A Multifunctional Protein Encoded by Turkey Herpesvirus Suppresses RNA Silencing in Nicotiana benthamiana

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Many plant and animal viruses counteract RNA silencing-mediated defense by encoding diverse RNA silencing suppressors. We characterized HVT063, a multifunctional protein encoded by turkey herpesvirus (HVT), as a silencing suppressor in coinfiltration assays with green fluorescent protein 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 (dsRNA). 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 the HVT063 protein showed that each selected single amino acid contributed to the suppression activity of HVT063 and region 1 (residues 138 to 141) was more important, because three of four single amino acid mutations in this region could abolish the silencing suppressor activity of HVT063. Moreover, HVT063 seemed to induce a cell death phenotype in the infiltrated leaf region, and the HVT063 dilutions could decrease the silencing suppressor activity and alleviate the cell death phenotype. Collectively, these results suggest that HVT063 functions as a viral suppressor of RNA silencing that targets a downstream step of the dsRNA formation in the RNA silencing process. 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, since HVT063 has been demonstrated to be an RNA binding protein.

Small interfering RNA (siRNA)-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 proteins into small RNA duplexes of 21 to 24 nucleotides (nt) (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 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, and RNA targeting and amplification (7, 28, 29, 36, 56). For example, the P14 protein of Potnos latent aureusvirus, the P38 protein of Turnip crinkle virus (TCV), the 2b protein of Tomato aspermy virus, and the 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). The p19 protein of Tomato bushy stunt virus (TBSV) and influenza A virus NS1 protein prevent RNA silencing by siRNA sequestration through binding siRNA in a size-specific manner (18, 47). The 2b protein of Cucumber mosaic virus (CMV) and the P0 protein of Potato leaf roll virus target the AGO protein to prevent RISC assembly (9). The P38 protein of TCV and the P1 protein of Sweet potato mild mottle virus interact with AGO proteins by the glycine/tryptophan (GW/GW) residues (4, 22). The human immunodeficiency virus type 1 (HIV-1) Tat protein and the core protein of hepatitis C virus interact with Dicer to ablate the effect of RNAi (6, 42, 60). Thus, 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 not cell autonomous; it is initially induced...
at the single-cell level and then transmitted to the remote cells or tissues to cause systemic RNA silencing. In plants, the silencing signal moves from cell to cell through plasmodesmata and over long distances via the vascular system (24, 40, 53, 54, 57). Recent studies suggest that the short-distance spread of RNA silencing is mediated by 21-nt siRNAs; however, the role of small RNAs in long-distance signaling remains to be elucidated (19). Roth et al. reported that several suppressors, such as CMV 2b, TBSV p19, Tomato spotted wilt virus (TSVW) Ns proteins, and PVX P25 could prevent gene silencing in systemic tissues (44). Among many of the known mammalian viral suppressors, the B2 protein of H1V (30) and the influenza A virus NS1 protein (8, 17) first exhibit RNA silencing suppressor activity in plant cells. For this reason, RNA silencing represented as an important antiviral defense 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 understanding RNA silencing mechanisms, as well as virus-host interactions. Here, we present HVT063 encoded by turkey herpesvirus is a strong RSS. HVT is a dsDNA avian virus, and it is classified as the third serotype of the Marek’s disease virus 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, has some features that make it a good RSS candidate, such as binding RNA and shutting between nucleus and cytoplasm (sequence information is available from the National Center for Biotechnology Information [NCBI]). Our results showed that HVT063 suppressed both local and systemic RNA silencing induced by sense RNA or dsRNA. Moreover, HVT063 protein could reverse local silencing with a preexisting silenced transgene, and its RSS activities were dosage dependent. Also, the single alanine substitutions of arginine or lysine residues indicated that the RSS activity of HVT063 was probably related to RNA or siRNA binding ability.

**MATERIALS AND METHODS**

**Plant materials and plasmid constructs.** Green fluorescent protein (GFP) transgenic *N. benthamiana* line 16c plants were grown at 24 ± 2°C under a 16-h illumination (34-W fluorescent bulbs) and 5-h darkness regimen. The HVT063 protein (NP_073349) used in this experiment was PCR amplified from the HVT genome using the specific primers HVT063-F and HVT063-R (data not shown). Furthermore, the pp24 gene (ABF27222), pp38 gene (ABF72309), and MEQ gene (ABF72204) were PCR amplified from the Marek’s disease virus genome with the specific primer pairs pp24-F/pp24-R, pp38-F/pp38-R, and MEQ-F/MEQ-R, and the N gene (AC03757;3) was amplified by reverse transcription-PCR (RT-PCR) from total RNA extracted from Avian infectious bronchitis virus with the specific primer pair N-F/N-R (data not shown). These constructs were digested with XbaI and BamHI restriction enzymes and cloned into the binary vector pBI121 to generate 35S-p19 as a positive control in various experiments.

**Total RNA and siRNA isolation for Northern blot analysis.** Approximately 1 g of frozen fresh samples of leaf infiltrated zones or systemic silencing leaves were ground into powder in liquid nitrogen and then transferred to 15-mL tubes containing 2 to 3 mL of hot phenol buffer (extraction buffer was composed of 0.1 M LiCl, 100 mM Tris-Cl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulfate [SDS]; to prepare the phenol buffer, extraction buffer was mixed with equal volumes of phenol and water and preheated at 80°C for 5 min). A 1/4 volume of chloroform was added, followed by vortexing for 20 s. The tubes were centrifuged at 10,000 × g for 10 min at room temperature, the aqueous phase was transferred to new tubes, and then an equal volume of 4 M LiCl was added. The contents were gently mixed and left at −20°C overnight. The total RNA pellets were obtained by centrifugation at 10,000 × g for 15 min at 4°C (keeping the supernatant for small RNA isolation). The pellets were resuspended in 300 μL of diethyl pyrocarbonate (DEPC)-treated TE buffer (10 mM Tris-Cl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate [SDS]; to prepare the TE buffer, 10 mM Tris-Cl, 1 mM EDTA, and 0.1% SDS were mixed with equal volumes of water and hot preheated at 80°C for 5 min) and then washed twice with 75% ethanol. The pellets were air dried and dissolved in 50 to 100 μL of DEPC-treated H2O.

Low-molecular-weight RNAs were isolated from mixtures of total and siRNAs. First, a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the supernatant, followed by incubation at −20°C for 15 min. The RNA pellets were precipitated by centrifugation at 10,000 × g for 15 min at 4°C, washed with 70% ethanol, and resuspended in 50 to 100 μL of DEPC-treated H2O.

**Total RNA Northern blot analysis.** For Northern blot analysis of high-molecular-weight RNA, 10 μg of total RNA was separated on a 1% agarose-morpholinepropanesulfonic acid (MOPS)-formaldehyde gel in 1× MOPS buffer and transferred to Hybond-N+ membranes (Amersham) for Northern blot analysis as previously described (43). Next, the membrane was UV cross-linked and treated at 80°C for at least 2 h and then stored at 4°C until used. The membrane was briefly washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.105 M sodium citrate) for 1 min at room temperature. The wash solution was then discarded,
RESULTS

HVT063 blocks local RNA silencing triggered by both sense RNA and dsRNA. To screen potential RNA silencing suppressors encoded by avian viruses, the HVT063 protein of HVT, the pp24, pp38, and MEQ proteins of Marek’s disease virus, and the N protein of Avian infectious bronchitis virus were tested using an Agrobacterium coinfiltration assay. Transgenic *N. benthamiana* line 16c plants infiltrated with cultures harboring 35S-GFP and cultures harboring either an empty vector, 35S-HVT063, 35S-GFP and cultures harboring either an empty vector, 35S-HVT063, 35S-GFP and 35S-mHVT063 (in which the initiating codon ATG of HVT063 ORF was replaced with GTG) or a vector containing 35S-p19 were used as a negative or positive control, 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, the GFP fluorescence reached the highest level in the infiltrated zone 2 to 3 days postinfiltration (dpi) and then declined to almost disappear at 6 dpi as a consequence of RNA silencing activation (Fig. 1A). The same results were observed in leaves coinfiltrated with sense GFP and any avian viral protein constructs (data not shown) except for the HVT063 construct. Leaves coinfiltrated with cultures carrying the GFP reporter gene plus that expressing HVT063 showed a marked increase in green fluorescence in infiltrated area at 3 dpi and remained at a high level until 6 dpi, as observed under a hand-held UV light or under a confocal microscope (Fig. 1A). The GFP fluorescence in regions coinfiltrated with 35S-GFP and 35S-mHVT063 was slightly higher than that in areas coinfiltrated with the established plant viral silencing suppressor p19 of TBSV at 3 dpi (Fig. 1A). However, at 6 dpi, GFP intensity in leaves coinfiltrated with 35S-GFP and 35S-HVT063 was substantially weaker than that in coinfiltrations 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 all coinfiltrations with 35S-GFP with 35S-HVT063 or 35S-p19 accumulated to a markedly higher abundance than that of 35S-GFP and 35S-mHVT063 coinfiltrations at both 3 and 6 dpi (Fig. 1B). Moreover, the level of GFP mRNA accumulation from regions coinfiltrated with 35S-GFP and 35S-HVT063 was higher than that from leaves coinfiltrated with 35S-GFP and 35S-HVT063.
35S-p19 at 3 dpi, whereas GFP mRNA levels of HVT063 coinfiltrations were significantly lower than that of p19 at 6 dpi (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 coinfiltrated with 35S-GFP and 35S-mHVT063 was correlated with the accumulation of GFP-specific 21- and 24-nt siRNAs, which were especially abundant at 6 dpi (Fig. 1B). However, this accumulation was drastically reduced to an undetectable level in tissues infiltrated with the different strains indicated above each lane at 3 and 6 dpi. Ethidium bromide-stained rRNA and tRNA are shown as the loading controls for mRNA and siRNA, respectively.

35S-p19 at 3 dpi, whereas GFP mRNA levels of HVT063 coinfiltrations were significantly lower than that of p19 at 6 dpi (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 coinfiltrated with 35S-GFP and 35S-mHVT063 was correlated with the accumulation of GFP-specific 21- and 24-nt siRNAs, which were especially abundant at 6 dpi (Fig. 1B). However, this accumulation was drastically reduced to an undetected level in tissues infiltrated with 35S-GFP/35S-HVT063 or 35S-p19 at either 3 or 6 dpi (Fig. 1B).

The inverted repeats are considered as the strong inducers of RNA silencing since they produce a high level of functional siRNAs. To test whether HVT063 could interfere with GFP silencing initiated by a dsGFP (i.e., an inverted repeat generating GFP dsRNA) inducer, leaves of 16c were coinfiltrated 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 coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-mHVT063 at 3 dpi (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 6 dpi (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 coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-mHVT063 or 35S-p19 than those coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-HVT063 at both 3 and 6 dpi (Fig. 2B). Further analysis of siRNA indicated that by 3 dpi the GFP siRNAs had accumulated to high levels in leaves coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-mHVT063. They reached much higher levels in these leaves by 6 dpi (Fig. 2B). However, the GFP-specific siRNA levels were remarkably low in leaves coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-HVT063 or 35S-p19 at either 3 or 6 dpi (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 coinfiltrated with 35S-GFP and PVX-mHVT063 or PVX-HVT063, respectively. Weak or absent GFP fluorescence was observed in tissues coinoculated with 35S-GFP and PVX-mHVT063 at either 3 or 6 dpi, whereas fluorescence was maintained in inoculated patches when HVT063 was expressed from the PVX vector until 6 dpi (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 the barely detectable levels observed in leaves coinfiltrated with PVX-mHVT063 (Fig. 3B). Furthermore, the level of PVX RNA was significantly higher in leaves coinfiltrated with PVX-HVT063 than that in leaves
coinfiltrated with PVX-mHVT063 at 6 dpi (Fig. 3B). More genomic PVX RNA in the presence of PVX-HVT063 indicated that the HVT063 open reading frame cloned was retained in the viral progeny and played the role of an RSS. Taken together, these results again indicate that HVT063 presents efficient RSS activity in plant cells. Interestingly, coinfiltration of 35S-GFP and PVX-HVT063 induced the cell death phenotype as early as 7 dpi and completely dry necrosis by 10 dpi (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 coinfiltration 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 first, and then a PVX vector (pGR106) carrying HVT063 or mHVT063 was inoculated on the upper leaves at 18 dpi when silencing occurred throughout the plant. The leaves infiltrated with PVX carrying HVT063 recovered green fluorescence under UV light at 7 dpi, while the leaves infiltrated with PVX-mHVT063 showed a 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.

These findings were also demonstrated using the pBI121 vector expressing HVT063. By 7 dpi, intense green fluorescence could be observed in the leaves of GFP-silenced plants coinfiltrated with pBI121 carrying HVT063 or p19. In contrast, in tissues infiltrated with the pBI121-mHVT063, green fluorescence was not detected (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 inhibits a function required for the maintenance of the silenced state.

HVT063 inhibits systemic RNA silencing triggered by sense RNA or dsRNA. If the silencing signal delivered from the coinfiltrated area, the cell-to-cell movement of the signal should cause shutting down of GFP expression. These 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 plants were coinfiltrated in parallel with 35S-GFP plus either 35S-mHVT063, 35S-HVT063, or 35S-p19. The infiltrated plants were monitored under UV light for the initia-
tion of systemic silencing in the newly emerging leaves. At 13 dpi, plants infiltrated with 35S-GFP and 35S-mHVT063 showed the characteristic vein-proximal GFP silencing in new leaves (Fig. 5A). Limited systemic silencing was observed in plants infiltrated with 35S-GFP and either 35S-HVT063 or 35S-p19 (Fig. 5A). Furthermore, ca. 75% of the plants infiltrated with 35S-GFP and 35S-HVT063 consistently showed strong green GFP fluorescence in the newly emerging leaves even at 35 dpi. By that time, GFP expression had been absolutely lost in plants coinfiltrated with 35S-p19 (data not shown). Systemic silencing was further explored by determining the GFP mRNA levels in the newly emerging leaves of the plants coinfiltrated with the different strains indicated above each lane at 13 dpi. Ethidium bromide-stained rRNA was used as a loading control for mRNA.

Moreover, strong GFP expression was only seen in leaves coinfiltrated with 35S-GFP and 35S-p19 at 13 dpi, whereas the fluorescence in leaves coinfiltrated with 35S-GFP plus either 35S-mHVT063 or 35S-p19 declined significantly and became almost undetected by 13 dpi (Fig. 5A). Our visual observations were confirmed by Northern blot analysis of GFP mRNA isolated from local and systemic leaves of the plants coinfiltrated with the different strains indicated above each lane at 13 dpi. Ethidium bromide-stained rRNA was used as a 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 A. tumefaciens harboring 35S-GFP and either 35S-mHVT063 (mHVT063, an untranslatable mutant of HVT063 protein) or constructs expressing HVT063 and p19 at 13 dpi. (B) Northern blot analysis of GFP mRNA isolated from local and systemic leaves of the plants coinfiltrated with the different strains indicated above each lane at 13 dpi. Ethidium bromide-stained rRNA was used as a loading control for mRNA.

FIG. 6. HVT063 suppresses systemic GFP silencing triggered by GFP dsRNA. (A) Coinfiltration 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 constructs expressing HVT063 and p19, and photographs were taken under UV or normal light at 13 dpi. (B) Northern blot analysis of GFP mRNA isolated from local and systemic leaves of the plants coinfiltrated with the different strains indicated above each lane at 13 dpi. Ethidium bromide-stained rRNA was used as a loading control for mRNA.

HVT063 also became chlorotic as early as 7 dpi, 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 13 dpi (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 coinfiltrated 16c leaves with 35S-GFP, 35S-dsGFP, and either 35S-mHVT063, 35S-HVT063, or 35S-p19. Our results showed that systemic GFP silencing occurred in all of the plants coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-mHVT063, showing prominent vein-proximal GFP silencing in the newly emerging leaves at 13 dpi (Fig. 6A). In contrast, there was strong GFP fluorescence in the newly emerging leaves of plants whose primary leaves were coinfiltrated with 35S-GFP, 35S-dsGFP, and 35S-HVT063. The leaves infiltrated with 35S-GFP, 35S-dsGFP, and 35S-HVT063 had been completely dry at necrosis and did not show any fluorescence at 13 dpi (Fig. 6A).

The GFP fluorescence in local and systemic silencing leaves at 13 dpi 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 GFP 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 13 dpi (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 with 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 that did not lack 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 concentrations, and the death phenotype induced by HVT063 is dosage dependent. 16c plants were coinfiltrated with 35S-GFP and 35S-HVT063 or diluted 35S-HVT063 (1:2 to 1:50). Our results showed that by 3 dpi, the green fluorescence was much brighter in tissues infiltrated with 35S-GFP and 35S-HVT063 or 35S-mHVT063 than that infiltrated with 35S-GFP and 35S-mHVT063 (data not show). Moreover, at 7 dpi, the leaves infiltrated with 35S-GFP and 35S-mHVT063 showed red fluorescence under UV light, indicating the induction of local GFP silencing. The plants coinfiltrated with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2 to 1:50) dramatically increased GFP expression; however, the green fluorescence intensity in the patches infiltrated with 35S-GFP and 35S-HVT063 (1:50) declined (Fig. 7A). In the next 2 weeks, infiltration of the leaves with 35S-GFP and 35S-HVT063 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 7 dpi, leaves coinfiltrated with 35S-GFP and 35S-mHVT063 displayed a drastic decrease in the level of GFP mRNA. There was enhanced accumulation of GFP mRNA in 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 GFP 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 13 dpi (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 with 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 that did not lack silencing suppression capability.

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the leaves infiltrated with HVT063 or HVT063 (1:2 to 1:20), 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 to 1:2) (Fig. 7B). This phenomenon was possibly due to the reduced cell death caused by HVT063 (1:5) compared to HVT063 (1:1 to 1:2) and the still strong RSS activity of HVT063 (1:5). Furthermore, the HVT063 dilutions induced a weaker cell death phenotype than did undiluted HVT063 (data not shown).

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 (see also Fig. 7B). The GFP siRNAs showed high levels of accumulation in leaves infiltrated with 35S-GFP and 35S-mHVT063 at 7 dpi. However, this accumulation was remarkably reduced in samples infiltrated with 35S-GFP and 35S-p19, 35S-HVT063, or 35S-HVT063 (1:2 to 1:5). In contrast, a clearly increased siRNAs was observed in patches coinfiltrated with 35S-GFP and 35S-HVT063 (1:10 to 1:50). Consequently, HVT063 dilutions revealed a notable effect on GFP siRNAs, i.e., reappearance with increasing dilutions of HVT063.

Coinfiltration of 35S-GFP with either 35S-mHVT063 or 35S-p19 did not induce the cell death phenotype, whereas infiltrations of 35S-GFP with HVT063 or diluted (1:2 to 1:50) HVT063 induced different degrees of the 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 the coinfiltrated zone, indicating that necrosis is likely the type of cell death rather than programmed cell death (data not shown). At 11 dpi, 100% of the plants infiltrated with 35S-GFP and 35S-HVT063 evolved to chlorotic and necrotic mottling. By that time, 20 to 80% of the plants coinfiltrated with 35S-GFP and 35S-HVT063 (1:2 to 1:20) predicted in the cell death phenotype, and <10% of the plants coinfiltrated with 35S-GFP and 35S-HVT063 (1:50) progressed to the chlorotic phenotype (Table 1).

This indicated that HVT063 at all of the concentrations discussed above could cause cell necrosis, and high levels of 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 a low concentration of HVT063 lost its suppressor activity against systemic silencing, GFP expression levels were monitored in the newly emerging leaves of plants infiltrated with 35S-GFP and 35S-HVT063 or 35S-HVT063 (1:2 to 1:50) (Table 2). By 10 dpi, systemic GFP silencing occurred in 100% of the plants infiltrated with 35S-GFP and 35S-mHVT063 and in 26% of the plants coinfiltrated with 35S-GFP and 35S-HVT063 (1:50) (Table 2). In contrast, no plants coinfiltrated with 35S-GFP and 35S-HVT063 or 35S-p19 were systemically silenced, even at 20 dpi. The plants infiltrated with 35S-GFP and 35S-HVT063 (1:2 to 1:50) progressively lost systemic RNA silencing suppressing activity, and 33 to 100% of the plants showed systemic silencing at 24 dpi (Table 2).

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

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 to 141) and region 2 (residues 185 to 195) are both located near the N terminus of the protein (Fig. 8A). These regions were analyzed by single alanine substitutions. Thus, four single amino acid substitutions were made in region 1 (K138A, R139A, R140A, and R141A), and six were made in region 2 (R185A, R188A, K189A, R190A, R191A, R192A).

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**TABLE 2. Suppression of systemic RNA silencing by HVT063 or HVT063 dilutions**

<table>
<thead>
<tr>
<th>Infiltration (dilution)*</th>
<th>Total no. of plants showing systemic RNA silencing at various times postinfiltration (dpi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S-GFP+mHVT063</td>
<td>0  8  15</td>
</tr>
<tr>
<td>35S-GFP+p19</td>
<td>0  0  0  0  0  0  0  0  1  2</td>
</tr>
<tr>
<td>35S-GFP+HVT063</td>
<td>0  0  0  0  0  0  0  0  0  0</td>
</tr>
<tr>
<td>35S-GFP+HVT063 (1:2)</td>
<td>0  0  0  0  0  1  1  2  3  5</td>
</tr>
<tr>
<td>35S-GFP+HVT063 (1:5)</td>
<td>0  0  0  1  6  11 13 14 14</td>
</tr>
<tr>
<td>35S-GFP+HVT063 (1:10)</td>
<td>0  0  1  2  9  12 14 14 15</td>
</tr>
<tr>
<td>35S-GFP+HVT063 (1:20)</td>
<td>0  0  2  6  13 14 15</td>
</tr>
<tr>
<td>35S-GFP+HVT063 (1:50)</td>
<td>0  0  4  9  15</td>
</tr>
</tbody>
</table>

* Undiluted HVT063 was the Agrobacterium culture which grown to an OD_{600} of 1.0, and the Agrobacterium strain carrying HVT063 was sequentially diluted by a factor of 2, 5, 10, 20, and 50 (mHVT063, an untranslatable mutant of HVT063 protein). dpi, days postinfiltration.

* A total of 15 plants were coinfiltrated for each of the different combinations indicated in column 1.

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The substitutions were coinfilted with 35S-GFP into 16c leaves, along with the negative control 35S-GFP and 35S-mHVT063 (Fig. 8A).

By 3 dpi, in leaves infiltrated with 35S-GFP plus the empty vector or 35S-mHVT063 the green fluorescence decreased as a consequence of RNA silencing activation. In contrast, in tissues coinfilted with 35S-GFP and 35S-p19 or 35S-HVT063, intense green fluorescence was observed. HVT063 mutations showed different RNA silencing suppressor activities (Fig. 8B). The GFP intensity was very low in tissues coinfilted 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 the HVT063 mutations failed to induce the characteristic cell death phenotype even at 13 dpi (data not shown).

Northern blot analysis of GFP mRNA was consistent with the above observations. At 3 dpi, the 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 coinfiltrations 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 coinfiltrated with 35S-GFP and the empty vector or 35S-mHVT063, while slightly lower level of GFP mRNA accumulation was observed in leaves coinfilted 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 coinfiltrations were higher than that of K138A, R139A, R140A, R191A, or R193A constructs but significantly lower than that of p19 and HVT063 (Fig. 8C).

To investigate whether HVT063 mutants are active in blocking systemic silencing, GFP expression was monitored in the newly emerging leaves of plants infiltrated with 35S-GFP and the respective test or control constructs. We found that no plants that were infiltrated with 35S-GFP and 35S-HVT063 developed GFP silencing at 13 dpi; 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 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 195R), are more critical for the silencing ability of HVT063, and that region 1 (residues 138 to 141) might be an important site for the silencing suppressor activity of HVT063. Nevertheless, all of the 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; it is a multistep process, including silencing initiation, spread, and maintenance (55, 57). To counteract the RNA silencing, viruses encode specific proteins vary-
ing from sequences to structures, which have the ability to target different stages of the RNA silencing pathway (14, 57). In the present study, we screened five avian viral proteins using a classical *A. tumefaciens* coinfiltration assay. Our findings indicated that HVT063 protein encoded by HVT was a novel RNA silencing suppressor, and its suppression activity is obviously dominated by protein rather than mRNA, since an untranslated mutant of HVT063 could not suppress RNA silencing in coinfiltration assays. In addition, the HVT063 protein could suppress local silencing triggered by sense RNA or dsRNA in coinfiltrated 16c plants. This indicated that HVT063 probably interferes with RNA silencing downstream of dsRNA formation, either by sequestering siRNAs or by interacting with components of RISC. If suppression only occurs before dsRNA formation, HVT063 will no longer suppress RNA silencing once dsRNA forms. 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 the 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 when expressed from transient constructs but not from the heterologous PVX vector (10, 43, 49). An explanation for this phenomenon could be that these kinds of proteins effectively inhibit silencing at an early stage of the RNA silencing process, since the expression of these proteins from the transient constructs might occur earlier than that from the recombinant virus. Consequently, HVT063 protein may interfere with the spread and maintenance of RNA silencing, since it possesses the capacity to suppress RNA silencing in coinfiltration and the 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 coinfiltration. The silencing suppression ability of HVT063 protein was obviously stronger than p19 at 3 dpi, subsequently coinfiltration. The silencing suppression function of HVT063 was also confirmed using the 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 when expressed from transient constructs but not from the heterologous PVX vector (10, 43, 49). An explanation for this phenomenon could be that these kinds of proteins effectively inhibit silencing at an early stage of the RNA silencing process, since the expression of these proteins from the transient constructs might occur earlier than that from the recombinant virus. Consequently, HVT063 protein may interfere with the spread and maintenance of RNA silencing, since it possesses the capacity to suppress RNA silencing in coinfiltration and the 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 coinfiltration. The silencing suppression ability of HVT063 protein was obviously stronger than p19 at 3 dpi, subsequently declined significantly and was even weaker than p19 at 6 dpi, as estimated from the intensity of GFP fluorescence or the GFP mRNA levels in coinfiltrated regions. This phenomenon may be related to the death phenotype triggered by HVT063. During the *Agrobacterium* coinfiltration 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 13 dpi. At the early phase of coinfiltration assays (especially 3 dpi), HVT063 protein exerted strong suppressor activity in the infiltrated area, whereas 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 coinfiltrated region. Intriguingly, the RNA silencing suppression activity of HVT063 at 1:5 is weaker than that of HVT063 at 1:1 to 1:2, as judged by the 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 coinfiltrated 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 compared to HVT063 (1:1 to 1:2), although its suppression activity decreased. However, the enhanced GFP expression was not observed in HVT063 (1:10 to 1:50) coinfiltrated tissues, since 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 has also been reported previously, such as P0 protein expressed by *Sugarcane yellow leaf virus*, which progressed so rapidly and severely that the infected areas appeared to be chlorotic and necrotic at as early as 1 dpi (34). Unlike the scenario described for HVT063, P0 dilutions did not cause the local silencing suppression activity but failed to induce the cell death phenotype. We propose that the cell death phenotype was due to RSSs, which have certain cytotoxic components or could interact with host cellular components of the 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. First, 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 the RSS 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 40 dpi (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* also only recovered GFP fluorescence again in the inoculated leaves (39), while PVY HC-Pro could restore the 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 accounting 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 C1 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 did not cause severe symptoms of PVX in infected plants even at 20 dpi, compared to 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 coinfiltration, while the PVX-HVT063 induced the cell death...
phenotype at as early as 7 dpi and completely dry necrosis at 10 dpi, which might decrease the accumulation of PVX and prevent PVX spreading 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, and so on, which contain 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 played a key role in the dsRNA binding region (8, 13, 25, 51, 58). For instance, three alanine substitutions of lysine residues at positions 173 to 175 of the NS3 protein lost both its siRNA binding capacity and RNA silencing suppression activity (26); the inhibitory effect of B2 was almost abolished when lysine was replaced 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 the 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 acid-rich regions—region 1 (residues 138 to 141) and region 2 (residues 185 to 195) of HVT063—demonstrated that all HVT063 mutations affected its RNA silencing suppression activities. In particular, three of four single alanine mutagenesis in region 1 destroyed HVT063 suppression activities, suggesting that 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 prevent RNA silencing through interacting with RNA silencing components, for example, 2b and p38 inhibit RNA silencing by binding siRNAs or interacting with AGO1 (9), and Tat has been found to sequester dsRNA or inhibit the activity of Dicer. Whether the HVT063 protein has similar characteristics remains to be tested.

Our observations unequivocally demonstrated that HVT063 possesses strong silencing suppression activity, and it is prospectively to be developed as a novel tool in gene expression research 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 shown to suppress silencing in mammalian cells (62). HSV-1 infection suppressed EGF-specific silencing, as demonstrated by increased enhanced GFP (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 the genes of HSV-1 (1, 50). It may be assumed that HVT, which also belongs to the alphaherpesvirus subfamily, could probably 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 established experimental protocols (23, 35).

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