Virology 433 (2012) 104–115

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Triticum mosaic poacevirus enlists P1 rather than HC-Pro to suppress RNA silencing-mediated host defense

Satyanarayana Tatineni [a,*], Feng Qu [b], Ruhui Li [c], T. Jack Morris [d], Roy French [a]

[a] United States Department of Agriculture-Agricultural Research Service and Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, NE 68583, United States
[b] Department of Plant Pathology, Ohio State University, Wooster, OH 44691, United States
[c] United States Department of Agriculture-Agricultural Research Service, National Germplasm Resources Laboratory, Beltsville, MD 20705, United States
[d] School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, United States

ARTICLE INFO

Article history:
Received 1 June 2012
Returned to author for revisions
7 July 2012
Accepted 18 July 2012
Available online 9 August 2012

Keywords:
RNA silencing suppressor
Sugarcane streak mosaic virus
Wheat
Systemic silencing
Potyviridae
Poacevirus
P1
HC-Pro
Inhibition

ABSTRACT

Triticum mosaic virus (TriMV) is the type species of the newly established *Poacevirus* genus in the family *Potyviridae*. In this study, we demonstrate that in contrast to the helper component-proteinase (HC-Pro) of *Potyvirus* species, the P1 proteins of TriMV and *Sugarcane streak mosaic poacevirus* function in suppression of RNA silencing (SRS). TriMV P1 effectively suppressed silencing induced by single- or double-stranded RNAs (ss/ds RNAs), and disrupted the systemic spread of silencing signals at a step after silencing signal production. Interestingly, contrary to enhanced SRS activity of potyviral HC-Pro by co-expression with P1, the presence of TriMV HC-Pro reduced SRS activity of TriMV P1. Furthermore, TriMV P1 suppressed systemic silencing triggered by dsRNA more efficiently than the HC-Pro of *Turnip mosaic potyvirus*. Furthermore, TriMV P1 enhanced the pathogenicity of a heterologous virus. Our results established poaceviral P1 as a potent RNA silencing suppressor that probably employs a novel mechanism to suppress RNA silencing-based antiviral defense.

Published by Elsevier Inc.

Introduction

Viruses are intracellular obligate parasites that exploit host cellular and molecular mechanisms to propagate in host cells. Upon virus invasion, the virus hosts are known to deploy diverse mechanisms to combat viral infections at both the cellular level and throughout the entire organisms. In plants, RNA silencing is one of the most important defense mechanisms that target viruses, transposons, and overexpressed transgenes (Cogoni and Macino, 2000; Wang and Metzlaff, 2005). RNA silencing is triggered by double-stranded (ds) RNAs, which could originate from replicating viral RNAs or highly structured regions of single-stranded (ss) viral RNAs (Bass, 2000; Dalmay et al., 2000; Hannon, 2002). These dsRNAs are processed by Dicer-like RNases (DCLs) to produce short dsRNAs of 21–24 nucleotides (nt) in length known as small interfering (si) RNAs (Deleris et al., 2006; Dunoyer et al., 2005; Fire et al., 1998; Ketting et al., 2001; Xie et al., 2004). One strand of these siRNAs is then recruited by an Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC), which guides the cleavage or translational repression of homologous RNA molecules (Ding and Voinnet, 2007; Hammond et al., 2000).

Once induced, RNA silencing sends sequence-specific signals, presumably siRNAs, to neighboring cells and throughout the entire plant to confer non-cell autonomous silencing of homologous targets (Dunoyer et al., 2010; Kalantidis et al., 2008; Molnar et al., 2010; Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Voinnet et al., 1998). Hence, siRNAs generated locally not only reduce the number of viral RNAs at the infection sites, but also serve to promote plant recovery from virus infections at distal parts of the plant, leading to clearance of viral RNAs and viral disease symptoms. The recovered plants become resistant to super-infection by the same or closely related viruses (Lindbo et al., 1993; Mlotshwa et al., 2008; Ratcliff et al., 1997).

To counter RNA silencing-based host defense, viruses have evolved to encode diverse, yet functionally conserved proteins with silencing suppression activity, referred to as RNA silencing suppressors (RSSs) (Ding and Voinnet, 2007). Since RNA silencing is a multistep process, RSSs encoded by different viruses are also known to target distinct steps of the RNA silencing pathway. For example, *Tombusvirus* p19 interferes with siRNA incorporation

* Corresponding author. Fax: +1 402 472 4020.
E-mail address: satya.tatineni@ars.usda.gov (S. Tatineni).

0042-6822/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.virol.2012.07.016

into RISCs by binding to siRNAs (Lakatos et al., 2004; Silhavy et al., 2002), whereas *Cucumovirus* 2b inhibits host RNA silencing by physically interacting with AGOs (Zhang et al., 2006).

The family *Potyviridae* is the most populous plant virus family that consists of eight different genera, encompassing ~40% of the known plant RNA viruses (Adams et al., 2011). Notably, recent studies suggest that viruses belonging to different genera of *Potyviridae* evolved independently to task different viral proteins with RSS functions. It is well known that species of the *Potyvirus* and *Rymovirus* genera suppress RNA silencing with the multifunctional helper component-proteinase (HC-Pro) (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998; Lakatos et al., 2006; Llave et al., 2000; Mallory et al., 2001; Young et al., 2012). By contrast, *Ipomovirus* members all use the P1 as the RSS, even though these viruses exhibit substantial intra-genus diversity in their genome organization: while an HC-Pro homolog is present in *Sweet potato mild mottle virus* (SPMMV) and is completely absent in *Cassava brown streak virus* or *Cucurbit vein yellowing virus* (CVYV) (Giner et al., 2010; Mbanzibwa et al., 2009; Valli et al., 2006). Furthermore, CVYV encodes two copies of P1, referred to as P1a and P1b, with P1b serving as the RSS (Valli et al., 2006). Finally, *Wheat streak mosaic virus* (WSMV), the type species of the *Tritimovirus* genus, also utilizes P1 as RSS, despite the fact that WSMV shares a similar genome organization with members of the *Potyvirus* and *Rymovirus* genera (Young et al., 2012).

Poacevirus is a newly established genus in the family *Potyviridae* with *Triticum mosaic virus* (TriMV), an economically important virus in the Great Plains region, as its type species and *Sugarcane streak mosaic virus* (SCSMV) as an additional definitive member (http://ictvonline.org/virusTaxonomy.asp?version=2011; Adams et al., 2011; Burrows et al., 2009; Seifers et al., 2008, 2011; Tatineni et al., 2009; Xu et al., 2010). TriMV possesses a 10,266 nt long, single-stranded, positive-sense RNA genome with a similar genome organization to those of *Potyvirus*, *Tritimovirus*, and *Rymovirus* species (Fellers et al., 2009; Tatineni et al., 2009). In addition to a substantially divergent amino acid sequence of polyprotein, TriMV differs from other potyvirid species by having an unusually long (739-nt) 5′-leader sequence (Tatineni et al., 2009). In the field, TriMV is transmitted by the eriophyid mite *Aceria tosichella* Keifer (Seifers et al., 2009). The functions of TriMV-coded proteins remain largely unknown.

In this study, we identified the P1 of TriMV as an RSS. TriMV P1 effectively suppressed local and systemic RNA silencing triggered by ss and dsRNAs of green fluorescent protein (GFP). Furthermore, in contrast to previous reports of increased silencing suppression activity by potyviral HC-Pro in the presence of its cognate P1, the presence of TriMV HC-Pro actually weakened the silencing suppression activity of TriMV P1. Additionally, TriMV P1 enhanced the pathogenicity of a heterologous virus [*Potato virus X* (PVX)] in *Nicotiana benthamiana*. Collectively, our data demonstrate that TriMV P1 is a strong RSS that may function through a novel mechanism.

Results

TriMV P1 is a strong RSS

TriMV genomic RNA encodes a single large polyprotein of 3112 amino acids (Fellers et al., 2009; Tatineni et al., 2009), which is thought to be cleaved into 11 individual mature proteins presumably by three virus-encoded proteinases and by ribosomal frameshifting (Fig. 1A). These mature proteins are presumed to be analogous to those encoded by the members of *Potyvirus*, *Tritimovirus,* and *Rymovirus* genera, but with limited sequence identity (Fig. 1A; Tatineni et al., 2009). To determine whether any of the TriMV encoded proteins possess an RSS activity, we utilized agroinfiltration assays in GFP-transgenic *N. benthamiana* line 16c leaves (Voinnet and Baulcombe, 1997). Each transformed agrobacterial strain containing one of the TriMV cistrons (P1, HC-Pro, P3, CI, 6K2, NIa-VPg, NIa-Pro, NIb, or CP) was mixed with an equal volume of *Agrobacterium tumefaciens* suspension harboring a 35S –GFP construct (ssGFP) (Qu et al., 2003) and infiltrated into 16c leaves. *A. tumefaciens* harboring a *Turnip mosaic virus* (TuMV) HC-Pro-expressing construct and an empty vector (pCASS4; a variant of pCASS2; Shi et al., 1997) were used as positive and negative controls, respectively, in co-infiltration experiments with ssGFP.

At 2 day postinfiltration (dpi), prior to initiation of host RNA silencing, all *Agrobacterium*-infiltrated leaf patches reached maximum GFP expression as evidenced by strong green fluorescence (data not shown). By 3 dpi, the intensity of green fluorescence decreased substantially in leaf patches expressing most TriMV cistrons, but increased in patches expressing TriMV P1 as well as TuMV HC-Pro (Fig. 1B); strongly suggesting that P1 is the RSS of TriMV. By contrast, fluorescence in leaf patches with TriMV HC-Pro was similar to that of the empty vector control (Fig. 1B), suggesting that unlike potyviral HC-Pro, the HC-Pro of TriMV does not function in suppression of RNA silencing (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). Additionally, *N. benthamiana* leaves agroinfiltrated with a construct designed to express TriMV HC-Pro tagged with the hemagglutinin (HA) epitope at the N-terminus was readily accumulated at detectable levels (Fig. 1D, lane 1), suggesting that failure of HC-Pro to function as RSS is probably not due to inefficient expression and/or protein instability.

Suppression of RNA silencing by TriMV P1 would be expected to protect the GFP mRNA from being degraded, thus allowing GFP mRNA accumulation to higher levels. To confirm this prediction and also to correlate the increased GFP fluorescence in TriMV P1-expressing leaf patches with enhanced GFP mRNA accumulation, we carried out *Northern* blot analysis of total RNA extracted from agroinfiltrated leaf patches (Fig. 1C). As expected, GFP mRNA accumulated to very high levels in tissues co-expressing ssGFP and TriMV P1 (lane 1) or TuMV HC-Pro (lane 10). By contrast, the level of GFP mRNA was dramatically lower in tissues expressing all other TriMV proteins, including HC-Pro (Fig. 1C, lanes 2–9). These results are consistent with the intensity of GFP fluorescence, further confirming that TriMV P1, but not HC-Pro, serves as the RSS of this virus.

ssRNA-triggered silencing suppression activity of TriMV P1 is inhibited by HC-Pro

Previous studies have shown that for *potyviruses*, even though HC-Pro is the primary RSS, its activity is enhanced by the presence of P1 (Rajamäki et al., 2005; Valli et al., 2006). To determine if similar synergistic interaction exists between TriMV P1 and HC-Pro, we compared the silencing suppression activity of TriMV P1 with that of P1/HC-Pro by agroinfiltration of *N. benthamiana* 16c leaves with ssGFP plus P1/HC-Pro or P1 of TriMV. TuMV HC-Pro and P1/HC-Pro, and pCASS4 were used as positive and negative controls, respectively. As an additional negative control, we created a new construct, TriMV P1+1FS, which would be expected to transcribe P1 mRNA but not express the P1 protein due to a +1 frameshift (FS) within its ORF. As shown in Fig. 2A, GFP fluorescence in leaves containing negative controls (TriMV P1+1FS or pCASS4) was very weak at 3 dpi, and almost completely faded by 6 dpi (Fig. 2A). GFP fluorescence in leaf patches with TriMV P1 or P1/HC-Pro constructs were both bright at 3 dpi (Fig. 2A). However, by 6 dpi, GFP fluorescence in TriMV P1/HC-Pro-expressing leaf patches became slightly weaker than in leaf patches expressing P1 alone (Fig. 2A), suggesting that presence of

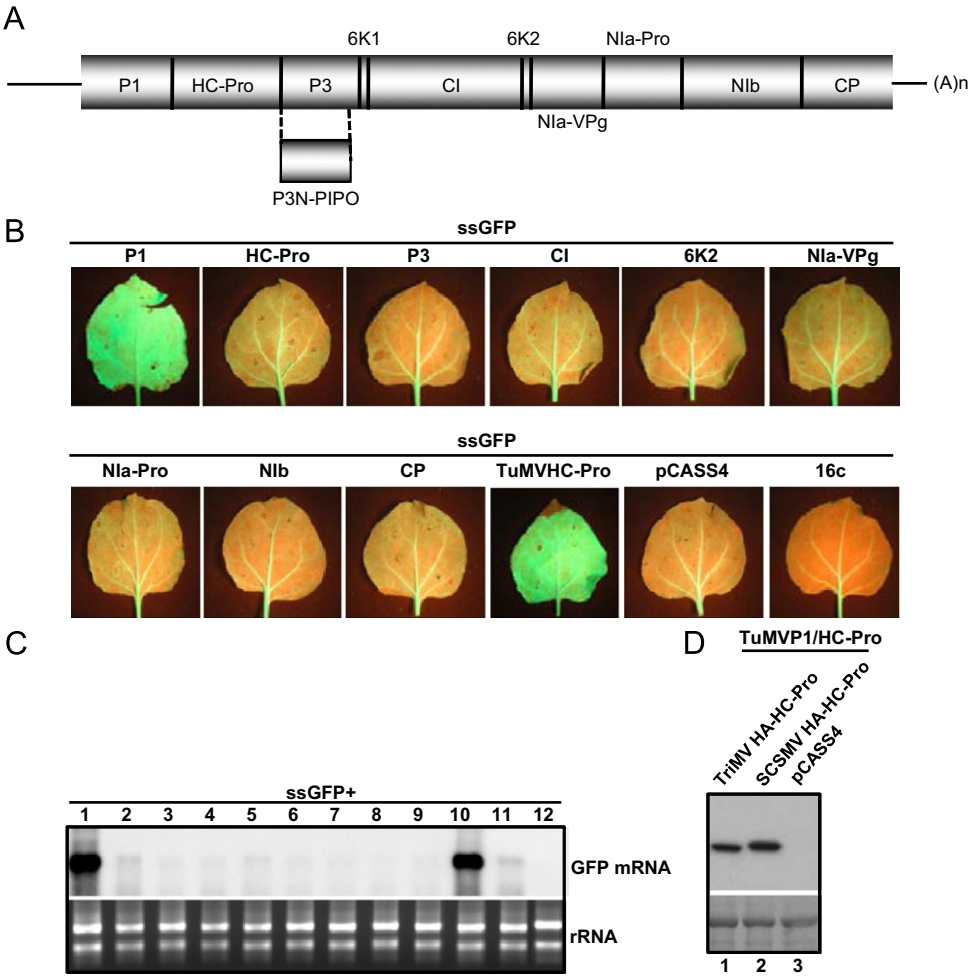


Fig. 1. Identification of TriMV P1 as a suppressor of RNA silencing triggered by ssGFP. (A) Genome organization of TriMV depicting coding proteins in cistrons and the position of predicted cleavage sites in polyprotein are indicated with solid lines. (B) Green fluorescent images of agroinfiltrated leaves of *N. benthamiana* line 16c under UV illumination at 3 dpi. The constructs used for agroinfiltration are indicated above the images. (C) Northern blot analysis of accumulation of GFP mRNA in leaf patches infiltrated with agrosuspensions harboring ssGFP plus one of the TriMV cistrons: P1 (lane 1), HC-Pro (lane 2), P3 (lane 3), CI (lane 4), 6K2 (lane 5), NIa-VPg (lane 6), NIa-Pro (lane 7), NIb (lane 8), and CP (lane 9). TuMV HC-Pro (lane 10), and pCASS4 (vector) (lane 11) and RNA from 16c plants (lane 12) were used as positive and negative controls, respectively. Ethidium bromide-stained rRNA is shown as sample loading controls. GFP plus-strand RNA-specific DIG-labeled riboprobe was used for Northern blot hybridization. (D) Western blot analysis of accumulation of HA-tagged TriMV HC-Pro (lane 1) and SCSMV HC-Pro (lane 2) in *N. benthamiana* leaf patches co-infiltrated with TuMV P1/HC-Pro at 3 dpi. Coomassie R-250-stained SDS-PAGE gel at the bottom of immuno-blot are host rubisco protein for sample loading controls.

HC-Pro with P1 as a polyprotein interfered with the silencing suppression function of P1. In contrast, GFP fluorescence in leaf patches containing TuMV HC-Pro or P1/HC-Pro was similar at both 3 and 6 dpi (Fig. 2A).

The difference in GFP fluorescence intensity in leaf patches as shown in Fig. 2A was further corroborated by Northern blot analysis of total RNA extracted from infiltrated leaves (Fig. 2B and C). GFP mRNA level in leaf patches expressing TriMV P1/HC-Pro was much lower than those expressing TriMV P1 at both 3 and 6 dpi (compare lanes 1 and 2). This is in stark contrast with the TuMV RSS: consistent with previous reports, TuMV P1/HC-Pro fusion permitted slightly higher levels of GFP mRNA accumulation than its HC-Pro alone (Fig. 2B and C, compare lanes 4 and 5). We next examined the levels of GFP-specific siRNAs in the same samples. As shown in Fig. 2D and E, GFP-specific siRNAs were more abundant in leaf patches expressing TriMV P1/HC-Pro (lane 1) than those expressing P1 alone (lane 2), at both 3 and 6 dpi. These results support the conclusion that in the presence of HC-Pro, the silencing suppression activity of TriMV P1 was weakened, allowing for the production of more silencing-mediating siRNAs.

The reduced silencing suppression activity of TriMV P1/HC-Pro might be caused by inefficient translation due to its larger size compared to that of P1. Therefore, we examined the protein levels of P1 and P1/HC-Pro by fusing the HA epitope to the N-terminus of TriMV P1 and P1/HC-Pro cistrons. The agrosuspensions containing HA-tagged P1 or P1/HC-Pro were infiltrated into wild-type *N. benthamiana* leaves. To rule out the possibility that RNA silencing might target these constructs differentially and cause varying levels of protein accumulation, the TuMV P1/HC-Pro was included in some of the infiltrations (as a suppressor of RNA silencing) (Fig. 2F). Total proteins extracted from 3 dpi leaves were analyzed by Western blot using an HA-monoclonal antibody (Sigma-Aldrich, St. Louis, MO). As shown in Fig. 2F, HA-tagged P1, derived from P1/HC-Pro (due to P1-mediated cleavage between P1 and HC-Pro), accumulated to slightly lower levels than HA-tagged P1 alone, regardless of the presence of an heterologous RSS (Fig. 2F, compare lanes 1, 2 with 3, 4; and 5, 6 with 7, 8). Furthermore, we found that in overloaded samples the P1 was efficiently cleaved from HC-Pro in P1/HC-Pro infiltrated leaves (Fig. 2F, compare lanes 10 and 11 with 12 and 13), suggesting that the reduced RSS activity of TriMV P1/HC-Pro is not due to inefficient cleavage of P1 from HC-Pro. Taken together, the drastically reduced RSS activity of TriMV P1/HC-Pro compared to that of P1 in *N. benthamiana* leaves is

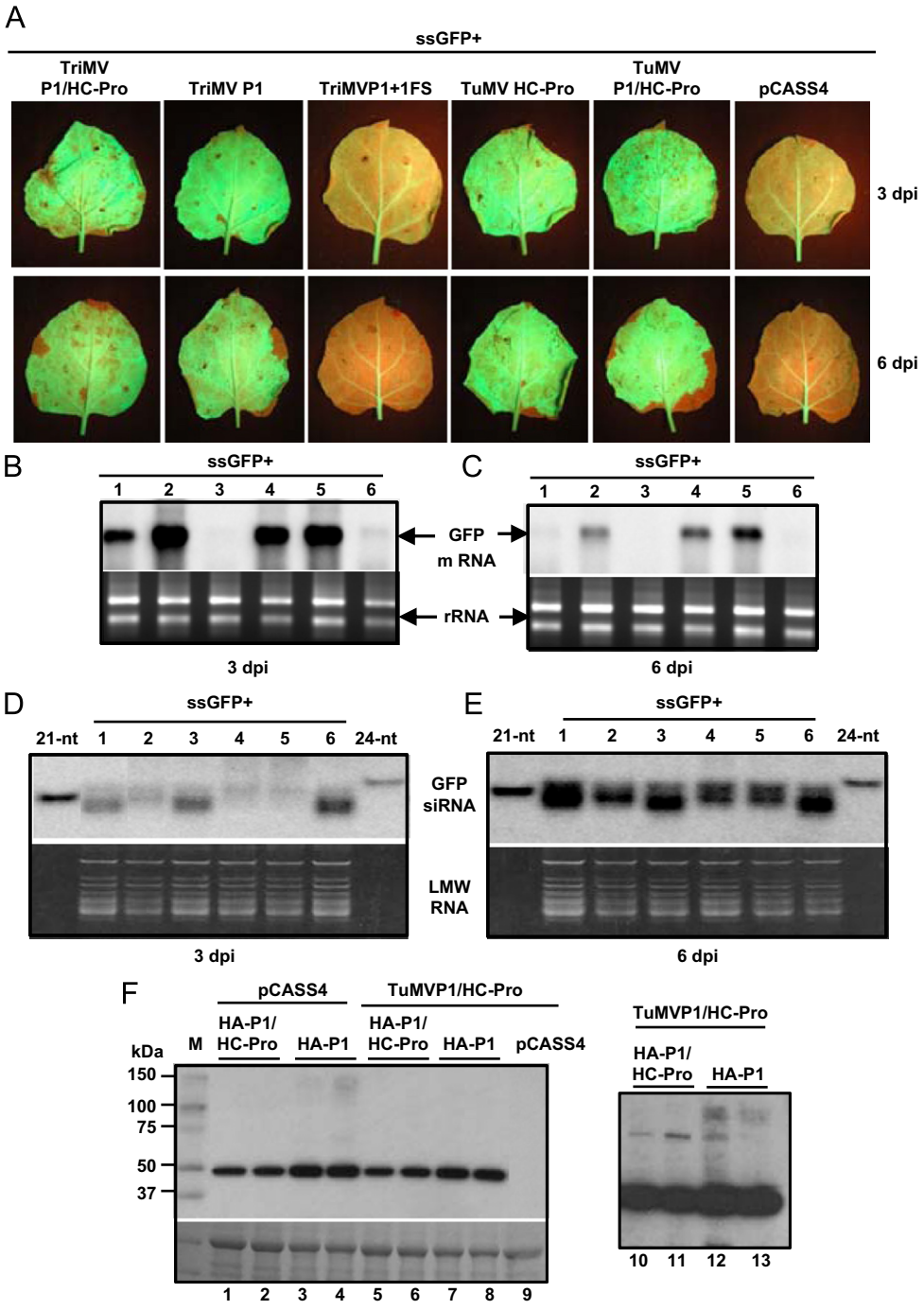


Fig. 2. TriMV HC-Pro interferes with ssRNA-triggered silencing suppression activity of P1 in *N. benthamiana* 16c leaves. (A) Green fluorescent images of agroinfiltrated leaf patches with ssGFP plus constructs as indicated at 3 and 6 dpi. (B–E) Northern blot analyses of accumulation of GFP mRNA (B and C) and GFP siRNAs (D and E) in agroinfiltrated leaf patches with ssGFP plus TriMV P1/HC-Pro (lane 1), TriMV P1 (lane 2), TriMV P1+1FS (lane 3), TuMV HC-Pro (lane 4), TuMV P1/HC-Pro (lane 5), or pCASS4 (lane 6) at 3 (B and D) and 6 (C and E) dpi. GFP-specific nonphosphorylated 21- and 24-nt RNA oligonucleotides were included as siRNA size markers in D and E. Ethidium bromide stained rRNA (B and C) and low molecular weight RNA (LMW) (D and E) are shown as sample loading controls. GFP plus-strand RNA-specific DIG-labeled riboprobe with (D and E) and without (B and C) hydrolysis was used for Northern blot hybridizations. (F) Western blot analysis of accumulation of HA-tagged TriMV P1/HC-Pro and TriMV P1 in agroinfiltrated *N. benthamiana* leaf patches at 3 dpi. *Agrobacterium* harboring HA-tagged TriMV P1/HC-Pro and TriMV P1 co-infiltrated with either pCASS4 (vector) (lanes 1–4) or TuMV P1/HC-Pro (as a suppressor of RNA silencing) (lanes 5–9), and higher amounts of protein (2.5-fold more than in lanes 1–8 with a longer exposure time) was loaded in lanes 10–13 to observe the presence of uncleaved P1/HC-Pro protein, if any. Lane M: Protein molecular weight standards. Two independent clones per construct were analyzed for accumulation of HA-tagged TriMV P1/HC-Pro and TriMV P1. Coomassie R-250-stained SDS-PAGE gel at the bottom of immuno-blot lanes 1–9 are host rubisco protein for sample loading controls.

not proportionate to the slightly reduced levels of P1 translation from the P1/HC-Pro construct. Thus, it appears that co-expression of HC-Pro from the P1/HC-Pro construct interferes with RSS activity of P1 in *N. benthamiana* leaves by an unknown mechanism.

TriMV P1 suppresses local RNA silencing triggered by dsRNA

The above experiments demonstrated that TriMV P1 is a strong suppressor of RNA silencing induced by ssRNA in the form of ssGFP. Since dsRNA is considered to be a more potent inducer

of RNA silencing, we examined whether TriMV P1 was able to suppress dsRNA-induced RNA silencing in *N. benthamiana* leaves. In these experiments, TriMV HC-Pro, TriMV P1+1FS and pCASS4 (empty vector) were included as negative controls. TuMV P1/HC-Pro and HC-Pro constructs were included as positive controls. In addition to the constructs expressing viral (TriMV or TuMV) gene products, all these co-infiltrations include the following two constructs: ssGFP serving as the visual reporter, and dsGFP which expresses GFP-derived dsRNA (Fig. 3A). GFP fluorescence was observed in co-infiltrations that contained TriMV P1, TuMV P1/HC-Pro, and TuMV HC-Pro, but not in leaf patches containing TriMV P1/HC-Pro or HC-Pro at 2 dpi, again confirming that TriMV HC-Pro is not an RSS (Fig. 3A). Lack of GFP fluorescence in leaf patches infiltrated with TriMV P1/HC-Pro plus ssGFP and dsGFP suggests that the HC-Pro interferes with the RSS activity of P1.

The observation of GFP fluorescence in agroinfiltrated leaf patches as shown in Fig. 3A was further confirmed by Northern blot hybridization (Fig. 3B) showing accumulation of GFP mRNA in leaf patches containing TriMV P1 (lane 3), TuMV P1/HC-Pro (lane 5), or TuMV HC-Pro (lane 6), but not TriMV P1/HC-Pro (lane 1), TriMV HC-Pro (lane 2), TriMV P1+1FS (lane 4), or pCASS4 (lane 7). We also examined accumulation of GFP-specific siRNAs in the same samples. Interestingly, the levels of siRNAs were higher (~1.5–4-fold) in leaf patches (Fig. 3C) that expressed TriMV P1 (lane 3), TuMV P1/HC-Pro (lane 5), and TuMV HC-Pro (lane 6) compared to negative controls (lanes 4 and 7). This likely suggests that these RSSs functioned at a step downstream of the processing of dsRNA into siRNAs, preventing both ssGFP and dsGFP from being degraded by siRNA-mediated cleavage. As a result, dsGFP RNA was stabilized, providing more substrate for DCL-mediated processing, hence the accumulation of more siRNAs.

The effect of HC-Pro on suppression of dsRNA-induced silencing activity by P1 was further examined by analyzing the levels of GFP protein in agroinfiltrated leaf patches at 5 dpi by Western-blot using GFP-specific monoclonal antibody (Clontech, Mountain View, CA). Strikingly, GFP protein was undetectable in leaf patches infiltrated with TriMV P1/HC-Pro, but was detected at high levels in leaf patches infiltrated with P1 (Fig. 3D). Conversely, approximately equal amounts of GFP accumulated in leaf patches containing TuMV P1/HC-Pro or HC-Pro alone (Fig. 3D). Taken together, these data demonstrated that TriMV HC-Pro inhibited the RSS activity of P1.

TriMV P1 inhibits systemic spread of RNA silencing signals

As described earlier, TriMV P1, like TuMV HC-Pro, is a strong RSS that reduces, but does not completely eliminate the

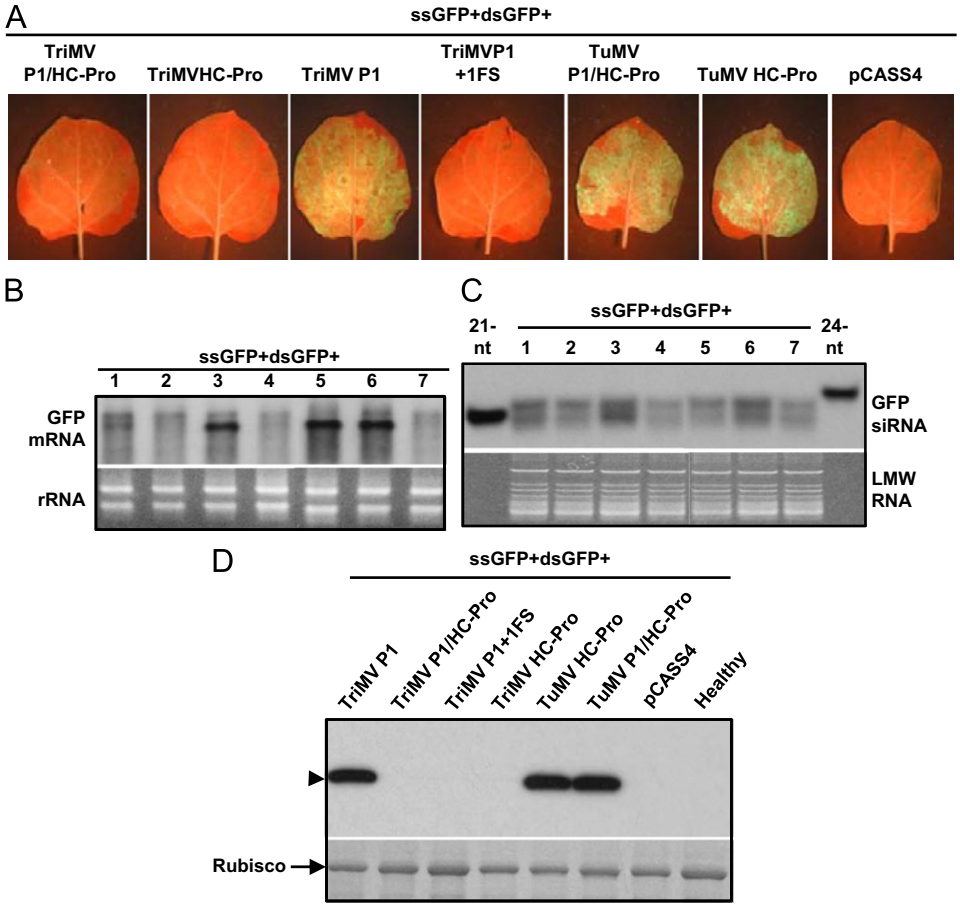


Fig. 3. TriMV P1 suppresses local silencing induced by GFP dsRNA. (A) Green fluorescent images of wild-type *N. benthamiana* leaf patches infiltrated with agrosuspensions harboring a combination of three constructs as indicated at 2 dpi. Northern blot analyses of accumulation of GFP mRNA (B) and GFP siRNA (C) in leaf patches infiltrated with agrosuspensions harboring ssGFP, dsGFP plus one of the constructs. The bottom ethidium bromide-stained rRNA (B) and low molecular weight RNA (LMW) (C) are shown as sample loading controls. GFP-specific nonphosphorylated RNA oligonucleotides (21- and 24-nt) were included as siRNA size markers in C. The numbers on top of Northern blots represent total RNA isolated from leaf patches infiltrated with agrosuspensions harboring ssGFP and dsGFP plus TriMV P1/HC-Pro (lane 1), TriMV HC-Pro (lane 2), TriMV P1 (lane 3), TriMV P1+1FS (lane 4), TuMV P1/HC-Pro (lane 5), TuMV HC-Pro (lane 6), or pCASS4 (lane 7). GFP plus-strand RNA-specific DIG-labeled riboprobe with (for C) and without (for B) hydrolysis was used for Northern blot hybridizations. (D) Western immuno-blot analyses of total proteins from leaf patches infiltrated with ssGFP and dsGFP plus suppressor proteins as indicated at 5 dpi. Coomassie R-250-stained SDS-PAGE gel at the bottom of immuno-blot showing host rubisco protein for sample loading controls. The position of GFP protein is indicated with an arrow head.

production of siRNAs. Since siRNAs are thought to be part of the systemic silencing signal that mediates the silencing of homologous mRNA in top young leaves, we examined whether TriMV P1 was able to prevent systemic silencing as well.

The top two fully expanded leaves of *N. benthamiana* 16c plants at the 6–8-leaf stage were infiltrated with a mixture of *A. tumefaciens* suspensions harboring ssGFP plus either TriMV P1 or P1/HC-Pro. We also infiltrated *A. tumefaciens* harboring ssGFP plus either TuMV P1/HC-Pro or HC-Pro as positive controls, and ssGFP plus TriMV P1+1FS, pCASS4, or buffer as negative controls. The agroinfiltrated plants were examined for systemic silencing in upper young leaves at 10 dpi. In negative controls, GFP fluorescence was lost in 95–100% of infiltrated plants, with the major and minor veins of most upper young leaves turning red under UV illumination, which indicated systemic RNA silencing (Fig. 4A and B). In contrast, GFP fluorescence persisted in most 16c plants infiltrated with ssGFP plus TriMV P1, with only 14% of plants displaying systemic silencing (Fig. 4A). Notably, GFP fluorescence faded in the young leaves of 79% of plants infiltrated with ssGFP plus TriMV P1/HC-Pro, indicating that TriMV HC-Pro negatively affected the P1 function in suppressing systemic silencing (Fig. 4A). As expected, suppression of systemic silencing by TriMV P1 (and TuMV HC-Pro) was accompanied by a complete loss of GFP-specific siRNAs in the systemic leaves (Fig. 4C). Together these results further confirm that TriMV P1 is a potent RSS that also interferes with the systemic spread of the silencing signals, while the presence of HC-Pro affected the efficiency of P1 suppression of systemic RNA silencing.

In addition to TriMV P1, the HC-Pro as well as P1/HC-Pro of TuMV were also assessed for their ability to interfere with

A

Constructs	Number of plants infiltrated	Number of plants systemically silenced	% plants systemically silenced
TriMV P1+ssGFP	35	5	14
TriMV P1/HC-Pro+ssGFP	24	19	79
TriMV P1+1FS+ssGFP	20	18	90
TuMV HC-Pro+ssGFP	32	6	16
TuMV P1/HC-Pro+ssGFP	25	6	24
pCASS4+ssGFP	21	20	95
Buffer+ssGFP	10	10	100

B

C

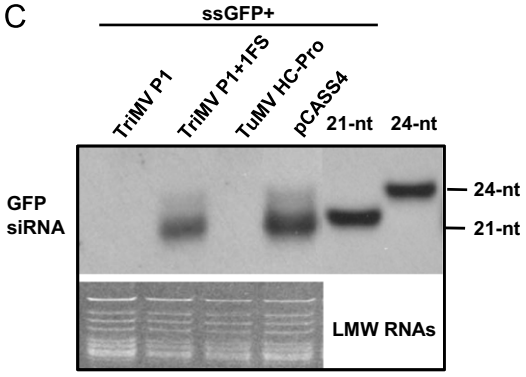


Fig. 4. TriMV P1 suppresses systemic RNA silencing triggered by ssGFP in *N. benthamiana* line 16c plants. (A) The number of *N. benthamiana* line 16c plants systemically silenced at 10 dpi. *N. benthamiana* line 16c plants co-infiltrated at the 6–8-leaf stage with a mixture of agrosuspensions carrying different combinations of constructs as shown. (B) Representative *N. benthamiana* line 16c plants infiltrated with agrosuspensions carrying ssGFP plus TriMV P1 (left; with no systemic silencing) or TriMV P1+1FS (right; with systemic silencing) at 10 dpi. *N. benthamiana* 16c plants were considered systemically silenced if the major and minor veins of upper young leaves of agroinfiltrated plants turned red under UV illumination. (C) Northern blot analysis of accumulation of GFP-specific siRNAs in top fully expanded *N. benthamiana* 16c leaves agroinfiltrated with different combinations of constructs as indicated. GFP-specific nonphosphorylated 21- and 24-nt RNA oligonucleotides were used as siRNA size markers. Ethidium bromide-stained low molecular weight (LMW) RNAs are included as loading controls. GFP plus-strand RNA-specific DIG-labeled riboprobe hydrolyzed to ~50 nt was used for Northern blot hybridization.

systemic trafficking of silencing signals. As shown in Fig. 4A, while the both TuMV-derived proteins suppressed systemic silencing, they allowed for the development of systemic silencing in a slightly higher percentage of plants (16% and 24% for HC-Pro and P1/HC-Pro, respectively). To further assess whether this difference reflected any mechanistic differences between TriMV P1 and TuMV HC-Pro, we then tried to induce systemic silencing with the dsGFP construct. We observed that less than 5% (one of 19) of the plants infiltrated with ssGFP, dsGFP, and TriMV P1 exhibited systemic silencing at 5 dpi and the number of plants systemically silenced increased to 70% and 90% at 7 and 10 dpi, respectively (Table 1). In contrast, plants infiltrated with ssGFP, dsGFP, and TuMV HC-Pro exhibited systemic silencing in 90% of plants at 5 dpi, which was similar to 95–100% of systemically silenced plants with negative controls (Table 1). By 7 dpi, 100% of plants infiltrated with ssGFP, dsGFP, and TuMV HC-Pro, TriMV+1FS, or pCASS4 displayed systemic silencing (Table 1). These data suggested that TriMV P1 inhibited systemic RNA silencing triggered by dsGFP more efficiently than the HC-Pro of TuMV.

Blocking of systemic silencing by TriMV P1 occur after the production of the signal

The above experiments suggested that TriMV P1 efficiently blocked systemic RNA silencing signals induced by ssRNA or dsRNA. We next examined the possible mechanism of P1-mediated suppression of systemic RNA silencing by expressing P1 and ssGFP from different positions in *N. benthamiana* line 16c plants using a procedure described by Guo and Ding (2002). The agrobacterial suspension harboring the suppressor gene (TriMV P1 or TuMV HC-Pro) and ssGFP were simultaneously infiltrated into the apical and basal portions of the same leaf (Fig. 5A, I, II), and onto the upper and lower leaves of the same plant (Fig. 5A, III, IV). The expectation was that the spread of the RNA silencing signal produced by ssGFP in the apical portion of the leaf and in the lower leaves would be blocked and/or inactivated by the suppressor at or near the basal region of the leaf and in the upper leaves, respectively. As a result, *N. benthamiana* line 16c plants would not be systemically silenced, and transient expression of GFP would continue.

The number and percentage of plants systemically silenced at 10 dpi is summarized in Fig. 5B. The infiltrations as shown in sets 2, 3, 8, and 9, in which TriMV P1 or TuMV HC-Pro was expressed in the tissue zone between the source and sink of silencing signals, resulted in suppression of systemic RNA silencing triggered by ssGFP (Fig. 5B). Notably, none of the 18 plants in set 3, in which the TriMV P1 was expressed in leaves directly above the sites of RNA silencing induction, were systemically silenced (Fig. 5B). Similarly, only two of 19 plants in set 2, in which the TriMV P1 was expressed on the basal side of the leaves of silencing induction, were systemically silenced. By contrast, systemic silencing was successfully established when the positions of TriMV P1 and GFP were reversed (sets 1 and 4). In control experiments, TuMV HC-Pro behaved in a manner similar to TriMV P1 with a slightly more number of plants systemically silenced (Fig. 5B, sets 8 and 9). These results suggested that TriMV P1 expressed at locations closer to the growth point of the plants blocked and/or inactivated the systemic silencing signal induced by ssGFP at a step after the signal production.

TriMV P1 enhances PVX symptoms in N. benthamiana

A characteristic feature of RSSs is that they act synergistically with unrelated viruses to enhance the severity of infections caused by these viruses (Cañizares et al., 2008; Cao et al., 2005; Xiong et al., 2009; Young et al., 2012). This characteristic of RSS was tested by expressing TriMV P1 in a modified recombinant virus pP2C2S, a *Potato virus X* (PVX)-based vector (Chapman et al., 1992). Sequences encoding TriMV HC-Pro, TriMV P1, and TriMV P1+1FS were inserted into pP2C2S. As a positive control, a cistron encoding *Tobacco etch virus* (TEV) HC-Pro was also inserted into pP2C2S. *In vitro* transcripts of PVX with TriMV cistrons or with TEV HC-Pro were inoculated onto *N. benthamiana* leaves at the 4–6-leaf stage.

N. benthamiana plants inoculated with *in vitro* transcripts of the PVX derivatives all induced chlorotic spots on inoculated leaves at 4–6 day after inoculation (dai), followed by systemic veinal chlorosis with mild inward rolling of leaves at 6–8 dai. By 10–12 dai, plants inoculated with wild-type PVX developed mosaic symptoms with a few green islands, which subsequently became milder (Fig. 6A). PVX expressing TriMV P1 or TEV HC-Pro induced severe inward leaf rolling and necrosis at 8 dai, followed by severe leaf and apical shoot necrosis at 16 dai (Fig. 6A), resulting in death of the apical shoots and eventual plant death by 18 dai. *N. benthamiana* plants inoculated with control constructs containing TriMV HC-Pro or TriMV P1+1FS inserts initially produced mild mosaic symptoms at 10 dai, followed by mild or no visible symptoms in subsequent leaves (Fig. 6A). These data demonstrated that co-expression of TriMV P1 with PVX synergistically acted to cause more severe disease symptoms, which is consistent with TriMV P1 being an RSS.

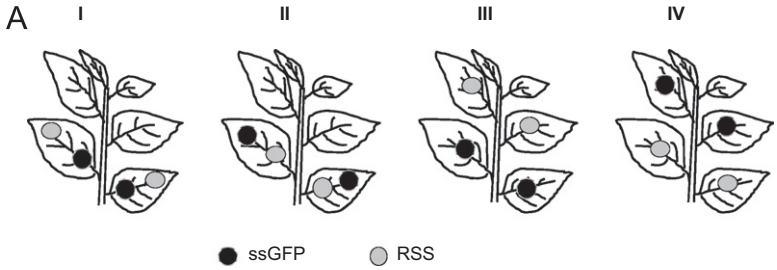
To correlate the more severe symptoms with the accumulation levels of viral RNAs, we used Northern blot hybridization to determine the steady-state level accumulation of PVX-specific RNAs from all the constructs included in this study at 10 and

Table 1
TriMV P1 suppresses systemic RNA silencing induced by GFP dsRNA in *N. benthamiana* line 16c plants[a].

Construct	5 dpi		7 dpi		10 dpi	
	# of plants silenced[b]/# of plants infiltrated	% of plants systemically silenced	# of plants silenced/# of plants infiltrated	% of plants systemically silenced	# of plants silenced/# of plants infiltrated	% of plants systemically silenced
TriMV P1+ssGFP+dsGFP	1/20	5	14/20	70	18/20	90
TriMV P1+1FS+ssGFP+dsGFP	12/12	100	12/12	100	12/12	100
TuMV HC-Pro+ssGFP+dsGFP	18/20	90	20/20	100	20/20	100
pCASS4+ssGFP+dsGFP	19/20	95	20/20	100	20/20	100

[a] The top two fully expanded *N. benthamiana* 16c leaves infused with agrosuspensions carrying ssGFP, dsGFP plus a suppressor gene as indicated.
[b] *N. benthamiana* 16c plants were considered systemically silenced if the major and minor veins of upper young leaves of agroinfiltrated plants turned red under UV illumination.

A



B

set	Group	Construct	# of plants infiltrated	# of plants systemically silenced	% of plants systemically silenced
1	I	TriMV P1(Ap)/ssGFP(Ba)	20	18	90
2	II	TriMV P1(Ba)/ssGFP(Ap)	19	2	11
3	III	TriMV P1(Up)/ssGFP (Lo)	18	0	0
4	IV	TriMV P1(Lo)/ssGFP(Up)	20	20	100
5	II	TriMV P1+1FS(Ba)/ssGFP(Ap)	13	10	92
6	III	TriMV P1+1FS(Up)/ssGFP(Lo)	11	10	91
7	I	TuMV HC-Pro(Ap)/ssGFP(Ba)	11	10	91
8	II	TuMV HC-Pro(Ba)/ssGFP(Ap)	10	5	50
9	III	TuMV HC-Pro(Up)/ssGFP(Lo)	12	1	8
10	IV	TuMV HC-Pro(Lo)/ssGFP(Up)	12	12	100

Fig. 5. Positional effect of silencing suppressors and silencing inducer on systemic silencing of *Nicotiana benthamiana* 16c plants. (A) Schematic diagrams of *N. benthamiana* 16c plants showing the position of infiltrations with *Agrobacterium* suspensions harboring ssGFP (dark-colored dots) and RNA silencing suppressor (RSS) (gray-colored dots) constructs in groups I, II, III, and IV. (B) The number of *N. benthamiana* 16c plants systemically silenced at 10 day postinfiltration in sets 1–10. *N. benthamiana* 16c leaves agroinfiltrated with ssGFP (silencing inducer) and TriMV P1 or TuMV HC-Pro (silencing suppressors) as indicated in sets 1–10. TriMV P1 with a +1 frameshift (TriMV P1+1FS) was used as a negative control. 'Ap' and' Ba' indicate the apical and basal portions of infiltrated 16c leaves, and 'Up' and 'Lo' indicate the upper and lower leaves of 16c plants, respectively.

14 dai (Fig. 6B and C). At 10 and 14 dai, PVX-specific RNAs from *N. benthamiana* plants infected with wild-type PVX, PVX-TriMV HC-Pro, or PVX-TriMV P1+1FS accumulated similar levels of viral genomic as well as subgenomic RNAs (Fig. 6B and C, lanes 1, 2, and 4). However, plants infected with PVX-TriMV P1 or PVX-TEV HC-Pro contained substantially higher levels of PVX-specific RNAs than the control constructs described above (Fig. 6B and C, compare lanes 3 and 5 with 1, 2, and 4). In summary, our results illustrated that TriMV P1 as a strong RSS is capable of synergistically enhancing the infection severity of a different virus.

The silencing suppression function of TriMV P1 is shared by another Poacevirus

In light of the identification of TriMV P1 as the RSS encoded by this virus, we examined whether using P1 as the RSS is a shared feature of all *poaceviruses*. We hence tested the P1 of SCSMV, another *Poacevirus* (Xu et al., 2010), for its potential to suppress RNA silencing using the agroinfiltration assay. Infiltration of *N. benthamiana* 16c leaves with *Agrobacterium* suspensions harboring ssGFP plus SCSMV P1 resulted in strong GFP fluorescence at 3 and 6 dpi (Fig. 7A). Conversely, patches infiltrated with agrobacterial suspensions harboring ssGFP plus SCSMV HC-Pro failed to emit GFP fluorescence (Fig. 7A). As expected, strong GFP fluorescence was observed in leaf patches infiltrated with ssGFP plus TriMV P1 or TuMV HC-Pro (positive controls), while no fluorescence was observed in leaf patches infiltrated with pCASS4 (negative control) (Fig. 7A).

Northern blot analysis revealed that large amounts of GFP mRNA accumulated in leaf patches infiltrated with ssGFP plus SCSMV P1, TriMV P1, or TuMV HC-Pro at 3 dpi (Fig. 7B, lanes 1, 3, and 4). However, the amount of GFP mRNA was decreased slightly by 6 dpi (Fig. 7C). The increased accumulation of GFP mRNA in leaf patches infiltrated with ssGFP plus SCSMV P1 was correlated with reduced accumulation of GFP siRNAs at 3 dpi (Fig. 7D, lane 1). At 6 dpi, the accumulation of GFP siRNAs slightly increased in all infiltrated leaf patches (Fig. 7E). Furthermore, HA-tagged HC-Pro of SCSMV was readily detected in agroinfiltrated *N. benthamiana* leaves (Fig. 1D, lane 2), suggesting that failure of HC-Pro to function as an RSS is probably not due to inefficient expression and/or protein instability. These data suggested that the P1 protein of SCSMV, not HC-Pro, is a suppressor of RNA silencing, similar to that of TriMV P1 in the genus *Poacevirus*.

Discussion

Plant viruses encode RSS proteins as the most effective means to overcome host RNA silencing, a major defense response targeting virus genomes. These proteins differ extensively in their amino acid sequences and sizes, as well as their modes of action. In the family *Potyviridae*, HC-Pro was first identified as an RSS protein of a *potyvirus* (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Recently, the P1b or P1 of *Ipomovirus* and *Tritimovirus* genera were shown to have RNA silencing suppression activity (Giner et al., 2010; Valli et al., 2006; Young et al., 2012). In this study, we have identified the P1 of the newly established genus *Poacevirus* in the family *Potyviridae* as a potent suppressor of RNA silencing. We revealed that TriMV P1 suppressed both local and systemic RNA silencing

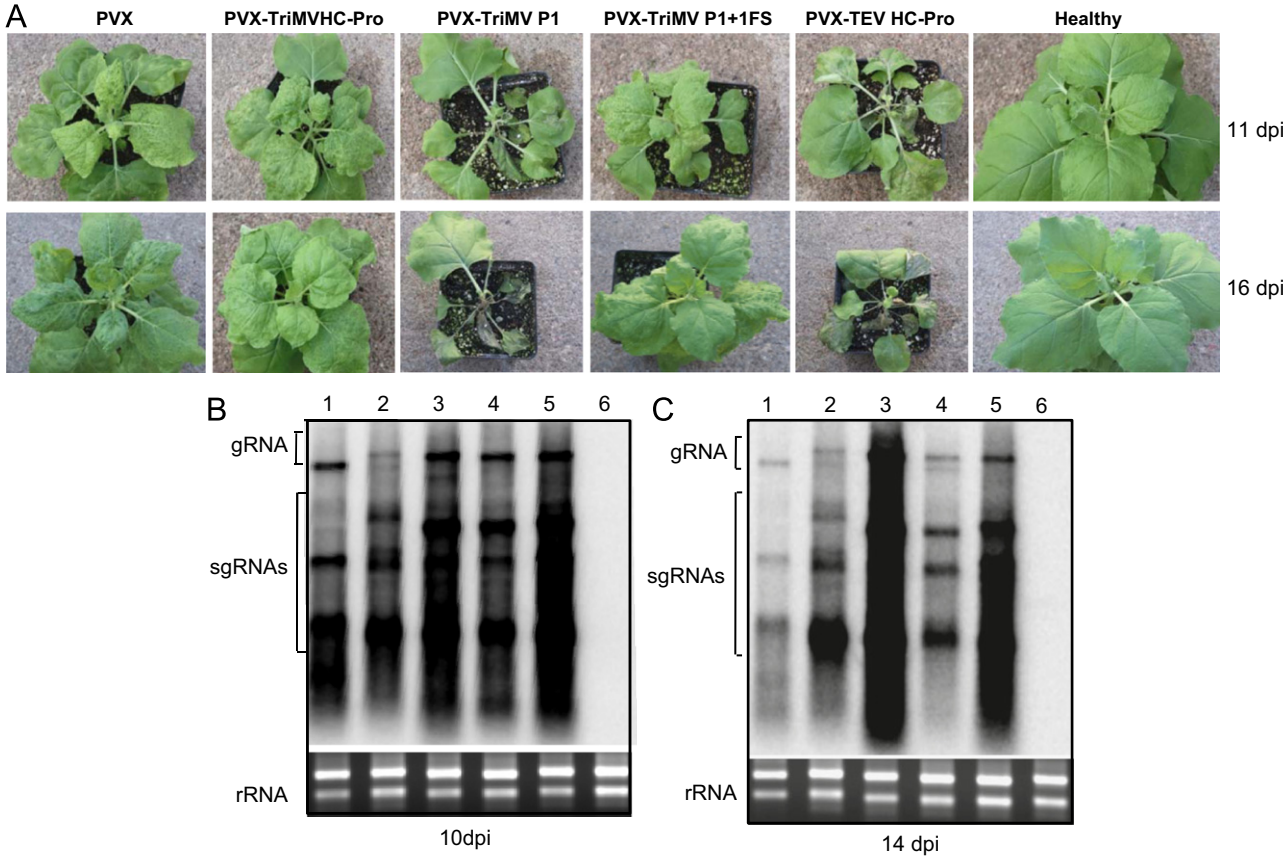


Fig. 6. TriMV P1 is a strong pathogenicity determinant. (A) Symptom phenotypes of wild-type PVX, PVX with TriMV HC-Pro, TriMV P1, TriMV P1+1FS, or TEV HC-Pro in wild-type *N. benthamiana* plants at 11 and 16 day after inoculation (dai). Buffer inoculated *N. benthamiana* was used as a negative control (healthy). (B and C) Northern blot analysis of PVX RNAs accumulation in *N. benthamiana* plants infected with PVX with TriMV cistrons. Total RNA isolated at 10 (B) and 14 dai (C) was separated (250 ng per lane) through 1.0% agarose-formaldehyde gel and the Northern blot was hybridized with a plus-strand RNA-specific DIG-labeled riboprobe corresponding to the 3′ end of PVX genomic RNA. Lane 1, wild-type PVX; lane 2, PVX-TriMV HC-Pro; lane 3, PVX-TriMV P1; lane 4, PVX-TriMV P1+1FS; lane 5, PVX-TEV HC-Pro; and lane 6, healthy. The bottom ethidium bromide-stained agarose gels in B and C are showing the rRNA as sample loading controls.

triggered by ssRNA and dsRNA, and likely prevented ssRNA-induced systemic silencing by inhibiting and/or blocking silencing signals. We also demonstrated the ability of TriMV P1 to synergistically enhance the disease symptoms of a different virus. Finally, we verified that the P1 of SCSMV, a different *poacevirus*, was also capable of suppressing RNA silencing. Therefore, using P1 as the RSS is likely a conserved feature for members of the *Poacevirus* genus. Notably, both TriMV and SCSMV P1s reduced, but did not abolish, the accumulation of siRNAs, suggesting that these RSS proteins did not affect siRNA biogenesis. These results are in agreement with observations made with potyviral HC-Pro and ipomoviral P1 and P1b (Anandalakshmi et al., 1998; Giner et al., 2010; Kasschau and Carrington, 1998; Valli et al., 2006), and suggest that TriMV P1 acts downstream of the siRNA biogenesis.

Although potyviral HC-Pro alone functions as a suppressor of RNA silencing (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998), the presence of P1 with HC-Pro substantially increased its silencing suppression activity (Rajamäki et al., 2005; Valli et al., 2006). In contrast, the poaceviral HC-Pro appears to interfere with the RNA silencing suppression function of P1 as GFP mRNA accumulation decreased substantially when TriMV P1 was replaced with that of TriMV P1/HC-Pro. Under the same experimental conditions, TuMV P1/HC-Pro efficiently expressed GFP and permitted slightly increased levels of GFP mRNA accumulation compared to that of HC-Pro. Recently, Carbonell et al. (2012) reported that the silencing suppression activity of P1a/P1b fusion of CVYV was also weaker than P1b alone, which was attributed to incomplete self-cleavage of P1a from P1b in *N. benthamiana*. However, TriMV P1 was efficiently cleaved from HC-Pro in P1/HC-Pro infiltrated leaf patches. It is possible that the P1 and HC-Pro proteins of TriMV from P1/HC-Pro construct may interact with each other to down-regulate the RSS activity of P1, as the P1 of WSMV has been shown to interact with other WSMV proteins including HC-Pro (Choi et al., 2000). In the course of a normal TriMV infection, other viral protein–protein interactions may also occur to mitigate the inhibitory effect of HC-Pro on P1 RSS function.

TriMV P1 also suppressed dsRNA-triggered systemic RNA silencing. This is intriguing because, at the level of infiltrated leaves, dsRNA-triggered RNA silencing was suppressed to a similar extent by both TriMV P1 and TuMV HC-Pro. Yet, in contrast to TriMV P1, the HC-Pro of TuMV was unable to suppress dsRNA-triggered systemic RNA silencing under the same experimental conditions. This observation further suggests that there may be mechanistic differences between these two RSSs. Indeed, while potyviral HC-Pro is known to interact with double-stranded siRNAs, the P1 protein encoded by an *Ipomovirus* (SPMMV) has recently been shown to interact with AGO proteins through conserved WG/GW motifs (Giner et al., 2010; Lakatos et al., 2006). Further studies are needed to determine whether TriMV P1 shares the same silencing suppression mechanism with SPMMV P1, although it should be noted that TriMV P1 does not contain the WG/GW motifs identified in SPMMV P1.

TriMV P1 efficiently suppressed systemic RNA silencing triggered by ss and dsRNAs. The mechanisms involved in suppression of systemic RNA silencing signals have been examined for only a

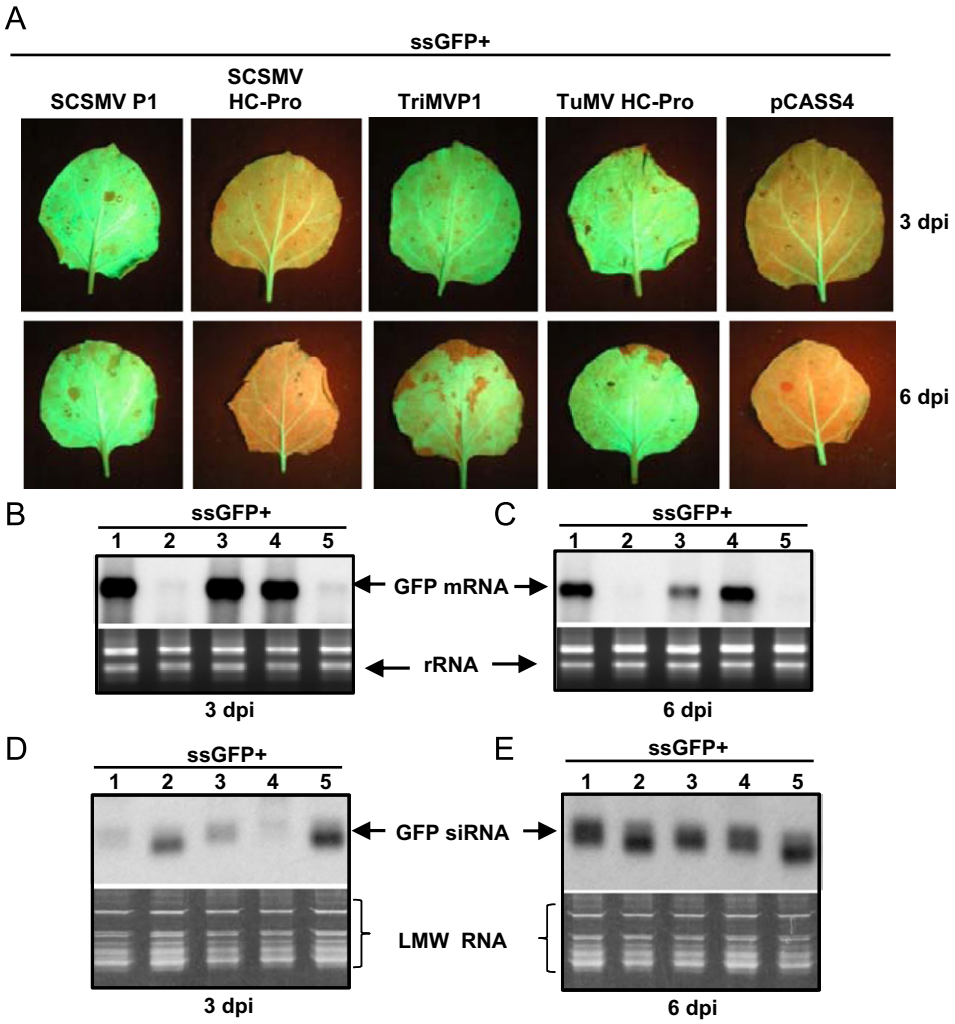


Fig. 7. The P1 of *Sugarcane streak mosaic virus* (SCSMV) is a strong suppressor of RNA silencing triggered by ssGFP in *N. benthamiana* 16c leaves. Green fluorescent images of agroinfiltrated leaf patches as indicated at 3 and 6 dpi. Northern blot analyses of GFP mRNA (B and C) and GFP siRNA (D and E) from agroinfiltrated leaf patches with ssGFP plus SCSMV P1 (lane 1), SCSMV HC-Pro (lane 2), TriMV P1 (lane 3), TuMV HC-Pro (lane 4), or pCASS4 (lane 5). Ethidium bromide-stained rRNA (B and C) and low molecular weight RNA (LMW) (D and E) are shown as sample loading controls. GFP plus-strand RNA-specific DIG-labeled riboprobe with (for D and E) and without (for B and C) hydrolysis was used for Northern blot hybridizations.

few viruses (for e.g. Cao et al., 2005; Guo and Ding, 2002; Xiong et al., 2009). The positional expression of P1 and ssGFP in *N. benthamiana* 16c leaves suggests that TriMV P1 is capable of blocking the spread of silencing signals after their production when P1 is expressed between the recipient and source tissues of the silencing signals. The efficient blocking of systemic silencing in top young leaves when P1 and ssGFP (silencing initiator) were expressed in upper and lower leaves, respectively, of the same plant, suggests the possibility that the P1 exerts its effects by trafficking from expressing cells to the main stem, where it may physically interact with silencing signals that are being transported from the lower leaves. Perhaps, the transported P1 could bind to siRNAs at the interface, thus inhibiting systemic spread of RNA silencing in upper young leaves. This hypothesis is consistent with the recent finding that silencing suppressor proteins bind to siRNAs as a strategy to suppress RNA silencing (Lakatos et al., 2006).

Most viral proteins originally identified as pathogenicity determinants were later identified as suppressors of RNA silencing (Brigneti et al., 1998; Pruss et al., 1997). However, the silencing suppressor proteins elicited different levels of symptoms in heterologous systems. This could be due to the fact that some silencing suppressors do not affect the miRNA pathway but inhibit RNA interference exclusively (Dunoyer et al., 2004), which suggests that RSS proteins can differentially affect pathogenicity. PVX with TriMV P1 or TEV HC-Pro developed more severe symptoms, resulting in systemic veinal chlorosis/necrosis, followed by apical shoot necrosis and death of plants. These dramatically intensified disease symptoms with increased steady-state accumulation of PVX-specific RNAs suggest that TriMV P1 is a strong symptom determinant that synergistically interacts with a heterologous virus. Together, our data establishes P1 as a potent RSS of viruses in the *Poacevirus* genus of the *Potyviridae* family.

Materials and methods

Construction of binary plasmids

TriMV cistrons coding for P1/HC-Pro, P1, HC-Pro, P3, CI, 6K2, NIa-VPg, NIa-Pro, NIb, and CP were amplified by PCR using an infectious cDNA clone (S. Tatineni, unpublished data; GenBank accession number FJ669487) as a template. Oligonucleotides were designed such that the translation initiation (AUG) and termination (UAG) codons were introduced at the 5′ and 3′ ends of each cistron, respectively, and fused to the 3′ end of TEV leader

sequence (L) (Carrington and Freed, 1990) by the overlap extension PCR method (Ho et al., 1989). All PCR amplifications were performed with Herculase II Fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The overlap extension PCR products comprising TEV-L, followed by TriMV cistrons were digested with *Sac*I (engineered in reverse primers) and ligated into pCASS4 between *Stu*I and *Sac*I restriction sites. Similarly, P1/HC-Pro, HC-Pro, and P1 cistrons of SCSMV were amplified from a cDNA clone (Xu et al., 2010) by overlap extension PCR and ligated into pCASS4 as described above. As positive controls, HC-Pro and P1/HC-Pro of TuMV were ligated into pRTL2 (Carrington and Freed, 1990) and an expression cassette comprising 35S promoter, TEV-L, TuMV P1/HC-Pro or HC-Pro and 35S terminator were then transferred to pPZP212 (Hajdukiewicz et al., 1994). A dsGFP expression cassette was generated by ligating sense and antisense sequences of GFP in tandem separated by a 100 nt spacer derived from *Turnip crinkle virus* (TCV) into pRTL2 and the expression cassette was subsequently transferred into pPZP212 to obtain 35S-dsGFP. The 35S –GFP in pPZP212 construct was described previously (Qu et al., 2003). TEV-L sequence and sequence encoding HA-epitope (YPYDVPDYA) were fused in tandem to the 5′ of TriMV P1, P1/HC-Pro and HC-Pro, and SCSMV HC-Pro cistrons by overlap extension PCR (Ho et al., 1989), and PCR fragments were ligated into pCASS4 as described above. All plasmid DNAs used in this study were sequenced to confirm the presence of intended sequences at the University of Florida ICBR Core DNA Sequencing Facility using an Applied Biosystems 3730 model sequencer. Oligonucleotide sequences used in this study will be provided upon request.

Agroinfiltration assays and GFP imaging

Binary plasmids with TriMV, SCSMV, or TuMV cistrons were chemically transformed into *A. tumefaciens* strain EHA105. All *A. tumefaciens* cultures were incubated at 28 °C. Overnight-grown *A. tumefaciens* cultures were centrifuged at 4300*g* for 15 min, and the pellets were resuspended in 10 mM MES, pH 5.5 containing 10 mM $MgCl_2$ and 100 μM acetosyringone to an optical density of 1.0 at 600 nm. The agrobacterial suspensions were incubated at room temperature for 3–4 h prior to infusion. In co-infiltration experiments, equal volumes of each *A. tumefaciens* suspension with an optical density of 1.0 at 600 nm were mixed prior to infusion. Wild-type or line 16c (GFP transgenic) *N. benthamiana* plants at the 6–8-leaf stage were infiltrated with *A. tumefaciens* suspensions, and infiltrated plants were incubated in a growth chamber at 24 °C maximum and 22 °C minimum temperatures, with a 14-h photoperiod. Green fluorescence was observed under long-wavelength UV light (Black-Ray Model B-100A, San Gabriel, CA) and photographed using a Nikon Digital D70 camera with an orange filter. All agroinfiltrations, and subsequent Northern and Western blot analyses (see below) were repeated at least 3–4 times.

RNA extraction and Northern blot hybridization

Total RNA was extracted from 400 mg agroinfiltrated leaf patches as described previously (Tatineni et al., 2010). Total RNA was quantified using a NanoPhotometer (Implene Inc., Westlake Village, CA), and separated through 1.0% agarose gels containing formaldehyde (for GFP mRNA) or through 15% acrylamide-urea gels (for siRNAs), followed by electro-transfer to nylon membranes (Roche, Indianapolis, IN). Nylon membranes were probed for GFP mRNA with a digoxigenin (DIG)-labeled GFP plus-strand RNA-specific riboprobe. This probe was further hydrolyzed into ~50 nt long RNA pieces by treatment with sodium carbonate buffer as described by Dalmay et al. (2000), and used for Northern-blot hybridization of GFP-specific siRNAs. Prehybridization and hybridization were carried out in a hybridization buffer containing 50% formamide, 5X SSC, 2% blocking solution (Roche), 0.02% SDS, and 0.1% *N*-lauroylsarcosine at 65 °C (for GFP mRNA) or at 41 °C (for GFP siRNAs) for 2–3 h and overnight, respectively. The nylon membranes were developed immunologically using an anti-DIG-ALP conjugate (Roche) essentially as described by the supplier, except that washings with 0.1X SSC plus 0.1% SDS were carried out at 65 °C (for GFP mRNA) or at 45 °C (for GFP siRNAs). The GFP-specific mRNA, siRNA, and protein bands (see below) captured on X-ray film (BioMax Light Film, Kodak, Rochester, NY) were quantified with the Molecular Imager ChemiDoc XRS system (Bio-Rad). Nonphosphorylated synthetic RNAs of 21 and 24 nts were used as siRNA size markers in Northern blot hybridizations in Figs. 2–4. However, Dicer-generated siRNAs contain a 5′ phosphate (Siomi and Siomi, 2009), so they migrate slightly faster than nonphosphorylated synthetic RNAs of the same size.

Western-blot analysis

Wild-type *N. benthamiana* plants at the 6–8-leaf stage were infiltrated with *A. tumefaciens* suspensions and plants were incubated in a growth chamber at 24 °C maximum and 22 °C minimum temperatures, with a 14-h photoperiod. Extraction of total soluble proteins from agroinfiltrated *N. benthamiana* leaf patches, followed by SDS-PAGE separation and immuno-blot analysis was carried out as described previously (Tatineni et al., 2011a). Monoclonal antibodies specific to HA epitope (Sigma-Aldrich) and GFP (Clontech) were used at 1:5000 and 1:10,000 dilution, respectively.

Cloning TriMV cistrons into PVX and analysis

TriMV P1 and P1 with a +1 FS were amplified from a full-length infectious cDNA clone (S. Tatineni, unpublished data), and ligated into the PVX vector pP2C2S (Chapman et al., 1992) at the *Cla*I restriction site. Translation initiation (AUG) and termination (UGA) codons were introduced at the 5′ and 3′ ends of cistrons, respectively. As a positive control, TEV HC-Pro was amplified and ligated into the same PVX vector. *In vitro* transcripts were generated from linearized PVX plasmids as described previously (Tatineni et al., 2011b), and inoculated mechanically to *N. benthamiana* plants at the 4–6-leaf stage and incubated in a growth chamber at 23 °C maximum and 20 °C minimum temperatures, with a 14-h photoperiod. Total RNA was isolated from *N. benthamiana* plants infected with PVX containing TriMV or TEV cistrons, and analyzed for accumulation of PVX-specific RNAs using a PVX 3′ end (~700 nt) plus-strand RNA-specific DIG-labeled riboprobe.

Acknowledgments

We thank Stephen N. Wegulo for editorial assistance, David C. Baulcombe for providing *Nicotiana benthamiana* line 16c seeds, and Bryce Falk for sharing the PVX vector. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

References

Adams, M.J., Zerbini, F.M., French, R., Rabenstein, F., Stenger, D.C., Valkonen, J.P.T., 2011. *Potyviridae*. In: Andrew, M.Q., King, Michael J., Adams, Eric B., Carstens, Lefkowitz, Elliot J. (Eds.), Virus Taxonomy. Elsevier, Oxford, pp. 1069–1090.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V.B., 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA 95, 13079–13084.

Bass, B.L., 2000. Double-stranded RNA as a template for gene silencing. Cell 101, 235–238.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., Baulcombe, D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO J. 17, 6739–6746.

Burrows, M., Franc, G., Rush, C., Blunt, T., Ito, D., Kinzer, K., Olson, J., O'Mara, J., Price, J., Tande, C., Ziems, A., Stack, J., 2009. Occurrence of viruses in wheat in the Great Plains region 2008. Plant Health Prog., doi:10.1094/PHP-2009-0706-01-RS.

Cañizares, M.C., Navas-Castillo, J., Moriones, E., 2008. Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, *Tomato chlorosis virus*. Virology 379, 168–174.

Cao, X., Zhou, P., Zhang, X., Zhu, S., Zhong, X., Xiao, Q., Ding, B., Li, Y., 2005. Identification of an RNA silencing suppressor from a plant double-stranded RNA virus. J. Virol. 79, 13018–13027.

Carbonell, A., Dujovny, G., Garcia, J.A., Valli, A., 2012. The *Cucumber vein yellowing virus* silencing suppressor P1b can functionally replace HC-Pro in *Plum pox virus* infection in a host-specific manner. Mol. Plant–Microbe Interact. 25, 151–164.

Carrington, J.C., Freed, D.D., 1990. Cap-independent enhancement of translation by a plant *potyvirus* 5′ nontranslational region. J. Virol. 64, 1590–1597.

Chapman, S., Kavanagh, T., Baulcombe, D.C., 1992. Potato virus X as a vector for gene expression in plants. Plant J. 2, 549–557.

Choi, I.R., Stenger, D.C., French, R., 2000. Multiple interactions among proteins encoded by the mite-transmitted wheat streak mosaic tritimovirus. Virology 267, 185–198.

Cogoni, C., Macino, G., 2000. Posttranscriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. 10, 638–643.

Dalmay, T., Hamilton, A.J., Mueller, E., Baulcombe, D.C., 2000. Potato virus X amplicons in *Arabidopsis* mediate genetic and epigenetic gene silencing. Plant Cell 12, 369–380.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K.D., Carrington, J.C., Voinnet, O., 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science 313, 68–71.

Ding, S.W., Voinnet, O., 2007. Antiviral immunity directed by small RNAs. Cell 130, 413–426.

Dunoyer, P., Lecellier, C.H., Parizotto, E.A., Himber, C., Voinnet, O., 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell 16, 1235–1250.

Dunoyer, P., Himber, C., Voinnet, O., 2005. DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. Nat. Genet. 37, 1356–1360.

Dunoyer, P., Schott, G., Himber, C., Meyer, D., Takeda, A., Carrington, J.C., Voinnet, O., 2010. Small RNA duplexes function as mobile silencing signals between plant cells. Science 328, 912–916.

Fellers, J.P., Seifers, D., Ryba-White, M., Martin, T.J., 2009. The complete genome sequence of *Triticum mosaic virus*, a new wheat-infecting virus of the High Plains. Arch. Virol. 154, 1511–1515.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Giner, A., Lakatos, L., García-Chapa, M., López-Moya, J.J., Burgyán, J., 2010. Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog. 6, e1000996.

Guo, H.S., Ding, S.W., 2002. A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO J. 21, 398–407.

Hajdukiewicz, P., Svab, Z., Maliga, P., 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. 25, 989–994.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293–296.

Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using polymerase chain reaction. Gene 77, 51–59.

Kalantidis, K., Schimacher, H.T., Alexiadis, T., Helm, J.M., 2008. RNA silencing movement in plants. Biol Cell 100, 13–26.

Kasschau, K.D., Carrington, J.C., 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95, 461–470.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., Plasterk, R.H.A., 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *Caenorhabditis elegans*. Genes Dev. 15, 2654–2659.

Lakatos, L., Szittya, G., Silhavy, D., Burgyan, J., 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. EMBO J. 23, 876–884.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P., Dolja, V.V., Calvino, L.F., López-Moya, J.J., Burgyan, J., 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J. 25, 2768–2780.

Lindbo, J.A., Silva-Rosales, F., Proebsting, W.M., Dougherty, W.G., 1993. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5, 1749–1759.

Llave, C., Kasschau, K.D., Carrington, J.C., 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proc. Natl. Acad. Sci. USA 97, 13401–13406.

Mallory, A.C., Ely, L., Smith, T.H., Marathe, R., Anandalakshmi, R., Fagard, M., Vaucheret, H., Pruss, G., Bowman, L., Vance, V.B., 2001. HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. Plant Cell 13, 571–583.

Mbanzibwa, D., Tian, Y., Mukasa, S.B., Valkonen, J.P.T., 2009. *Cassava brown streak virus* (*Potyviridae*) encodes a putative Maf/HAM1 pyrophosphatase implicated in reduction of mutations and a P1 proteinase that suppresses RNA silencing but contains no HC-Pro. J. Virol. 83, 6934–6940.

Mlotshwa, S., Pruss, G.J., Vance, V., 2008. Small RNAs in viral infection and host defense. Trends Plant Sci. 13, 375–382.

Molnar, A., Melnyk, C.W., Bassett, A., Hardcastle, T.J., Dunn, R., Baulcombe, D.C., 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science 328, 872–875.

Palauqui, J.C., Elmayan, T., Pollien, J.M., Vaucheret, H., 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 16, 4738–4745.

Pruss, G., Ge, X., Shi, X.M., Carrington, J.C., Vance, V.B., 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell 9, 859–868.

Qu, F., Ren, T., Morris, T.J., 2003. The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. J. Virol. 77, 511–522.

Rajamäki, M.L., Kelloniemi, J., Alminaite, A., Kekarainen, T., Rabenstein, F., Valkonen, J.P., 2005. A novel insertion site inside the *potyvirus* P1 cistron allows expression of heterologous proteins and suggests some P1 functions. Virology 342, 88–101.

Ratcliff, F., Harrison, B.D., Baulcombe, D.C., 1997. A similarity between viral defense and gene silencing in plants. Science 276, 1558–1560.

Seifers, D.L., Martin, T.J., Harvey, T.L., Fellers, J.P., Stack, J.P., Ryba-White, M., Haber, S., Krokhin, O., Spicer, V., Lovat, N., Yamchuk, A., Standing, K.G., 2008. *Triticum* mosaic virus: A new virus isolated from wheat in Kansas. Plant Dis. 92, 808–817.

Seifers, D.L., Martin, T.J., Harvey, T.L., Fellers, J.P., Michaud, J.P., 2009. Identification of the wheat curl mite as the vector of *Triticum mosaic virus*. Plant Dis. 93, 25–29.

Seifers, D.L., Martin, T.J., Fellers, J.P., 2011. Occurrence and yield effects of wheat infected with *Triticum mosaic virus* in Kansas. Plant Dis. 95, 183–188.

Shi, B.J., Ding, S.W., Symons, R.H., 1997. Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. J. Gen. Virol. 78, 1181–1185.

Siomi, H., Siomi, M.C., 2009. On the road to reading the RNA-interference code. Nature 457, 396–404.

Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M., Burgyan, J., 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 21, 3070–3080.

Tatineni, S., Ziems, A.D., Wegulo, S.N., French, R., 2009. *Triticum mosaic virus*: a distinct member of the family *Potyviridae* with an unusually long leader sequence. Phytopathology 99, 943–950.

Tatineni, S., Graybosch, R.A., Hein, G.L., Wegulo, S.N., French, R., 2010. Wheat cultivar-specific disease synergism and alteration of virus accumulation during co-infection with *Wheat streak mosaic virus* and *Triticum mosaic virus*. Phytopathology 100, 230–238.

Tatineni, S., McMechan, J.A., Hein, G.L., French, R., 2011a. Efficient and stable expression of GFP through *Wheat streak mosaic virus*-based vectors in cereal hosts using a range of cleavage sites: formation of dense fluorescent aggregates for sensitive virus tracking. Virology 410, 268–281.

Tatineni, S., Van Winkle, D.H., French, R., 2011b. The N-terminal region of Wheat streak mosaic virus coat protein is a host- and strain-specific long-distance transport factor. J. Virol. 85, 1718–1731.

Valli, A., Martín-Hernández, A.M., López-Moya, J.J., Gárcia, J.A., 2006. RNA silencing suppression by a second copy of the P1 serine protease of *Cucumber vein yellowing Ipomovirus*, a member of the family *Potyviridae* that lacks the cysteine protease HCPro. J. Virol. 80, 10055–10063.

Voinnet, O., Baulcombe, D.C., 1997. Systemic signaling in gene silencing. Nature 389, 553.

Voinnet, O., Vain, P., Angell, S., Baulcombe, D.C., 1998. Systemic spread of sequence-specific transgene RNA degradation is initiated by localized introduction of ectopic promoterless DNA. Cell 95, 177–187.

Wang, M.B., Metzlaff, M., 2005. RNA silencing and antiviral defense in plants. Curr. Opin. Plant Biol. 8, 216–222.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., Carrington, J.C., 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. 2, E104.

Xiong, R., Wu, J., Zhou, Y., Zhou, X., 2009. Characterization and subcellular localization of an RNA silencing suppressor encoded by Rice stripe tenuivirus. Virology 387, 29–40.

Xu, D.L., Zhou, G.H., Xie, Y.J., Mock, R., Li, R., 2010. Complete nucleotide sequence and taxonomy of sugarcane streak mosaic virus, member of a novel genus in the family *Potyviridae*. Virus Genes 40, 432–439.

Young, B.A., Stenger, D.C., Qu, F., Morris, T.J., Tatineni, S., French, R., 2012. Tritimovirus P1 functions as a suppressor of RNA silencing and an enhancer of disease symptoms. Virus Res. 163, 672–677.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., Chua, N.H., 2006. Cucumber mosaic virus-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. Genes Dev. 20, 3255–3268.