERENCES


RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J. 25: 2768-2780.


38. Merai, Z., Z. Kerenyi, A. Molnar, E. Barta, A. Valoczi, G. Bisztray, Z. Havelda,


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**Reference List**


**Figure Legend**

FIG. 1. Identification of HVT063 as an RNA silencing suppressor by Agrobacterium co-infiltration assay. (A) Co-infiltration of GFP-transgenic N. benthamiana line 16c leaves with Agrobacterium carrying 35S-GFP and 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or with constructs expressing HVT063 and p19. Photographs taken under UV illumination and under a confocal microscopy of N. benthamiana line 16c leaves at 3 and 6 days post infiltration (dpi). (B) Northern blot analysis of GFP mRNA and siRNA extracted from the patches co-infiltrated with the different strains indicated above each lane, at 3 and 6dpi. Ethidium bromide-stained
FIG. 2. HVT063 counteracts local RNA silencing triggered by sense GFP RNA and GFP dsRNA. (A) Leaves of GFP-transgenic *N. benthamiana* line 16c plants were infiltrated with three strains of *Agrobacterium* carrying 35S-GFP, 35S-dsGFP, and either 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or with constructs expressing HVT063 and p19 respectively. GFP expression in the infiltrated tissue was monitored at 3 and 6 days post infiltration (dpi) under UV illumination. (B) Northern blot analysis of GFP mRNA and siRNA extracted from the patches co-infiltrated with the different strains indicated above each lane, at 3 and 6dpi. Ethidium bromide-stained rRNA and tRNA was shown as loading control for mRNA and siRNA, respectively.

FIG. 3. Suppression of local GFP silencing when HVT063 expressed from the heterologous vector PVX. Co-infiltration of GFP-transgenic *N. benthamiana* line 16c with 35S-GFP and PVX-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or PVX-HVT063, photographs were taken at 6 days post infiltration (dpi) under UV light (A) and at 10dpi under normal light (C). (B) Northern blot analysis of GFP mRNA and genomic PVX RNA isolated from the patches co-infiltrated with the different strains indicated above each lane at 6dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA.
FIG. 4. Reversal of established RNA silencing by HVT063. GFP-transgenic *N. benthamiana* line 16c was infiltrated with an *Agrobacterium* strain harboring 35S-GFP and then the plants were silenced for GFP at 18 days post infiltration (dpi). (A) PVX-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or PVX-HVT063 were infiltrated into upper leaves of these plants, images of these leaves were taken 7 days after the second infiltration under UV illumination. (B) Northern blot analysis of GFP mRNA and genomic PVX RNA isolated from infiltrated local leaves (A) at 7dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA. (C) Images of these plants were taken 20 days after the second infiltration under UV illumination. (D) The 35S-mHVT063, 35S-HVT063 or 35S-p19 were infiltrated into upper leaves of the silenced plants, images of these plants were taken 7 days after the second infiltration under UV illumination. (E) Northern blot analysis of GFP mRNA isolated from infiltrated local leaves (D) at 7dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA.

FIG. 5. Suppression activity of HVT063 against systemic GFP silencing triggered by GFP sense RNA. (A) Photographs taken under UV light and normal light of GFP transgenic *N. benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* harbouring 35S-GFP, and either 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein), or with constructs expressing HVT063 and p19, at 13 days post infiltration (dpi). (B) Northern blot analysis of GFP mRNA isolated from local and systemic leaves of the plants co-infiltrated with the different strains indicated above.
each lane, at 13dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA.

FIG. 6. HVT063 suppresses systemic GFP silencing triggered by GFP dsRNA. (A) Co-infiltration of GFP transgenic *N. benthamiana* 16c leaves with three strains of *Agrobacterium* carrying 35S-GFP, 35S-dsGFP, and either 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or with constructs expressing HVT063 and p19, and the photographs were taken under UV light or normal light at 13 days post infiltration (dpi). (B) Northern blot analysis of GFP mRNA isolated from local and systemic leaves of the plants co-infiltrated with the different strains indicated above each lane, at 13dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA.

FIG. 7. Effect of HVT063 on local and systemic GFP silencing. Co-infiltration of GFP transgenic *N. benthamiana* 16c leaves with 35S-GFP, and either 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or with constructs expressing p19, HVT063 (undiluted) and HVT063 diluted (1:2, 1:5, 1:10, 1:20 and 1:50). Note that undiluted HVT063 was the *Agrobacterium* culture which grown to an optical density OD$_{600}$=1.0, and the *Agrobacterium* carrying HVT063 was sequentially diluted by a factor of 2, 5, 10, 20 and 50. The photographs were taken at 7 days post infiltration (dpi) (A) and 28dpi (C) under UV light. (B) Northern blot analysis of GFP mRNA and GFP siRNA isolated from the local leaves co-infiltrated
FIG. 8. Suppression of GFP silencing in 16c leaves by single alanine substitution mutants of HVT063. (A) Schematic representation of HVT063 single alanine mutagenesis that were used in the preliminary screening for silencing suppressor activity against local sGFP-PTGS in GFP transgenic *N. benthamiana* line 16c (mHVT063, an untranslatable mutant of HVT063 protein). (B) Co-infiltration of GFP transgenic *N. benthamiana* 16c leaves with *Agrobacterium* carrying 35S-GFP plus either the empty vector (signed as vector) or with 35S-p19, 35S-HVT063, 35S-mHVT063 and the HVT063 mutant constructs (K138A, R139A, R140A, R141A, R185A, R188A, K189A, R190A, R191A, R193A, R195A), and the photographs were taken at 3 days post infiltration (dpi) under UV light. (C) Northern blot analysis of GFP mRNA and siRNA isolated from local leaves of the plants co-infiltrated with the different strains indicated above each lane, at 3dpi. Ethidium bromide-stained rRNA and tRNA was shown as loading control for mRNA and siRNA, respectively.

### Tables

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<th>Table 1. Induction of cell death phenotype determined by HVT063 or the HVT063...</th>
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<td>with the different strains indicated above each lane, at 7dpi. Ethidium bromide-stained rRNA and tRNA was shown as loading control for mRNA and siRNA, respectively.</td>
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<td>(D) Northern blot analysis of GFP mRNA extracted from systemic leaves of the plants co-infiltrated with the different strains indicated above each lane, at 28dpi. The ethidium bromide-stained rRNA was used as loading control for mRNA.</td>
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<td>FIG. 8. Suppression of GFP silencing in 16c leaves by single alanine substitution mutants of HVT063. (A) Schematic representation of HVT063 single alanine mutagenesis that were used in the preliminary screening for silencing suppressor activity against local sGFP-PTGS in GFP transgenic <em>N. benthamiana</em> line 16c (mHVT063, an untranslatable mutant of HVT063 protein). (B) Co-infiltration of GFP transgenic <em>N. benthamiana</em> 16c leaves with <em>Agrobacterium</em> carrying 35S-GFP plus either the empty vector (signed as vector) or with 35S-p19, 35S-HVT063, 35S-mHVT063 and the HVT063 mutant constructs (K138A, R139A, R140A, R141A, R185A, R188A, K189A, R190A, R191A, R193A, R195A), and the photographs were taken at 3 days post infiltration (dpi) under UV light. (C) Northern blot analysis of GFP mRNA and siRNA isolated from local leaves of the plants co-infiltrated with the different strains indicated above each lane, at 3dpi. Ethidium bromide-stained rRNA and tRNA was shown as loading control for mRNA and siRNA, respectively.</td>
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### Table 2. Suppression of systemic RNA silencing by HVT063 or the HVT063 dilutions

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<th>infiltrations</th>
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<tr>
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<td>6</td>
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<tr>
<td>sGFP+mHVT063</td>
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<tr>
<td>sGFP+p19</td>
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<td>sGFP+HVT063(1:10)</td>
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<tr>
<td>sGFP+HVT063(1:50)</td>
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*aUndiluted HVT063 was the Agrobacterium culture which grown to an optical density OD$_{600}$=1.0, and the Agrobacterium carrying HVT063 was sequentially diluted by a factor of 2, 5, 10, 20 and 50 (mHVT063, an untranslatable mutant of HVT063 protein). b15 plants co-infiltrated with the different strains indicated above each lane.

dpi: days post infiltration.
15 plants co-infiltrated with the different strains indicated above each lane. dpi: days post infiltration.
A 6 dpi
35S GFP+ PVX-mHVT063 PVX-HVT063

B
PVX-mHVT063 PVX-HVT063
GFP mRNA
PVX gRNA
rRNA

C 10 dpi
35S GFP+ PVX-mHVT063 PVX-HVT063
### A

<table>
<thead>
<tr>
<th>35S GFP+</th>
<th>13 dpi</th>
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<tr>
<td>mHVT063</td>
<td>HVT063</td>
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![Image of leaf samples](image)

## B

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<td>HVT063 p19</td>
<td>mHVT063 HVT063 p19</td>
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<td>rRNA</td>
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### A

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| 35S GFP+   | 35S dsGFP+ |

### B

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</table>

**GFP mRNA**

**rRNA**