Viral suppressors of RNA silencing
Wan Xiang Li and Shou Wei Ding*

The suppression of RNA silencing by plant viruses represents a viral adaptation to a novel host antiviral defense. Three types of viral suppressors have been identified through the use of a variety of silencing suppression assays. The first two types of suppressor are capable of a complete or partial reversal of pre-existing RNA silencing; the third type does not reverse RNA silencing but can instead prevent its systemic signaling.

Addresses
Department of Plant Pathology, University of California, Riverside, California 92521, USA; *e-mail: shou-wei.ding@ucr.edu

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Abbreviations
CMV cucumber mosaic virus
PTGS post-transcriptional gene silencing
PVX potato virus X
RdRP RNA-dependent RNA polymerase
RISC RNA-induced silencing complex

Introduction
RNA silencing is a novel gene regulatory mechanism that operates at the level of RNA. Upon activation, this mechanism can degrade viral and transgenic RNAs as well as endogenous mRNAs in a sequence-specific manner [1–3]. RNA silencing occurs in a variety of eukaryotic organisms and has been variously described as post-transcriptional gene silencing (PTGS) and RNA-mediated virus resistance (RMVR) in plants, quelling in Caenorhabditis elegans and Drosophila. Available data support a general mechanism for RNA silencing in which double-stranded RNA is the initiator [4–6] and is subsequently processed by a ribonuclease (RNase) III into short segments, first referred to as 25-nucleotide (nt) RNAs [7,8••,9••,10,11••,12]. These 25 nt RNAs, recently shown to be 21–23 nt in length [8••,9••], may then serve as guide sequences that instruct an RNase complex, the RNA-induced silencing complex (RISC), to destroy homologous RNA species [7,8••,9••,10,11••,12]. Additional evidence to support a conserved RNA silencing pathway comes from the recent isolation of homologous genes from Arabidopsis [13••,14••,15•], Neurospora [16,17•] and C. elegans [18,19] that are essential for PTGS/RMVR, quelling and RNAs, respectively. A fascinating feature of RNA silencing in plants is that after initiation of local silencing, a signal is produced that can move between cells through plasmodesmata and over long distances through the vascular system to direct specific RNA silencing in the whole plant [20–23,24••].

An important milestone in this area of research was the discovery in 1998 that plant viruses encode proteins that are suppressors of RNA silencing [25–28]. This finding provided not only the strongest support that RNA silencing functions as a natural defense mechanism against viruses [29–32], but also yielded valuable tools for the dissection of the RNA silencing pathway. This review highlights the key features of assays used in the analysis of the silencing suppression activities of viral proteins, discusses the mechanisms of silencing suppression employed by distinct viral suppressors, and considers the potential interactions of viral suppressors with other host defense pathways. Length restrictions prevent discussion of other related aspects of RNA silencing and we refer the reader to three recent reviews [1,33,34].

Silencing suppression assays
A key component in silencing suppression assays is a reporter transgene that undergoes RNA silencing. The transgene may be silenced constitutively [35] or upon induction by infiltration with an Agrobacterium tumefaciens strain carrying a Ti plasmid encoding the same transgene, referred to as agro-infiltration [21,22]. To assay for silencing suppression activity, the candidate protein can be introduced into the silenced plants by a replicating virus vector, genetic crosses or agro-infiltration. When the reporter transgene used is silenced constitutively, a viral suppressor expressed from a stably integrated transgene may be introduced into the silenced plants by genetic crosses [25,28]. However, both the constitutive and the de novo induced silenced transgenes can be used as reporters when the suppressor is delivered into the silenced plants by the persistent infection of a virus vector [26,27]. The third method of suppressor delivery is by agro-infiltration [24••,36•]. In this approach, a suppressor expression cassette is cloned within the transfer DNA (T-DNA) of a Ti plasmid and the Agrobacterium transformed with this plasmid is introduced into plants by localized leaf injections. As a result, expression of the suppressor protein in this assay is transient and localized, which is in contrast to the persistent and systemic expression achieved through the use of virus vectors or from stably integrated transgenes.

A critical consideration in assaying for silencing suppression is the status of the transgene reporter when the suppressor is introduced into plants. When a candidate suppressor protein is expressed after RNA silencing of a transgene is complete, suppression of transgene RNA silencing occurs only if this protein is able to reverse RNA silencing [26,27,36•]. A suppressor that targets only the early stages of the RNA silencing pathway is likely to be inactive in the silencing reversal assay. Identification of these suppressors will require their expression either before or during the initiation of transgene RNA silencing. This is achieved in the recently reported transient silencing suppression assay [24••], in which the transgenes encoding the inducer and suppressor of RNA silencing are simultaneously introduced by agro-infiltration.

In addition to transgenes, a replicating viral RNA may also be used as the reporter for assaying silencing suppression. For example, replication of the potato virus X (PVX) genomic RNA — either after inoculation of wild-type plants with virus particles or from an integrated amplicon transgene in transgenic tobacco and Arabidopsis plants — is known to trigger PVX-specific RNA silencing [33,37,38] and leads to the accumulation of PVX 25 nt RNAs [7]. When the PVX genome is engineered to express a marker protein, such as green fluorescent protein (GFP), the silencing of viral RNAs can also be assessed visually by a loss of GFP fluorescence. A visual assessment of viral RNA silencing can also be made on the basis of the expression of host nuclear genes, because plant infection by PVX vectors carrying fragments of transgenes or endogenous genes induces RNA silencing of the corresponding target genes, a process termed virus-induced gene silencing (VIGS) [39].

To assay for VIGS suppression, a suppressor can be engineered to be expressed from the same VIGS vector before it is used for triggering VIGS in plants [25]. Alternatively, viruses encoding a suppressor of interest can be used directly to infect amplicon plants containing the replicating PVX RNA [38•]. VIGS suppression can also be carried out in a transient assay in which the agro-infiltrated inducer of the GFP transgene silencing, instead of a cauliflower mosaic virus 35S promoter-driven GFP construct (35S-GFP), is an amplicon transgene construct encoding a virus RNA that is replication-competent but deficient in both virus movement and silencing suppression, such as 35S–PVX:GFPΔ25KΔCP [24••].

It is important to note that proteins that are suppressors of transgene RNA silencing may not necessarily be active in assays that use a replicating virus RNA as the initiator of RNA silencing. This is because although an Arabidopsis gene homologous to the tomato RNA-dependent RNA polymerase (RdRP) [40] is required for transgene RNA silencing, it is not required for silencing triggered by several viruses examined (with the exception of cucumber mosaic virus; CMV). This observation suggests that these two pathways may be independent before converging at, or before production of, the 25 nt RNAs [13••,24••].

**Mechanisms of suppression by plant viral proteins**

Three types of viral suppressors of RNA silencing have been distinguished using a silencing reversal assay (Figure 1). The assay involves transgenic Nicotiana benthamiana plants carrying a highly expressed GFP transgene [27]; systemic RNA silencing of the GFP transgene is induced to completion by agro-infiltration with 35S–GFP before the plants are infected with viruses carrying a suppressor.

**HC-Pro**

HC-Pro, P1, and AC2 (encoded by potyviruses, rice yellow mottle sobemovirus and African cassava mosaic geminivirus, respectively) were able to activate GFP expression in all tissues of the previously silenced GFP plants [27,41]. Further work showed that transient expression of HC-Pro by agro-infiltration is sufficient to inhibit RNA silencing of a Gus transgene in N. tabacum [36•]. Interestingly, suppression of RNA silencing by HC-Pro was associated with a significantly reduced accumulation of the 25 nt RNAs [36•,42•], but did not prevent production and systemic signaling of the silencing signal [42•]. These data suggest that HC-Pro targets a maintenance step of the RNA silencing pathway that is upstream to the production of the 25 nt RNAs but downstream to the signal production. HC-Pro may suppress RNA silencing via an in vivo protein–protein interaction with a calmodulin-related protein (rgs-CaM); over-expression of rgs-CaM also resulted in silencing suppression in a manner similar to that produced by HC-Pro [43••].

**Cmv2b**

Cmv2b of CMV [44] was found to produce a distinct silencing suppression pattern in the same silencing reversal assay used for HC-Pro [27]. Expression of Cmv2b from either its own or the PVX genome resulted in GFP expression in those leaves that had newly emerged from the growing points, but not in the older tissues in which RNA silencing had already been established before virus infection [27]. The introduction of either Tav2b or p19 (encoded by tomato aspermy cucumovirus and tomato bushy stunt tombusvirus, respectively) into the silenced GFP plants produced a similar suppression pattern [41,45]. Thus, in contrast to HC-Pro/P1/AC2, this second type of viral suppressor is not able to reverse RNA silencing once silencing is established, indicating that they target an earlier stage of RNA silencing than HC-Pro. As the growing points (the meristematic zones) do not exhibit RNA silencing in this assay system even when there is extensive silencing in the rest of the plant [22], the observed suppression pattern suggests that Cmv2b may suppress RNA silencing in the new systemic leaves by blocking transport of the silencing signal and/or inhibiting the signal-mediated de novo induction of transgene RNA silencing. Recent data demonstrate that Cmv2b encodes a functional nuclear localization signal [46•,47] and nuclear targeting is critical to the suppressor activity of Cmv2b in the silencing reversal assay [46•]. This result indicates that suppression of RNA silencing by Cmv2b may occur in the nucleus or that nuclear trafficking is essential for Cmv2b to be a functional suppressor. A cDNA clone which encoded a protein that interacted with Cmv2b in a yeast two-hybrid assay was isolated from tobacco, although a functional link with silencing has yet to be established [48].

**p25**

The 25K protein (p25) of PVX displayed no detectable suppressor activity in the silencing reversal assay [27], which is consistent with the fact that PVX is an efficient vector for VIGS [39]. However, systemic RNA silencing did not occur in the majority of the transgenic GFP plants co-infiltrated with 35S–25K, which encodes p25, and either 35S–GFP or 35S–PVX:GFP, unlike those infiltrated with 35S–GFP or 35S–PVX:GFP alone [24••]. This work clearly demonstrates, for the first time, a role for a viral protein in interfering with the systemic signaling of RNA silencing.
inhibited by p25, it seems likely that p25 targets a step in the systemic silencing signal and is sensitive to p25 [24**]. This finding not only reveals a further complexity in the molecular strategies employed by pathogens and their hosts for defense and counter-defense, but also indicates the effectiveness in targeting the viral suppressors of RNA silencing for the control of viral diseases.

A recent study has documented a novel activity of Cmv2b in the inhibition of salicylic acid (SA)-mediated virus resistance in tobacco [58*]. SA is necessary and sufficient for the induction of systemic acquired resistance (SAR) effective against a broad range of plant pathogens including bacteria, fungi and viruses [59]. How SA regulates virus resistance is not well understood. There is evidence from both pharmacological and genetic studies, however, for a virus-specific branch of SAR that is independent of the expression of genes encoding the pathogenesis-related proteins, unlike resistance to bacterial and fungal pathogens [60,61]. Using a tobacco line expressing the NahG transgene that degrades SA, it was found that SA removal reduced virus resistance but did not fully compensate for the loss of Cmv2b [58*]. This result indicates that SA acts as a positive modifier rather than as an essential component of the Cmv2b-sensitive antiviral defense. These findings support the proposed model [58*] in which Cmv2b has a single target in the RNA silencing antiviral pathway and it displays an inhibitory activity to SA-mediated virus resistance most likely because SA enhances virus resistance by potentiating RNA silencing (Figure 1).

Conclusions

The discovery of p25 as a suppressor of systemic RNA silencing [24**] has several important implications in addition to those discussed above. p25 is essential for the cell-to-cell movement of PVX in its host, in contrast to other known suppressors such as Cmv2b and HC-Pro that facilitate long-distance movements of the virus and disease symptoms expression. Thus, it is likely that the PVX-specific silencing signal is produced before the secondary infection occurs and has the potential to contain the invading virus in the primary infected cells. It will be of interest to determine if other viral cell-to-cell movement proteins, in particular those incapable of plasmodesmata gating, have a p25-like activity. In addition, identification of a suppressor from a virus (PVX) previously thought not to encode this activity, further supports the idea that active suppression represents a general viral counter-defensive strategy in response to the RNA silencing antiviral defense in plants. As the RNA silencing pathway is conserved in a wide range of eukaryotic organisms and as viruses of plant and animal hosts encode homologous proteins, it seems feasible to look for animal viral suppressors using the

![Figure 1](image_url)

**Figure 1**

Targeting distinct stages of RNA silencing pathways by viral suppressors (see text for details). RdRP, RNA-dependent RNA polymerase; RISC, RNA-induced silencing complex. (The figure was adapted from [13] and [24] with permission.)

[24**], which may also explain the transient nature of VIGS mediated by PVX [49]. The identification of p25-like suppressors will provide valuable tools for dissecting the various steps involved in the systemic signaling of RNA silencing, which may include signal production, signal transport and signal-mediated induction of RNA silencing. Interestingly, although p25 arrested systemic RNA silencing by either inducer, it inhibited localized RNA silencing induced by the 35S–GFP transgene but not by the replicating virus [49]. The replication of PVX RNA genome in N. benthamiana plants triggers two independent branches of RNA silencing [13**,24**]: one branch is p25-insensitive and the other is similar to the transgene-induced RNA silencing that leads to production of the systemic silencing signal and is sensitive to p25 [24**]. As both local and systemic transgene RNA silencing are inhibited by p25, it seems likely that p25 targets a step either at or upstream to the signal production [3,24**].

Notably, neither HC-Pro nor Cmv2b interfere with transcriptional gene silencing (TGS), further supporting the notion that PTGS and TGS might operate through distinct pathways [50*]. It will be of interest to determine if these suppressors are able to block a novel type of TGS that, similarly to PTGS, is triggered by double-stranded RNA and associated with the accumulation of 25 nt RNAs in silenced plants [51*].

### Interaction of viral suppressors with other defense pathways

The suppression of RNA silencing by plant viruses represents a viral adaptation to a novel host antiviral defense mechanism and many of the viral suppressors identified were previously shown to be required for virulence determination [52–57]. It is interesting in this regard that Tav2b, but not Cmv2b, is recognized in N. tabacum as a target of another distinct host defense mechanism akin to the gene-for-gene disease resistance, although both are silencing suppressors (or virulence factors) in a related host species, N. benthamiana [45]. This finding not only reveals a further complexity in the molecular strategies employed by pathogens and their hosts for defense and counter-defense, but also indicates the effectiveness in targeting the viral suppressors of RNA silencing for the control of viral diseases.
silencing suppression assays established in plants. The first candidates for such a role will be those animal viral proteins that share an evolutionary origin with the known plant viral suppressors [52].

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References and recommended reading
Papers of particular interest, published within the annual period of review, critical reading of the manuscript.


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