INDUCTION AND SUPPRESSION OF RNA SILENCING: INSIGHTS FROM VIRAL INFECTIONS

Olivier Voinnet

Abstract | In eukaryotes, small RNA molecules engage in sequence-specific interactions to inhibit gene expression by RNA silencing. This process fulfils fundamental regulatory roles, as well as antiviral functions, through the activities of microRNAs and small interfering RNAs. As a counter-defence mechanism, viruses have evolved various anti-silencing strategies that are being progressively unravelled. These studies have not only highlighted our basic understanding of host–parasite interactions, but also provide key insights into the diversity, regulation and evolution of RNA-silencing pathways.

Post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in animals are manifestations of an evolutionarily conserved process known as 'RNA silencing'. Although RNA silencing operates through diverse pathways, it invariably relies on a set of core reactions that are triggered by dsRNA, which is processed into RNA duplexes that are 21 bp–24 bp in length by the RNaseIII enzyme Dicer and its homologues[1]. These reactions were first characterized in studies of experimental RNAi[2], now widely used as a powerful technology for gene knockdown[3,4]. In RNAi, a long and perfectly complementary dsRNA is cleaved by Dicer into small interfering RNAs (siRNAs)[5–7]. On ATP-dependent unwinding[8], one siRNA strand is incorporated into the multi-subunit RNA-induced silencing complex (RISC) and guides the complex to degrade cellular RNA molecules that are identical in sequence to the siRNA[9,10].

The first indication of a biological role for RNAi was provided when virologists attempted to overexpress certain plant genes from recombinant viral vectors[11]. Unexpectedly, degradation rather than overexpression of the engineered mRNA caused symptoms that phenocopied those of knockout mutations in the corresponding gene, a phenomenon that is termed virus-induced gene silencing (VIGS)[12]. The *trans*-acting nature and sequence-specificity of the process, together with the fact that dsRNA is a common product of virus replication, prompted the idea that VIGS recapitulates a plant antiviral-defence response that is mechanistically related to RNA silencing[13]. Viral-derived small RNAs (vsRNAs) that are similar to siRNAs were indeed subsequently found in virus-infected plants[5,14]. Presumably, they are incorporated into a RISC complex that retrieves and destroys viral RNAs. If the viral genome has sequence homology to a plant mRNA, both viral and plant transcripts are affected[11,12].

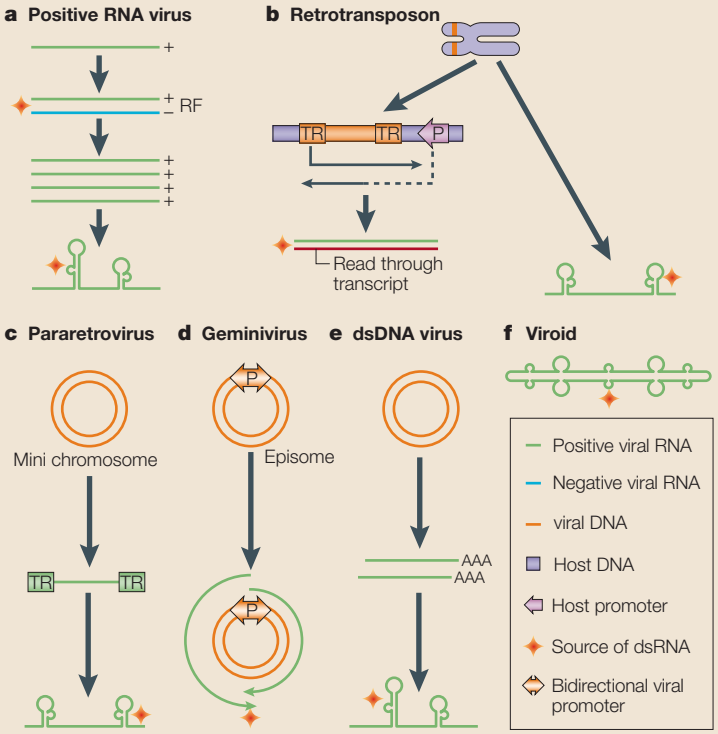
A surprising outcome of VIGS was that viruses that were engineered with promoter rather than transcribed sequences triggered transcriptional gene silencing (TGS) through sequence-specific alterations of DNA and chromatin[15,16]. The full significance of these observations was later appreciated when it was found that nuclear small RNAs (sRNAs) repress mobilization of transposable elements by promoting epigenetic modifications of the corresponding DNA[17–19], a second defensive role of RNA silencing in several organisms.

In parallel to this work on viruses, research on RNAi led to the discovery that nearly all eukaryotes express non-coding RNAs that are similar in size to siRNAs (reviewed in REF. 20). These microRNAs (miRNAs) derive from longer, single-stranded and imperfect RNA hairpins that are transcribed from non-coding nuclear genes and are processed by Dicer or its homologues.

Institut de Biologie Moléculaire des Plantes du CNRS, 12 Rue du Général Zimmer, 67084 Strasbourg Cedex, France.
e-mail: olivier.voinnet@ibmp-ulp.u-strasbg.fr
doi:10.1038/nrg1555
Published online
10 February 2005

Box 1 | **Possible primary sources of RNA silencing as elicited by viral and sub-viral pathogens**

RNA silencing is ubiquitously triggered by dsRNA. The sources of this molecule,which is produced during the replication cycles of the different classes of virus and sub-viral pathogens discussed in this review, are described here. In the figure, sources of dsRNA are indicated by the yellow symbols. For simplicity, full replication cycles are not shown in each case; see Supplementary information S1 (box) for full details.



Viruses with RNA genomes
Most plant viruses, and some animal viruses, have genomes of positive, ssRNA that are replicated within the cytoplasm of the host (**a**). Viral RNA-dependent RNA polymerases (vRdRPs) synthesize complementary negative-stranded genomic RNA, from which numerous copies of positive-stranded RNA are reproduced. Partial or complete annealing of positive and negative RNA strands constitutes the replicative form (RF), which provides one source of dsRNA. A second source is provided by the folding of replicated, single-stranded genomic RNA, which forms secondary double-stranded structures.

Viruses with genomes of negative ssRNA (not shown) follow a similar strategy, but their genomic RNA must be first copied into a complementary, plus-stranded mRNA before proteins can be synthesized. Such viruses are widespread in animals (for example, the influenza virus), but are less common in plants.

Retrotransposons
Retrotransposons (**b**) resemble animal retroviruses (not shown here) in genome organization and replication, but are not infectious. They consist of an RNA genome, which is reverse-transcribed to produce dsDNA that integrates into host DNA through a process that requires their terminal repeats (TR; in the figure, the viral DNA that is shown is already integrated into the host genome). Integration can occur in the vicinity of host genes, potentially resulting in dsRNA synthesis owing to read-through transcription. In addition, the terminal repeats form stem-loop structures at each end of the viral genome, which provide an extra source of dsRNA.

Viruses with DNA genomes
Pararetroviruses (**c**) are the only known dsDNA viruses of plants, and their genomes are circular and EPISOMAL. When they are transcribed, the terminal repeats at the ends of the viral mRNAs form secondary double-stranded structures that could function as triggers for RNA silencing.

Plant geminiviruses (**d**) are ssDNA viruses with genomes that are replicated in the nucleus through a rolling-circle mechanism that generates dsDNA intermediates, which are the templates for both replication and transcription. Transcription is bidirectional, and the presence of complementary RNA strands provides a source of dsRNA.

Adenovirus and Epstein–Barr virus (EBV) (**e**) are dsDNA viruses with linear genomes, which are replicated in the nucleus by the host DNA replication machinery or viral-encoded enzymes (not shown in the figure). They are maintained as episomes, from which viral gene expression occurs. Intramolecular interactions that occur within viral transcripts form double-stranded regions that, in the case of EBV, are known to be recognized and processed by the RNA-silencing machinery.

Viroids
Viroids (**f**) are subviral pathogens that consist of single-stranded, covalently closed RNA molecules, which usually adopt a quasi-rod-like secondary structure with extensive double-stranded regions.

Plant and animal miRNAs modulate the expression of mRNAs that orchestrate cell differentiation, development and probably many other cellular functions in a sequence-specific manner[20]. Most animal miRNAs are thought to inhibit the translation of their target mRNAs through imperfect complementarity to the 3′ untranslated region[21], although the mechanism that is involved is unknown. By contrast, many plant miRNAs have near perfect complementarity to the coding region of their targets[22] — most of which encode transcription

EPISOMES
Genetic elements that can replicate independently in eukaryotic nuclei.

factors — which they cleave on incorporation into a RISC complex[23]. So, in addition to its defensive role, RNA silencing also functions in the regulation of endogenous gene expression.

Interests in the regulatory and defensive roles of RNA-silencing are now converging through the study of antiviral RNA silencing, as it is becoming clear that viruses not only use elaborate strategies to suppress the effects of defensive RNA silencing, but also redirect or interfere with cellular functions that are orchestrated by endogenous small RNAs. These new aspects of host–virus interactions have strong evolutionary implications, both in terms of the continuing molecular arms race between hosts and pathogens, and in terms of the extent to which defence and gene regulation by RNA silencing might have influenced each other. This review highlights these concepts, presenting the latest advances in understanding how viruses and related parasitic genetic elements induce RNA silencing, how they suppress or evade this process and what the consequences of this are for the host. Finally, I also discuss whether, beyond the therapeutic applications that have been proposed for RNA silencing in vertebrates, this process also has antiviral roles in those organisms.

RNA-silencing pathways: a viral perspective

The principal subdivision of viruses is based on the nucleic-acid content of their genomes. Below, I describe the pathways that are involved in RNA silencing through replication of both DNA and RNA viruses (BOX 1), and highlight how understanding these mechanisms has provided insights into the functions and evolution of RNA silencing.

RNA-virus-induced gene silencing (RVIGS). Replication of RNA viruses produces double-stranded hybrids of positive (plus-stranded) and negative (minus-stranded) copies of genomic RNA, called replicative forms (BOX 1). Although it is widely believed that processing of replicative forms by Dicer-like enzymes (DCLs) forms the core of RVIGS in plants, experimental evidence points to a more complex situation. For instance, in tombusvirus-infected plants, vsRNAs are not distributed homogeneously along the viral genome, as a prevalent contribution of replicative forms would predict. Instead, they map preferentially to short, imperfect hairpins that result from interactions within the plus-stranded genomic RNA[14] (BOX 1). An important contribution of such intra-molecular base-pairing is directly supported by the fact that inverted repeats, as opposed to linear RNA fragments, induce more effective RVIGS when they are introduced into recombinant viral RNA vectors[24]. The vast majority of vsRNAs that accumulate in response to VIROIDS also derive from extensive intramolecular pairing of their circular RNA genome[25–27] (BOX 1).

This evidence indicates that RVIGS is probably triggered by the processing of both perfectly double-stranded RNA, such as replicative forms (which resemble the experimental triggers of RNAi), and single-stranded RNA hairpins which, at least in the case of tombusviruses, seem to be structurally related to plant miRNA precursors[14,22]. This raises the possibility that the functional diversification of DCLs that is seen in plants arose primarily as an adaptation to optimal recognition and processing of perfect and imperfect double-stranded forms of pathogenic RNAs. Specific sub-cellular localization of DCLs and further specialization might have evolved subsequently to fulfil regulatory roles in addition to defensive functions. This is highlighted by the four DCLs that function in *Arabidopsis thaliana*: DCL1 accumulates mainly in the nucleus, where it ensures the stepwise processing of miRNAs from their imperfect stem-loop precursors[27–29], whereas the nuclear DCL3 synthesizes specific small RNAs that guide epigenetic modifications of transposons and endogenous loci, which results in TGS (REF. 30) (FIG. 1). Although DCL2 is known to have a nucleo-cytoplasmic distribution[30], localization of DCL4 awaits characterization. Either of these two DCLs could account for the as yet unidentified activity that produces the siRNAs that direct experimental RNAi in plants.

Individual mutations that affect the nuclear DCL1 and DCL3 enzymes in *A. thaliana* do not compromise vsRNA accumulation from RNA viruses[30], which replicate exclusively in the cytoplasm — the compartment where viroid sRNAs also accumulate[27]. By contrast, plants that lack DCL2 function show increased susceptibility to turnip crinkle virus (TCV). However, this hyper-susceptibility correlates with a delay and not an elimination of vsRNA accumulation; it also seems to be TCV-specific, because replication of at least two other RNA viruses is unchanged in *dcl2* mutants[30]. So, no individual DCL has been compellingly linked to plant RVIGS so far, and this could have at least two explanations. First, DCLs — possibly including the uncharacterized DCL4 — might have partially overlapping functions in VIGS. Second, combinatorial interactions between distinct DCLs, perhaps mediated by the PIWI protein–protein interaction domain conserved among these factors[31], might in fact be required for optimal RVIGS (FIG. 1). For example, interaction between DCL2 and DCL1 could promote cytoplasmic re-localization of DCL1 and allow processing of viral hairpins that resemble miRNA precursors. DCL2, on the other hand, could process the perfectly double-stranded replicative forms. Implicit to this hypothesis is the prediction that sub-cellular redistribution of some DCLs might take place during virus infections, although this remains to be tested.

The accumulation of viral-derived siRNAs in silkmoths that are infected with the Sindbis RNA virus provides direct evidence that RVIGS also occurs in insects[32], where functional Dicer diversification is reminiscent of the situation in plants. So, *Drosophila melanogaster* Dicer 1 (DCR1) is required for miRNA biogenesis, but dispensable for siRNA-directed RNAi[33]. DCR1 function requires Argonaute 1 (AGO1)[34], a member of the PIWI-Argonaute-Zwille (PAZ) class of proteins that are broadly implicated in plant and animal RNA-silencing. Conversely, DCR2 and AGO2 are required for siRNA synthesis and RISC loading, respectively, but are dispensable for miRNA-guided functions[33,34]. Decreased

VIROID
An autonomously replicating subviral pathogen of plants with a circular, rod-like RNA genome that contains no ORF. Viroids account for some of the most devastating diseases of plants.

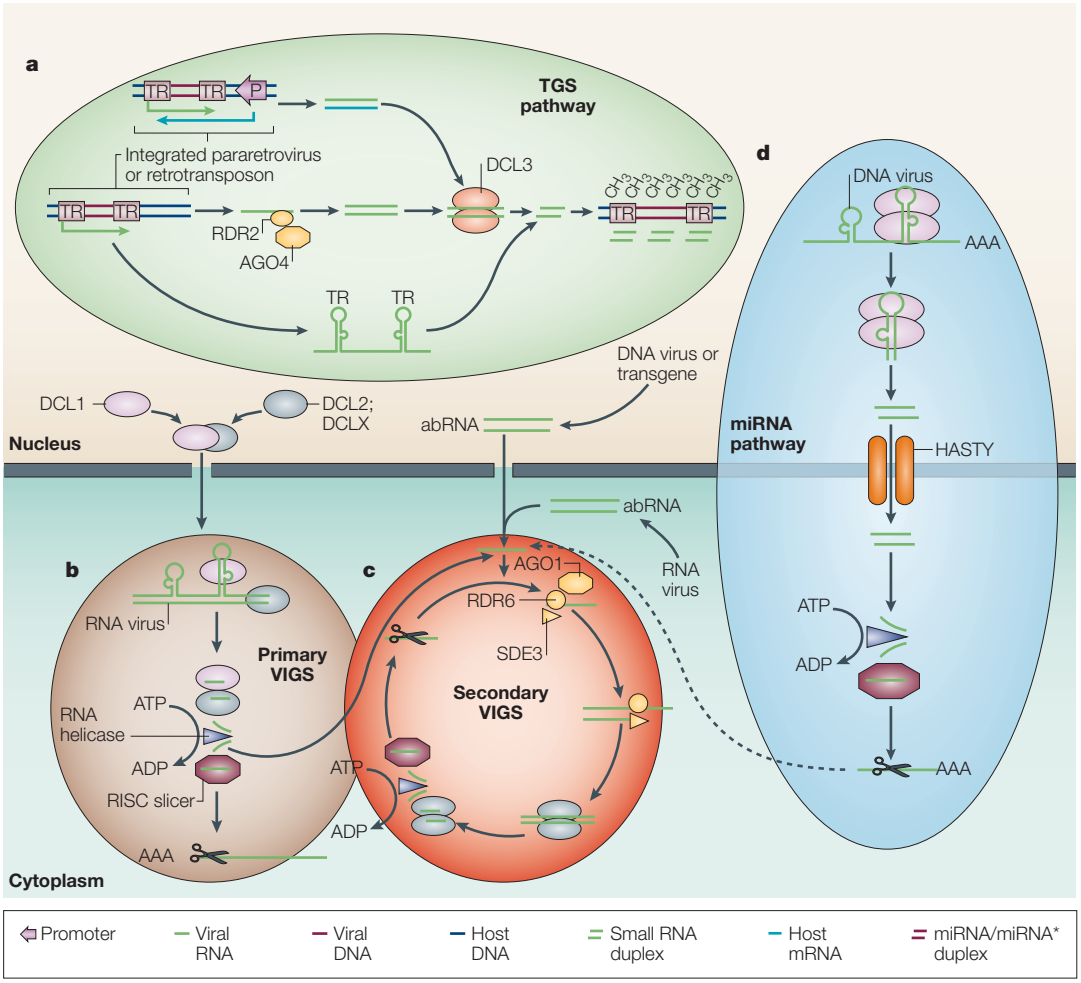


Figure 1 | **Antiviral RNA-silencing pathways in plants.** An integrated scheme showing pathways that have been either experimentally demonstrated in plants (solid arrows) — inferred from work on other organisms — or purely speculative (dotted arrows). **a** | In the nucleus, viruses and sub-viral pathogens that are integrated in the host genome can be subject to transcriptional gene silencing (TGS). In the situation in the upper part, read-through transcription leads to the production of dsRNA that is complementary to viral sequences, whereas in the situation shown in the middle, dsRNA is produced *de novo* through the activity of the argonaute protein AGO4 and the RNA-dependent RNA polymerase RDR2. Finally, the situation in the lower part shows dsRNA that is produced by intramolecular pairing of an RNA that contains terminal repeat (TR) sequences. In all cases, the dsRNA is recognized by DCL3, which results in the production of viral siRNAs. These then interact with the corresponding regions of the viral DNA within the host genome, directing epigenetic modifications (shown as methylation (CH_3)) to this region, which results in the silencing of gene expression. **b** | In the cytoplasm, silencing is initiated through the process of virus-induced gene silencing (VIGS). DCL2 is shown here as potentially interacting with DCL1 to promote its nuclear export and to facilitate processing of imperfect stem-loops that are found in RNA virus and viroid genomes, although this has not yet been tested. The resulting viral small interfering RNAs are unwound by an ATP-dependent RNA helicase and then incorporated into the RNA-induced silencing complex (RISC). The RISC complex is then directed to the corresponding viral mRNA, which is degraded. **c** | The primary signal can be amplified in the secondary VIGS pathway. Viral small RNAs produced in primary VIGS, or aberrant RNA (abRNA; for example, expressed from a transgene, or produced by a virus) are converted into dsRNA by the combined actions of the RNA-dependent RNA polymerase RDR6 (also known as SDE1), the AGO1 protein and SDE3, which might be an RNA helicase. In the same process that occurs in primary VIGS, these dsRNAs are then processed and lead to degradation of the corresponding viral or transgene mRNA. **d** | The miRNA pathway might also be involved in VIGS. There is evidence, from experiments in human cells, to suggest that viral dsRNAs can be processed in the nucleus by DCL1 and subsequently exported to the cytoplasm, where they then enter the antiviral RNA-silencing pathway. HASTY, the exportin 5 homologue of *Arabidopsis thaliana*.

AGO2 expression in *D. melanogaster* and mosquito cells results in hyper-susceptibility to the Flock House virus (FHV), and the Nodamura and O'nyong-nyong RNA viruses, which coincides with reduced vsRNA accumulation[35–37]. However, the contribution of the DCR1–AGO1 pathway in insect antiviral silencing remains unknown.

DNA-virus-induced gene silencing (DVIGS). DVIGS is best understood for geminiviruses, which do not go through a dsRNA phase. However, their DNA replicative forms are transcribed bi-directionally such that overlapping transcripts of opposite polarity might generate dsRNA through complementary base-pairing (BOX 1), as was shown for the C3 and coat protein (CP) transcripts of

the African cassava mosaic virus[38]. The DCL, or combination of DCLs, that is involved in the production of vsRNAs is unknown.

Because DNA viruses form nuclear episomes or integrate into host chromosomes, their genomes might be subject to the same epigenetic modifications that affect host genomes. So, RNA-silencing at the transcriptional level could in principle contribute to DVIGS. In *A. thaliana*, DVIGS from cabbage leaf curl geminivirus (CaLCuV) is not compromised in the defective DNA methylation 1 (*ddm1*) and maintenance of methylation 1 (*mom1*) mutants, which are both deficient in TGS of host-gene expression[39]. However, several additional pathways contribute to TGS in plants[40], and their involvement in DVIGS awaits evaluation. Notably, DCL3 cooperates with AGO4 to initiate a heterochromatic state for the *A. thaliana* AtSN1 retrotransposon (BOX 1), by recruiting AtSN1-derived sRNAs[17,18,30] (FIG. 1). This also requires the activity of RDR2, a putative RNA-dependent RNA polymerase (RdRP). This enzyme belongs to a class of proteins that are thought to initiate and amplify RNA-silencing by producing dsRNA from transposon-derived or virus-derived ssRNA (see below). By analogy, a similar vsRNA-directed pathway could be involved in the transcriptional inactivation of invading viral DNA. Potential targets of such a system include the recently identified endogenous pararetroviruses, which have become stably integrated into plant genomes[41] (BOX 1). As with retrotransposons, expression of their genomes is repressed by DNA methylation, but is often reactivated by stress[42]. Moreover, transgenes that are driven by enhancers of the tobacco endogenous pararetrovirus (TEPRV) become methylated in tobacco, but remain active in non-host species that are devoid of TEPRV sequences[43]. This indicates the involvement of a *trans*-acting, sequence-specific mechanism, which implies the contribution of vsRNA, although direct proof of this is still needed.

Recent findings in human lymphoma cells indicate that the miRNA pathway might also be involved in DVIGS. Epstein–Barr virus (EBV), a 172-kb dsDNA virus (BOX 1), produces nuclear-localized transcripts with partially dsRNA structures that are processed into smaller miRNA-like molecules[44]. This is the only example so far of small RNA production by a vertebrate virus, although it is likely that this might also be the case for other members of the Herpesviridae family and, perhaps, for other types of virus[45]. Both the processing reaction and the possible inhibitory effects of EBV miRNA on viral mRNA could contribute to limiting EBV infection. This might also apply to plant and insect DVIGS, and predicts, for example, that the miRNA-defective *dcl1* mutants of *A. thaliana*, although they have a normal response to RNA viruses, might show hyper-susceptibility to some DNA viruses (FIG. 1). However, this has yet to be tested.

Primary and secondary VIGS. The effects of the core RNA-silencing reactions might be further amplified by the action of host-encoded RdRPs (FIG. 1), which have been identified in plants, *Caenorhabditis elegans* and fungi, but not in insects or vertebrates[46–48]. Amplification occurs through at least two mechanisms, which are both relevant to antiviral defence. The first requires that primary small RNAs from a virus or transgene are recruited by an RdRP-containing complex to initiate the synthesis of new dsRNA. The occurrence of this small-RNA-directed process can be inferred from *in vitro* studies of the *Neurospora crassa* RdRP QDE1 (quelling defective 1) (REF. 48). It is also supported by experiments in plants, in which VIGS that is initiated by a vsRNA that corresponds to one part of a targeted mRNA leads to accumulation of siRNA that corresponds to the non-overlapping part[49]. In *A. thaliana,* this synthesis of secondary siRNAs requires the activities of RDR6 (an RdRP, also known as SGS2 or SDE1) and SDE3, a protein with RNA-helicase signatures[49,50].

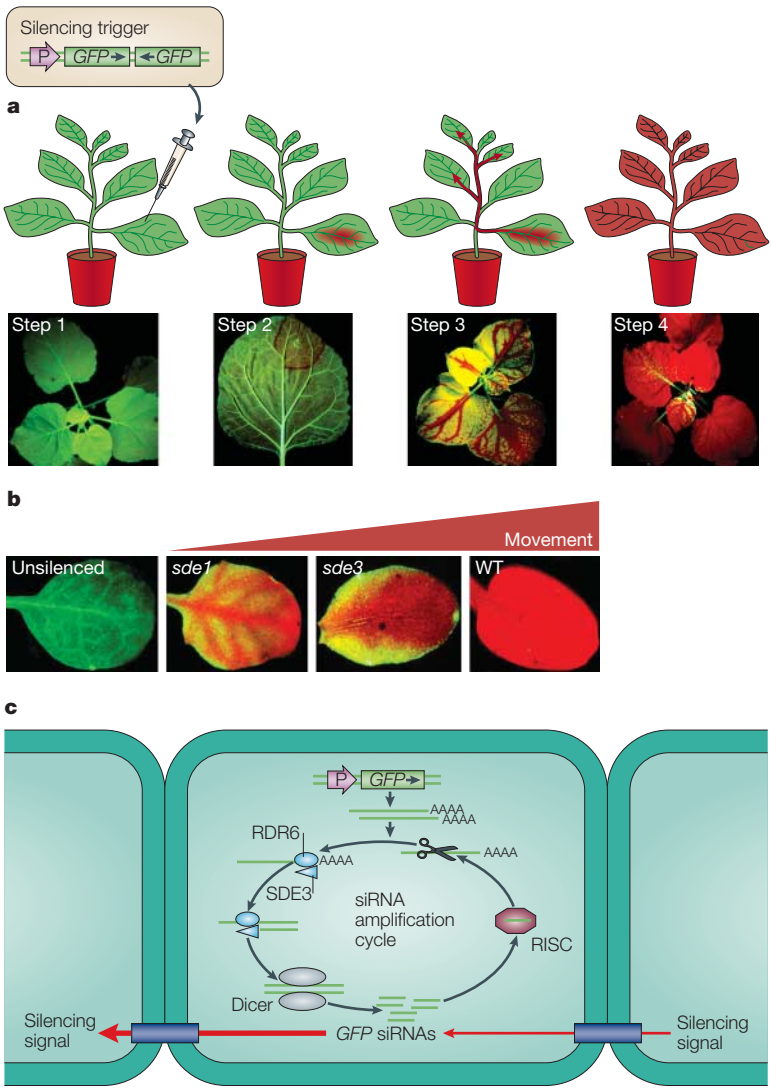
Together with AGO1 and the coiled-coil protein SGS3, RDR6 and SDE3 are also involved in a second mechanism, which, by contrast, does not require the presence of small RNA or dsRNA. This mechanism is thought to perceive aberrant RNAs (abRNAs) that are spuriously produced by sense transgenes[46], transposons or viruses[51] and to convert them *de novo* into dsRNA (FIG. 1). For example, the absence of a 5′ cap renders transgene mRNA susceptible to the activity of RDR6 (REF. 52), although other features, such as the lack of a polyA tail, might also differentiate normal RNA from abRNA. In principle, this second RdRP mechanism is not exclusive from the first one, as small RNA that results from abRNA processing could initiate further dsRNA synthesis from normal mRNAs. Similar reactions might take place that involve aberrant transcripts from cucumber mosaic virus (CMV, an RNA virus) and from CaLCuV, because *A. thaliana* mutants that lack individual components of the AGO1–RDR6–SGS3 pathway are hyper-susceptible to the former and show reduced DVIGS in response to the latter[39,53,54]. However, accumulation of other viruses remains unaffected by these mutations, indicating that there is some specialization in components of the RdRP pathways, similar to the DCLs. Consistent with this idea, knockdown of RDR1, a third *A. thaliana* RdRP that is distinct from RDR6 and RDR2, increases plant susceptibility to tobamo-, tobra- and potex-viruses, but not to CMV (REFS 55,56).

The involvement of RdRPs means that both RVIGS and DVIGS could be separated into primary and secondary reactions in plants. In this model, vsRNAs that are produced by viral replication would induce primary VIGS. This would in turn trigger host- rather than virus-directed secondary reactions, which might have evolved from a cellular mechanism whereby RdRPs normally prevent accumulation of aberrant endogenous transcripts. Some viruses could be more affected by primary VIGS because secondary structures in their genomes might be highly accessible to DCLs. Others might evade the primary response but remain susceptible to the host mechanisms that detect abRNA production, and therefore to secondary VIGS. In most cases, a full understanding of VIGS would require the ability to experimentally compromise both the primary and secondary reactions. Together with the possible redundancy and combinatorial interactions of plant DCLs,

Box 2 | **Silencing on the move**

Steps in systemic silencing

A spectacular aspect of RNA silencing in plants and *Caenorhabditis elegans* is its systemic nature. The sequence-specificity of this effect indicates that the silencing signal has a nucleic-acid component, probably RNA. Part **a** in the figure shows an experiment in which systemic RNA silencing was investigated in tobacco plants that express a *GFP* transgene. Plants were injected with a construct containing an inverted repeat of the *GFP* coding sequence, transcription of which results in a dsRNA that functions as a trigger for the silencing of the *GFP* transgene. The red and yellow colouring in steps 2–4 indicate loss of *GFP* expression (yellow indicates partial loss of expression and red indicates complete inhibition), as visualized under UV illumination.



Systemic silencing proceeds in the following way: first, there is local induction, triggered by the transiently expressed inverted-repeat construct (step 2). This is followed by long-distance movement of the signal within the vasculature (step 3), unloading of the signal into the leaves and amplification and cell-to-cell movement through plasmodesmata (step 4). This process is thought to provide a systemic antiviral response that immunizes tissues that are as yet uninfected. Signal amplification might be important because, having detected only a few pathogenic RNAs, the plant can mount a large systemic response against the invader.

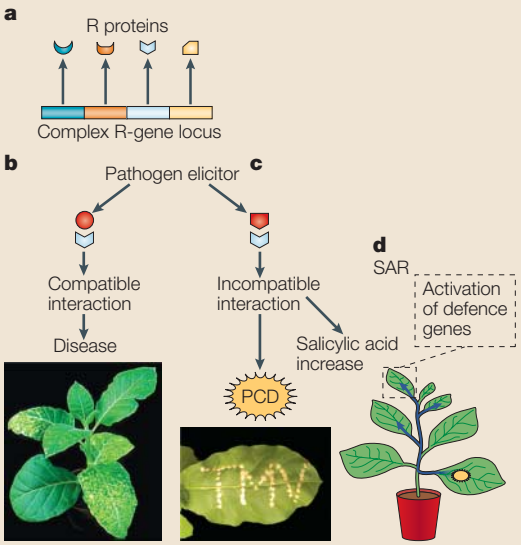
Molecular basis of systemic silencing

Part **b** in the figure shows an experiment in which silencing was specifically triggered within the vasculature of *Arabidopsis thaliana* plants that express transgenic *GFP*. In *sde1* mutants, in which the RNA-dependent RNA polymerase RDR6 is inactive, the movement of the silencing signal outside the veins is restricted to the 10–15 cells that are closest to the vasculature. This movement is more extensive in plants that are deficient for SDE3, and affects the entire lamina in wild-type (WT) plants.

The extensive movement of the silencing signal probably proceeds through the reiteration of short-distance signalling events, as shown by the model in **c**. The activities of RDR6 and SDE3 promote the amplification of the signal by catalysing the production of dsRNA from transgene mRNA. This dsRNA is recognized and processed by Dicer to produce 21-nt siRNAs[50]. In addition to directing cleavage of transgenic mRNA after becoming incorporated into the RISC complex, these siRNAs are thought to be able to spread from cell to cell through the plasmodesmata, and can therefore trigger either silencing or further signal amplification in neighbouring cells.

mentioned previously, this probably explains why no individual RNA-silencing component has so far been broadly implicated in antiviral defence in plants. Therefore, there might be many entry points into VIGS pathways (FIG. 1). Moreover, the number of RdRPs and DCLs probably varies between plant species, providing tremendous flexibility in the initiation and execution of VIGS.

Box 3 | **RNA silencing compared with innate immunity**



Innate immunity in plants relies on the rapid evolution of membrane-bound or cytosolic disease resistance proteins (R proteins) (a), which perceive pathogen protein signatures known as 'elicitors'[115]. In compatible plant–pathogen interactions (b), the elicitor escapes recognition by an R protein, leading to development of disease. In incompatible interactions (c), the elicitor is recognized by an R protein, triggering a cascade of defence reactions that culminate in a form of programmed cell death (PCD), the 'hypersensitive response' (in the plant shown, this was elicited by successive challenges with tobacco mosaic virus (TMV), and the areas of dead cells show points of infection with TMV). Coincident with the hypersensitive response is an increase in salicylic acid that triggers 'systemic acquired resistance' (SAR), whereby long-distance movement of an unidentified signal induces broadly effective antimicrobial activities in remote plant tissues[116] (d).

Although seemingly dissimilar, defence systems that are based on R proteins and on RNA silencing might be partly connected. Several viral silencing suppressors elicit a hypersensitive response (REFS 117,118) and salicylic acid induces expression of an RNA-dependent RNA polymerase that targets RNAs of several types of virus[55,56]. Furthermore, the cucumoviral 2b protein (TABLE 1) suppresses both the RNA-silencing and the salicylic acid pathways[119], indicating that salicylic acid might induce virus resistance by potentiating RNA-silencing-based antiviral defence. Perhaps even more striking is the finding that tobacco plants that express the potyviral helper component proteinase (HcPro) show enhanced resistance to a broad range of pathogens[120]. A proposed explanation is that HcPro suppresses the effects of endogenous small RNAs (for example, miRNAs) that are targeted against negative regulators of R-protein-mediated defence[120]. However, HcPro could also release a silencing-based mechanism that directly restricts constitutive R-gene expression, which is usually cytotoxic. Indeed, the complex genomic organization of R-gene loci is prone to negative epigenetic control through mechanisms that might well involve small RNAs[121].

A genetic immune system. It was anticipated soon after their discovery that RdRP systems might be key components of antiviral silencing mechanisms that are required to keep pace with high viral replication rates[57]: a few viral abRNAs or primary vsRNAs could be converted into many dsRNA molecules to reinforce the silencing response. This idea became even more attractive when it was realized that, in plants, RNA silencing in response to transgenes and viruses moves between cells and over long distances through a relay-amplification process[58,59] that is dependent on the activity of RDR6 and SDE3 (REF. 50). This non-cell-autonomous silencing probably forms the systemic arm of VIGS (BOX 2), because several viruses elicit the specific degradation of viral RNAs in tissues ahead of the infection front[50,60,61]. Furthermore, vsRNAs from the cucumber yellows virus accumulate in pumpkin phloem sap, suggesting the long-distance movement of these molecules[62].

Because it is highly adaptive, specific and systemic, RNA-silencing can therefore be seen as a form of immune system that operates at the nucleic-acid level. However, unlike immune systems that are mediated by proteins, the specificity of the RNA-silencing immune system is not programmed by the host. Instead, it is determined by features of and sequences within the pathogen's genome. It is noteworthy that RNA silencing integrates into a broader scheme of defence pathways that will also have to be considered in future studies of its defensive roles. It now seems that there might be overlap between nucleic-acid-mediated and protein-mediated resistance in several organisms. For instance, SALICYLIC ACID — an important component of the plant innate immune response to a broad range of pathogens (BOX 3) — is also implicated in antiviral defence through the induction of RDR1 expression in *A. thaliana* and tobacco[55,56].

SALICYLIC ACID
A compound that is involved in plant defence against insects and pathogens; it has intrinsic anti-microbial properties.

Viral suppression of RNA-silencing

Viruses have evolved a wide range of mechanisms to overcome RNA-silencing, providing yet another example of the continuing evolutionary arms race between hosts and parasites. Elucidating the mechanisms of suppression and evasion of silencing is also informative at a wider level, as important insights into the regulation of gene silencing and its roles in host gene expression has been gained through those studies.

Ubiquity and diversity of silencing suppressors. Initial work showed that the potyvirus-encoded helper component proteinase (HcPro) enhances the replication of many unrelated viruses[63,64]. One interpretation of this result is that HcPro inhibits a defence mechanism that is effective against a wide range of viruses. The finding that RNA-silencing is such a mechanism therefore prompted the idea that HcPro could suppress silencing, which was subsequently confirmed in tobacco[65–67]. Following the same rationale, the CMV-encoded 2b protein was also found to suppress RNA silencing[67]. On the basis of the widespread antiviral function of

Table 1 | **RNA-silencing suppressors encoded by plant, insect and vertebrate viruses**

Viral family	Virus	Suppressors	Other functions	References
Positive-strand RNA viruses in plants				
Carmovirus	Turnip Crinkle virus	P38	Coat protein	122
Cucumovirus	Cucumber mosaic virus; tomato aspermy virus	2b	Host-specific movement	67
Closterovirus	Beet yellows virus Citrus tristeza virus	P21 P20 P23 CP	Replication enhancer Replication enhancer Nucleic-acid binding Coat protein	123 124
Comovirus	Cowpea mosaic virus	S protein	Small coat protein	125
Hordeivirus	Barley yellow mosaic virus	γb	Replication enhancer; movement; seed transmission; pathogenicity determinant	126
Pecluvirus	Peanut clump virus	P15	Movement	127
Polerovirus	Beet western yellows virus; cucurbit aphid-borne yellows virus	P0	Pathogenicity determinant	103
Potexvirus	Potato virus X	P25	Movement	60
Potyvirus	Potato virus Y; tobacco etch virus; turnip yellow virus	HcPro	Movement; polyprotein processing; aphid transmission; pathogenicity determinant	65–67
Sobemovirus	Rice yellow mottle virus	P1	Movement; pathogenicity determinant	68
Tombusvirus	Tomato bushy stunt virus; cymbidium ringspot virus; carnation Italian ringspot virus	P19	Movement; pathogenicity determinant	68
Tobamovirus	Tobacco mosaic virus; tomato mosaic virus	P30	Replication	128
Tymovirus	Turnip yellow mosaic virus	P69	Movement; pathogenicity determinant	70
Negative-strand RNA viruses in plants				
Tospovirus	Tomato spotted wilt virus	NSs	Pathogenicity determinant	129
Tenuivirus	Rice hoja blanca virus	NS3	Unknown	
DNA viruses in plants				
Geminivirus	African cassava mosaic virus Tomato yellow leaf curl virus Mungbean yellow mosaic virus	AC2 C2 C2	Transcriptional activator protein (TrAP)	68,81
Positive-strand RNA viruses in animals				
Nodavirus	Flock house virus; nodamura virus	B2	Plaque formation	35
Negative-strand RNA viruses in animals				
Orthomyxovirus	Influenza virus A*	NS1	Poly(A) binding; inhibitor of mRNA export; PKR inhibitor	36,107,108
DNA viruses in animals				
Adenovirus	Adenovirus	VA1 RNA	PKR inhibitor	87
Poxvirus	Vaccinia virus*	E3L	PKR inhibitor	36

*These have only been demonstrated in heterologous systems (insects and plants). HcPro, helper component proteinase; PKR, an RNA-dependent protein kinase.

RNA-silencing, it was anticipated that the use of suppressors would be a shared strategy of many plant viruses. The realization that HcPro and 2b are PATHOGENICITY DETERMINANTS provided a rationale for identifying novel viral-encoded suppressors; the re-investigation of other factors of this type revealed that several effectively inhibit RNA-mediated silencing[68].

Many unrelated viral proteins have evolved silencing-suppressor activities in addition to their other functions, contributing to the remarkable diversity of these factors, which have now been identified for almost all types of plant virus (TABLE 1). Moreover, a single type of virus might encode several distinct suppressors, as was recently found with the citrus tristeza virus[124] (CTV, TABLE 1). Silencing suppression has also been documented in insect cells, and was discovered through the related expression strategies and functional similarities of the CMV suppressor 2b and the B2 protein of the insect virus FHV (TABLE 1). Deletion of the B2 ORF from FHV results in a drastic loss of virus accumulation in *D. melanogaster* S2 cells, which can be rescued by decreasing the cellular content of AGO2. Therefore, B2 suppresses the effect of the AGO2-dependent silencing response that normally restricts FHV accumulation[35].

PATHOGENICITY DETERMINANT
A factor that is not strictly required for virus replication, but is nevertheless needed for its efficient accumulation at the cellular or systemic level.

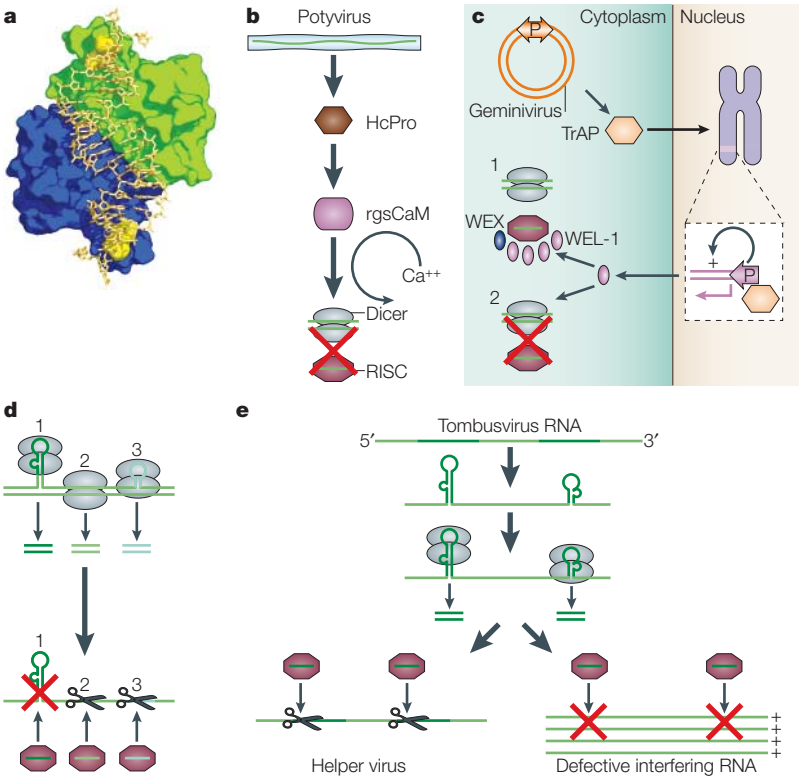


Figure 2 | **Viral strategies for suppression and evasion of RNA-silencing. a** | Direct interference with silencing-effector molecules is illustrated by the tombusviral P19 protein. The head-to-tail organization of P19 homodimers (blue and green) allows binding to small interfering RNA (siRNA) duplexes (yellow). Two sets of tryptophan residues (yellow) bind to the last set of base pairs on either end of the siRNA, leading to effective measurement of the duplex length, such that P19 selects siRNAs of 21nt for binding. The sequestered siRNA is prevented from entering the RNA induced silencing complex (RISC) and is therefore inactivated. **b** | Recruitment of endogenous negative regulators of RNA silencing is illustrated by the potyviral helper component proteinase (HcPro). HcPro interacts with the calmodulin-like protein rgsCaM (regulator of gene silencing CaM) to inactivate the RNA-silencing pathway through an unknown mechanism at an intermediate step that involves both RISC and Dicer. **c** | Geminiviral transactivator proteins (TrAPs) suppress RNA silencing by altering the host transcriptome so that proteins such as WERNER EXONUCLEASE-LIKE 1 (WEL1) that are homologues of components of the silencing pathway are produced at excessive levels. This can lead to dominant-negative effects by competing with positive effectors of silencing — in this case, WERNER SYNDROME-LIKE EXONUCLEASE (WEX) — for interaction with the core silencing machinery (case 1). The TrAP-induced factors might also directly inhibit the silencing machinery (case 2). **d** | Out-competition of RISC by unproductive viral-derived small RNA (vsRNA) could be a common feature of plant and insect virus infections. Stem-loop regions of the genome that are accessible to Dicer-like enzymes (DCLs) but inaccessible to RISC might generate unproductive vsRNA (case 1). If such regions are favoured as DCL substrates, as with tombusviruses, the resulting vsRNA could then outcompete productive vsRNA (2 and 3) for loading into RISC. **e** | Evasion of silencing by loss of silencing target sequences is illustrated by the generation of defective interfering RNA molecules from tombusviruses. Defective interfering RNA molecules result from skipping of the viral replicase at the junctions of stem-loop structures that are normally potent silencing inducers. Defective interfering RNA molecules are therefore devoid of silencing targets (bottom right) and have a strong selective advantage over the helper virus (bottom left). P, promoter.

Most of the original work on silencing suppression involved model systems that varied greatly in terms of the host, the virus or transgene used to induce silencing, the method used to deliver the suppressor protein and the timing and pattern of its expression. It was therefore difficult, if not impossible, to compare the outcomes of such studies. To resolve this issue, five unrelated silencing suppressors were recently expressed transgenically in the same *A. thaliana* ECOTYPE and their effects were analysed side-by-side[69]. RNAi of an endogenous gene, triggered by a corresponding inverted-repeat transgene, was used in these experiments to measure the impact of each suppressor on the siRNA pathway. All five proteins suppressed the inverted-repeat-induced silencing, indicating an effect on primary VIGS. However, they did this through distinct mechanisms. For example, the potyviral HcPro increased the stability of the dsRNA that triggered silencing, indicating the inhibition of DCL function. This inhibition was partial, however, as a significant amount of siRNA was still detected. By contrast, three of the other proteins that were tested strongly suppressed siRNA accumulation without enhancing dsRNA stability, indicating that they function downstream of DCLs. In the same study, the tombusviral P19 suppressor had no significant effect on either ds- or siRNAs, which is consistent with its ability to sequester small RNAs[69] (see below). By contrast, in a separate study, the P69 suppressor of turnip yellow mosaic virus (TYMV) did not affect inverted-repeat-induced silencing in *A. thaliana*, but efficiently suppressed silencing that was induced by sense transgenes, which, as mentioned previously, is dependent on AGO1–RDR6–SGS3 (REF. 70). Consequently, P69 might specifically inhibit secondary VIGS (FIG. 1). These analyses show that silencing suppressors target many distinct steps of the silencing pathway. These include non-cell-autonomous steps, as several suppressors specifically affect systemic as opposed to intracellular silencing[50,60,61]. Therefore, the functional diversity of these proteins mirrors their structural and sequence diversity.

Molecular basis of silencing suppression. The biochemical properties of several silencing suppressors have been described previously, and REFS 71,72 discuss this aspect in more detail. Here, I highlight the most significant advances in our understanding of the modes of action of these proteins, which also shows the range of strategies that can be visualized for silencing suppression.

An intuitive hypothesis for silencing suppression involves the inhibition of key components of RNA-silencing pathways, as highlighted by the function of the tombusviral P19 protein. *In vitro*, recombinant P19 specifically binds to chemically synthesized siRNA duplexes and shows an otherwise poor affinity for other nucleic acids, including long dsRNAs or single-stranded siRNAs[73]. siRNA binding also occurs *in vivo*, because the vast majority of tombusvirus-derived siRNAs co-fractionate with P19 in infected tissues[74] and transgenically expressed P19 co-immunoprecipitates with small RNAs in *A. thaliana*[69,75]. A decisive step in understanding the mode of action of P19 was the crystallization of P19 homodimers that are directly bound to siRNA duplexes[76,77], which highlights the extraordinary adaptation of viruses to the host silencing machinery (FIG. 2a). Because siRNAs are ubiquitously involved in RNA-silencing, these findings predicted that P19 would be effective in a broad range of organisms, which was verified by studies in human and *D. melanogaster* cells[36,69]. Presumably, physical sequestration of siRNAs

ECOTYPE
A population within a species that has developed distinct morphological or physiological characteristics as an adaptation to a specific environment, and which persists when individuals are moved to a different environment.

by P19 prevents their unwinding by an RNA helicase[8], a prerequisite for assembly of an active RISC complex. Although necessary, however, siRNA sequestration might not be sufficient for the effect of P19 *in vivo*, because we have isolated *A. thaliana* mutants in which P19-mediated silencing suppression is compromised. The P21 silencing suppressor of beet yellows virus (BYV; an RNA virus) also binds directly to siRNA duplexes, but whether this interaction is siRNA-specific, and whether P21 is structurally related to P19, remains undetermined[75].

A second silencing-suppression strategy involves the recruitment of endogenous negative regulators of RNA-silencing. For instance, screens for HcPro-interacting factors identified a calmodulin-related protein, rgsCaM (regulator of gene silencing CaM), the overexpression of which mimics silencing suppression by HcPro (REF. 78). rgsCaM could therefore function as an endogenous silencing suppressor through an as yet uncharacterized calcium-dependent pathway (FIG. 2b). Cellular inhibitors of RNA silencing have also been genetically identified in *C. elegans*. One of them, ERI-1 (Enhanced RNAi-1), defines a novel subfamily of evolutionary conserved DEDDh nucleases that process siRNAs into shorter, inactive forms[79]. It will therefore be interesting to evaluate the contribution of ERI-1 orthologues to silencing suppression by plant and animal viruses.

A third strategy relies on modifications of the host transcriptome, and is supported by studies of the geminivirus transcriptional-activator proteins (TrAPs), which have been identified as silencing suppressors. The nuclear localization and zinc- and DNA-binding activities of TrAPs are all required for their suppressor function, indicating that TrAPs function at the host-DNA level[80–82] (FIG. 2c). Indeed, genome-wide transcriptional profiling of *A. thaliana* PROTOPLASTS revealed that TrAPs from two geminiviruses induce a common set of ~30 host mRNAs, among which is the *WERNER EXONUCLEASE-LIKE 1* (*WEL1*) transcript[83]. Interestingly, the related proteins MUT-7 (mutator 7) and WERNER SYNDROME-LIKE EXONUCLEASE (WEX) are positive effectors of RNAi and transgene-induced RNA-silencing in *C. elegans* and *A. thaliana*, respectively[84,85]. So, the TrAPs-induced overaccumulation of WEL1 might result in dominant-negative effects by interfering with or competing for factors that are required for normal WEX function. Accordingly, transient overexpression of WEL1 suppresses transgene silencing in *Nicotiana benthamiana* leaves[83].

Silencing suppression could also be RNA- rather than protein-mediated and, paradoxically, this could involve vsRNAs. Indeed, some vsRNAs might not necessarily promote effective cleavage once loaded into the RISC complex if they are derived from portions of the pathogen's genome that are inaccessible to this complex. Considering the large amount of vsRNA in plant- and insect-infected cells[5,14], it is therefore conceivable that many of them are non-productive decoys for the RISC complex (FIG. 2d). Abundant vsRNAs might also outcompete endogenous small RNAs for the RISC, and therefore interfere with host biology — a possible cause of some of the symptoms of viral infection. Animal viruses provide good examples of RNA-based inhibitory functions that are targeted at defence systems. The human adenovirus non-coding RNA VA1 inhibits the activation of protein kinase R (PKR) by viral dsRNA[86], which normally leads to non-specific inhibition of translation (see online Supplementary Information S2 (figure) for details). Recent work indicates that VA1 is also a potent competitor of both exportin 5 and human dicer[87], which are involved, respectively, in the export of miRNA precursors from the nucleus and in miRNA maturation. An unanswered question is whether VA1 actually inhibits miRNA-mediated processes during the viral life cycle and whether this has any beneficial effect on virus replication, which could indicate a role for RNA-silencing in limiting adenovirus infection[87]. The production of VA1-like stem-loop RNAs could, in principle, contribute to the anti-silencing strategies of plant and insect viruses.

Silencing suppression and host gene expression

Besides of its antiviral role, RNA silencing has important functions in regulating host gene expression, and these might also be compromised by viral suppressors. For example, most soybean varieties have yellow seeds, but some produce seeds with dark, irregular streaks — an effect called 'mottling'. Mottling remained mysterious until it was found that the yellow seed coating is in fact a natural manifestation of RNA silencing targeted against chalcone synthase, which is involved in pigment synthesis, producing a dark seed colour[88]. Mottling results from the persistent infection of yellow seeds by two viruses of the potyviridae and cucumoviridae families, which, by suppressing silencing, cause the irregular pigmented pattern (FIG. 3a). This provides one of the clearest and simplest examples of the contribution of silencing suppression to viral symptoms and, therefore, to how viruses can affect host gene expression[89].

As antiviral and host gene-silencing pathways involve similar effector complexes and mediator molecules, it was expected that viral suppressors would interfere with processes that are orchestrated by endogenous miRNAs, many of which regulate the accumulation of transcription factors that control important developmental processes (FIG. 3b–c). Accordingly, *A. thaliana* plants that are either infected with potyvirus or that express HcPro transgenically show developmental abnormalities that resemble those of miRNA-deficient *dcl1* mutants[90]. HcPro inhibits the cleavage of miRNA targets but, curiously, it also non-specifically enhances the accumulation of miRNAs[69,90]. A possible explanation comes from the fact that the expression of DCL1 is itself negatively regulated by one of these miRNAs, miR162 (REF. 91). So, inhibition of miR162-directed cleavage by HcPro might cause enhanced miRNA processing by the stabilized DCL1 protein. Inhibition of miRNA-directed cleavage by HcPro indicates that this factor interferes with RISC function. Because HcPro also has a partial dsRNA-stabilizing effect, it is likely that it functions at the DCL–RISC interface (FIG 1), which is consistent with the evidence that these two silencing complexes interact *in vivo*[92].

PROTOPLAST
A plant cell from which the cell wall has been removed by mechanical or enzymatic means. Protoplasts can be prepared from primary tissues of most plant organs, as well as from cultured plant cells.

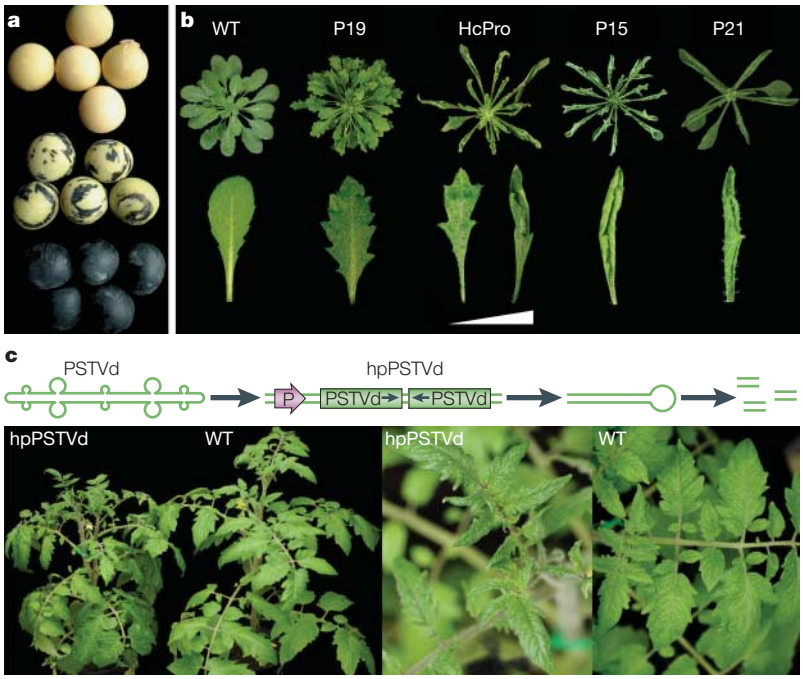


Figure 3 | **Viral symptoms and silencing suppression. a** | Mottling in soybean seeds (middle of panel) results from their persistent infection by potyviruses and cucumoviruses. Production of silencing suppressors by these viruses reverses silencing of chalcone synthase, an enzyme that is involved in pigment synthesis, which is naturally silenced in most soybean varieties, resulting in a pale seed colour (top of panel). The bottom of the panel shows seeds from an uninfected soybean variety in which chalcone synthase is not naturally silenced. **b** | Developmental symptoms in leaves of *Arabidopsis thaliana* that constitutively express the P19, helper component proteinase (HcPro), P15 or P21 silencing suppressors (TABLE 1). The similarity between those phenotypes is striking. Effects on leaves from a moderate (left) or strong (right) HcPro expresser indicates a continuum of developmental phenotypes that increase in severity as the penetrance of the effect of suppressors gets stronger. **c** | Transgenic expression of hairpins that are derived from the potato-spindle tuber viroid (PSTVd) genome leads to the development of leaf symptoms (curling and elongation) in tomatoes that phenocopy those of PSTVd-infected plants. hpPSTVd, hairpin potato spindle tuber viroid; P, promoter; WT, wild type.

Other viral suppressors cause anomalies that are strikingly similar to those elicited by HcPro (REFS 69,75), which are also attributable to the decreased cleavage of miRNA targets (FIG. 3b). These proteins are extremely diverse, and some, such as P19 and P15 (TABLE 1), are from viruses that do not naturally infect *A. thaliana*. So, these developmental symptoms cannot reflect a deliberate strategy of viruses to reprogramme host gene expression. Instead, they are probably an incidental consequence of the primary suppression of RVIGS at an intermediate step that is shared with the miRNA pathway, which probably involves a RISC complex (REFS 69,75). For example, the siRNA-binding properties of P19 and P21 explain their inhibitory effect on miRNA-directed cleavage because both proteins co-immunoprecipitate with the primary processing products of several miRNA precursors, which are known as miRNA/miRNA* duplexes[69,75]. Presumably, binding of these duplexes by P19 and P21 prevents their efficient use by the RISC complex, resulting in ectopic expression of the corresponding miRNA targets. Notably, two of the suppressors that have been investigated had little effect on miRNAs and did not cause visible symptoms, although they suppressed inverted-repeat-induced silencing[69]. This provides further evidence that siRNA and miRNA pathways are only partially overlapping in plants, and supports the idea that viral symptoms that are due to silencing suppression result from incidental cross-inhibition of the miRNA pathway. Note, however, that transgenically expressed suppressors do not necessarily reflect the way these proteins are synthesized or used during infection. Caution should therefore be exercised in making direct inferences about viral biology from these results.

Other viral responses to RNA silencing

Evasion of RNA silencing. There is evidence that some viruses might evade rather than suppress RNA silencing. For example, replication of Brome mosaic virus, which has an RNA genome, occurs in membrane-bound vesicles, keeping viral RNAs away from host ribonucleases[93], which might include silencing-related ribonucleases. Similarly, the chloroplastic replication of *Avsunviroidae* (a family of viroids) probably protects them from silencing[94], and transfected siRNAs are inefficient in targeting specific nuclear RNAs of the influenza virus A in human cells[95]. Silencing evasion can also result from loss of target sequences within viral genomes, owing to the high viral mutation rates. In lymphocytes, for example, the effects of anti-HIV siRNAs were progressively dampened by the emergence of viral quasi-species that harbour mutations within the siRNA target sequence[96,97]. In plants and animals, parasitic RNAs that are called defective-interfering RNAs are shortened forms of viral genomes that multiply at the expense of their progenitor helper viruses. Plant tombusviral defective-interfering RNAs arise through deletions that are caused by skipping of the replicase enzyme at the junctions of base-paired structures within the replicated RNAs of helper viruses[98] (FIG. 2e). As explained in a previous section, such hairpins contribute significantly to vsRNA production, and therefore defective interfering RNAs have strong selective advantages over helper viruses because they are largely devoid of target sequences[14].

Viral and sub-viral pathogens might also evade silencing because their genome is intrinsically resistant to the degradation machinery that is involved. For example, although the quasi-rod-shaped genomes of viroids are substrates for DCLs, viroid sequences are largely inaccessible to the RISC complex[99], presumably because their extensive intra-molecular folding cannot be resolved by this complex. Protection of viral genomes might also result from their association with proteins, as suspected for several cytoplasmic transcripts of the respiratory syncitial virus, which resist experimental RNAi in human cells[100]. Similarly, encapsidation might protect plant viral genomes from silencing[101]. Finally, viruses that replicate and spread at high rates might simply outcompete the capacity of the silencing machinery, at both the cellular and systemic levels.

RNA silencing to the benefit of viruses. Although it might seem counterintuitive, viruses might also exploit the host silencing response. As obligate parasites, they

must preserve the integrity of their hosts and, in this respect, silencing prevents the detrimental effects of their over-accumulation. An unusual RNA-silencing response, 'recovery', might represent an extreme representation of this idea. In recovery, plants that are initially highly symptomatic show a drastic reduction in viral levels in new growth, although the pathogen is never completely eliminated[102]. Recovery-inducing viruses often infect MERISTEMS (an unusual property among plant viruses), so this might represent an adaptation towards seed or pollen transmission: tolerating rather than suppressing RNA silencing would secure flowering and, ultimately, virus propagation.

In a previous section, processing of EBV-derived miRNAs was described from the viewpoint of host defence. However, searches for homology to human transcripts show that most EBV miRNAs could potentially target several classes of cellular mRNA, including those that encode regulators of cell proliferation and apoptosis, chemokines, transcription factors and signal-transduction components[44]. Therefore, EBV miRNAs might well reprogramme expression of specific host proteins to establish optimal infection conditions. This probably also applies to plant viroids, which do not encode any protein, but replicate, spread and induce symptoms in their hosts[94]. Many cellular changes that are elicited by viroids might actually result from viroid-induced silencing of host mRNAs, owing to homology between viroid small RNAs and plant genomes. Consistent with this idea, tomato plants that express viroid-derived inverted repeats (which produce viroid siRNAs) show symptoms that phenocopy those of viroid infections[99] (FIG. 3c). Considering the high probability of sequence matches between host and viral genomes, this ability of viruses to take advantage of silencing to directly modify host gene expression might, in fact, be widespread.

An antiviral role for silencing in vertebrates?

Cultured human cells can be immunized against incoming viruses by pre- or co-transfection with siRNAs[100,104]. More recently, systemic RNAi effectively cured adult mice of aggressive influenza strains[105,106]. Besides their significance for prophylaxis and therapy, these biotechnological breakthroughs raise a fundamental question: does RNA silencing naturally limit viral infections in vertebrates as it does in plants and insects?

An indirect approach to answering this question is the investigation of putative RNA-silencing suppressors that are encoded by vertebrate viruses. Recently, the influenza virus NS1 protein and the vaccinia virus E3L protein were found to have such inhibitory activity in plants and insect cells[36,107,108]. However, the interpretation of these results requires caution. First, both proteins are known to bind to dsRNA, a property that is directly related to their PKR-antagonizing function in mammalian cells. PKR, an RNA-dependent protein kinase, is activated by RNAs with double-stranded features such as those produced by viruses[86]. On auto-phosphorylation, PKR catalyses the intermolecular phosphorylation of the protein synthesis initiation factor eIF-2α, which shuts down translation of both host and viral mRNAs and triggers production of antiviral INTERFERONS (IFNs). Therefore, the effects of NS1 and E3L on RNA-silencing could result from a trivial competition for Dicer substrates or incidental binding of siRNA duplexes that result from their primary anti-PKR activities. Such non-specific effects have been documented for several dsRNA-binding proteins, including the RNaseIII enzyme of *Escherichia coli*, an organism in which RNA silencing does not operate at all[109].

A second caveat in these experiments is that they were carried out in non-vertebrate systems. Measuring the impact of NS1 or E3L on mammalian antiviral RNA silencing would entail the previous inactivation of the genes that encode them, which is complicated by the resultant considerable weakening of the viruses against the PKR-activated IFN response. A similar problem was encountered in addressing the significance of the inhibition of exportin 5 and dicer activities in human cells by the adenovirus VA1 RNA. Disruption of VA1 results in a marked decrease in adenovirus accumulation owing to strong activation of the PKR antiviral pathway, which precludes further analysis of possible RNA-silencing effects[87]. So, the FHV B2 protein remains the only RNA-silencing suppressor that is encoded by an animal virus, for which requirement in the infection process has been convincingly established in the context of the interaction between a virus and its natural host[35].

A second approach to studying antiviral RNA silencing in vertebrates is the cloning and sequencing of small RNAs that might accumulate during infection. However, it is remarkable that, despite considerable efforts, there has been no report of the presence of vsRNAs in either non-infected or severely infected vertebrate cells, with the notable exception of the EBV-derived miRNAs[44]. One interpretation is that, although they are operational, the effects of the vsRNA biosynthetic pathway are usually masked or supplanted in vertebrate cells by other defence systems. These include the HUMORAL IMMUNE RESPONSE and the IFN response. Not only would PKR strongly compete with dicer for viral dsRNA substrates, but the IFN response would also mask the specific effects of any putative vsRNAs produced. Perhaps, the occurrence and effects of vsRNAs will only be evident in vertebrate cells in which the IFN or humoral immune responses are compromised or naturally inactive, as in embryonic stem (ES) cells or pre-implanted embryos[110,111]. In fact, we could speculate that the vsRNA biosynthetic pathway might have been specifically preserved in ES cells as a primordial guard system, preventing the inheritance of viruses or transposon-induced genomic instability in all daughter-cell lineages.

Alternatively, the apparent lack of vsRNA in virus-infected vertebrate cells could indicate that antiviral silencing, as characterized in insects and plants, has been progressively lost in higher animals, owing to the emergence of more elaborate dsRNA-activated defence mechanisms. The lack of DCL diversification in mammals, as opposed to plants and insects, supports this hypothesis, which implies that only the miRNA regulatory pathway has been retained in vertebrates. However, even this hypothesis does not necessarily exclude the

MERISTEM
A plant tissue that is usually made up of small cells that can divide indefinitely. Meristems give rise either to similar cells or to cells that differentiate into mature tissues, including reproductive tissues that produce seeds and pollen.

INTERFERONS
(IFNs). A group of glycoproteins, produced by various cell types, that prevent viral replication in newly infected cells and, in some cases, modulate specific cellular functions. They are produced in response to a range of stimuli, including exposure to dsRNA.

HUMORAL IMMUNE RESPONSE
A bodily defence reaction that is mediated by antibodies (produced by B cells) that specifically neutralize invading antigens.

participation of RNA-silencing in antiviral responses. It could just be that vertebrate miRNAs, rather than siRNAs or vsRNAs, are the molecules that are involved in such responses, as shown for EBV. There are also indirect models that might implicate miRNAs in vertebrate antiviral defence. These include the negative control by miRNAs of basic viral compatibility factors, such as host proteins that are required for replication, or the recruitment of miRNAs in the mammalian innate immune response to viruses. Perhaps even more appealing is the recently proposed idea[112] that miRNAs and other endogenous small RNAs, in addition to their cellular functions, might constitute a repertoire of antiviral molecules by complementarity to incoming or resident parasitic nucleic acids. The perfect complementarity of miR127 and miR136 to the mouse retrotransposon Rtl1 (REFS 113,114) is a striking example that supports this concept.

Conclusion and future directions

Our understanding of antiviral silencing has considerably improved over the past few years, as exemplified by the explanation of the mode of action of several virus-encoded silencing suppressors. This research has also promoted the emergence of new and important concepts, such as the existence of cellular negative regulators of RNA silencing and the realization that many of the symptoms that are caused by viruses might result from usurpation of or interference with the regulatory functions of silencing.

The mechanisms that are involved in VIGS pathways are also becoming clearer, although the genetic approaches to understanding these pathways are quickly showing their limits and indicate that our initial views of the process were probably far too simplistic: VIGS probably consists of a multitude of intricate and partially redundant reactions. The sheer number of Argonaute-family proteins in several organisms is another challenging reminder of the complexity that is expected to emerge from the further genetic dissection of defensive RNA silencing.

Another challenge for the future will be to fully determine the extent to which RNA silencing is integrated within the interlinked and numerous layers of innate host defences, and to understand the impact of silencing suppression on these pathways. It also remains to be determined how many of the concepts and principles that have been developed in plant and insect models will apply to vertebrate systems. In any case, it is anticipated that immune systems that are based on small RNAs, either of pathogenic or cellular origin, are likely to be widespread.

1. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
2. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811 (1998).
3. Waterhouse, P. M. & Helliwell, C. A. Exploring plant genomes by RNA-induced gene silencing. *Nature Rev. Genet.* **4**, 29–38 (2003).
4. Hannon, G. J. & Rossi, J. J. Unlocking the potential of the human genome with RNA interference. *Nature* **431**, 371–378 (2004).
5. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
This paper reports the discovery of siRNAs and shows their probable role in limiting virus infection in plants.
6. Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33 (2000).
7. Elbashir, S. M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200 (2001).
8. Nykanen, A., Haley, B. & Zamore, P. D. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321 (2001).
9. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. *Nature* **404**, 293–296 (2000).
10. Liu, J. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441 (2004).
The authors demonstrate that AGO2 is the endonuclease that accounts for the catalytic activity of RISC during the RNAi reaction.
11. Kumagai, M. H. *et al.* Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl Acad. Sci. USA* **92**, 1679–1683 (1995).
12. Ruiz, M. T., Voinnet, O. & Baulcombe, D. C. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10**, 937–946 (1998).
13. Ratcliff, F., MacFarlane, S. & Baulcombe, D. C. Gene silencing without DNA: RNA-mediated cross protection between viruses. *Plant Cell* **11**, 1207–1215 (1999).
14. Szittya, G., Molnar, A., Silhavy, D., Hornyik, C. & Burgyan, J. Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. *Plant Cell* **14**, 359–372 (2002).
One of the few analyses of antiviral RNA silencing that has been carried out in a genuine infection context, which demonstrates the pivotal role of silencing in the selection of defective interfering RNAs in tombusvirus-infected plants.
15. Jones, L., Ratcliff, F. & Baulcombe, D. C. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.* **11**, 747–757 (2001).
16. Jones, L. *et al.* RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* **11**, 2291–2301 (1999).
17. Hamilton, A. J., Voinnet, O., Chappell, L. & Baulcombe, D. C. Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671–4679 (2002).
18. Zilberman, D., Cao, X. & Jacobsen, S. E. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**, 716–719 (2003).
19. Schramke, V. & Allshire, R. Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* **301**, 1069–1074 (2003).
20. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
21. Olsen, P. H. & Ambros, V. The *lin-4* regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671–680 (1999).
22. Rhoades, M. W. *et al.* Prediction of plant microRNA targets. *Cell* **110**, 513–520 (2002).
23. Llave, C., Xie, Z., Kasschau, K. D. & Carrington, J. C. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* **297**, 2053–2056 (2002).
24. Lacomme, C., Hrubikova, K. & Hein, I. Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant J.* **34**, 543–553 (2003).
25. Papaefthimiou, I. *et al.* Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. *Nucleic Acids Res.* **29**, 2395–2400 (2001).
26. Itaya, A., Folimonov, A., Matsuda, Y., Nelson, R. S. & Ding, B. Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. *Mol. Plant Microbe Interact.* **14**, 1332–1334 (2001).
27. Denti, M. A., Boutla, A., Tsagris, M. & Tabler, M. Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus. *Plant J.* **37**, 762–769 (2004).
28. Papp, I. *et al.* Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol.* **132**, 1382–1390 (2003).
29. Kurihara, Y. & Watanabe, Y. *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc. Natl Acad. Sci. USA* **101**, 12753–12758 (2004).
30. Xie, Z. *et al.* Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2**, e104 (2004).
A genetic dissection of RNA-silencing pathways in *A. thaliana* that clearly establishes the functional specialization and specific subcellular localization of Dicer-like enzymes in plants.
31. Tahbaz, N. *et al.* Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. *EMBO J. Rep.* **5**, 189–194 (2004).
32. Uhlirova, M. *et al.* Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proc. Natl Acad. Sci. USA* **100**, 15607–15612 (2003).
33. Lee, Y. S. *et al.* Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69–81 (2004).
34. Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M. C. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev.* **18**, 1655–1666 (2004).
35. Li, H., Li, W. X. & Ding, S. W. Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319–1321 (2002).
This provides the first demonstration that RNA silencing limits virus accumulation in insect cells.
36. Li, W. X. *et al.* Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc. Natl Acad. Sci. USA* **101**, 1350–1355 (2004).
37. Keene, K. M. *et al.* RNA interference acts as a natural antiviral response to O'nyong–nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA* (2004).
38. Chellappan, P., Vanitharani, R. & Fauquet, C. M. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* **78**, 7465–7477 (2004).

39. Muangsan, N., Beclin, C., Vaucheret, H. & Robertson, D. Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. *Plant J.* **38**, 1004–1014 (2004).
40. Bender, J. Chromatin-based silencing mechanisms. *Curr. Opin. Plant Biol.* **7**, 521–526 (2004).
41. Harper, G., Hull, R., Lockhart, B. & Olszewski, N. Viral sequences integrated into plant genomes. *Annu. Rev. Phytopathol*. **40**, 119–136 (2002).
42. Richert-Poggeler, K. R., Noreen, F., Schwarzacher, T., Harper, G. & Hohn, T. Induction of infectious petunia vein clearing (pararetro) virus from endogenous provirus in petunia. *EMBO J.* **22**, 4836–4845 (2003).
43. Mette, M. F. *et al.* Endogenous viral sequences and their potential contribution to heritable virus resistance in plants. *EMBO J.* **21**, 461–469 (2002).
44. Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304**, 734–736 (2004).
The authors used a cloning and sequencing approach to identify several microRNAs that are encoded in the genome of the Epstein–Barr virus, which potentially target many cellular mRNAs in human cells.
45. Bennasser, Y., Le, S. Y., Yeung, M. L. & Jeang, K. T. HIV-1 encoded candidate micro-RNAs and their cellular targets. *Retrovirology* **1**, 43 (2004).
46. Dalmay, T., Hamilton, A. J., Rudd, S., Angell, S. & Baulcombe, D. C. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**, 543–553 (2000).
47. Sijen, T. *et al.* On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
48. Makeyev, E. V. & Bamford, D. H. Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol. Cell* **10**, 1417–1427 (2002).
49. Vaistij, F. E., Jones, L. & Baulcombe, D. C. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* **14**, 857–867 (2002).
50. Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J.* **22**, 4523–4533 (2003).
51. Mourrain, P. *et al. Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542 (2000).
52. Gazzani, S., Lawerson, T., Woodward, D., Headon, R. & Sablowski, R. A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* **306**, 1046–1048 (2004).
The authors provided the first experimental support for the 'aberrant RNA' model for co-suppression in plants by showing that over-accumulation of non-capped mRNAs induces their sequence-specific turnover through an RDR6-dependent RNA-silencing pathway.
53. Dalmay, T. D., Horsefield, R., Braunstein, T. H. & Baulcombe, D. C. *SDE3* encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO J.* **20**, 2069–2078 (2001).
54. Boutet, S. *et al. Arabidopsis HEN1*: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.* **13**, 843–848 (2003).
55. Yu, D., Fan, B., MacFarlane, S. A. & Chen, Z. Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense. *Mol. Plant Microbe Interact.* **16**, 206–216 (2003).
56. Yang, S. J., Carter, S. A., Cole, A. B., Cheng, N. H. & Nelson, R. S. A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. *Proc. Natl Acad. Sci. USA* **101**, 6297–6302 (2004).
57. Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. & Dougherty, W. G. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* **5**, 1749–1759 (1993).
Although they largely pre-date the discovery of RNAi, the seminal experiments that are described in this classic paper have been used to formulate the first testable model for the mechanism and antiviral roles of RNA silencing in plants, with astonishing accuracy in retrospect.
58. Palauqui, J.-C., Elmayan, T., Pollien, J.-M. & Vaucheret, H. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* **16**, 4738–4745 (1997).
59. Voinnet, O. & Baulcombe, D. C. Systemic signalling in gene silencing. *Nature* **389**, 553 (1997).
60. Voinnet, O., Lederer, C. & Baulcombe, D. C. A viral movement protein prevents systemic spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167 (2000).
61. Havelda, Z., Hornyik, C., Crescenzi, A. & Burgyan, J. *In situ* characterization of Cymbidium Ringspot Tombusvirus infection-induced posttranscriptional gene silencing in *Nicotiana benthamiana*. *J Virol.* **77**, 6082–6086 (2003).
62. Yoo, B. C. *et al.* A systemic small RNA signaling system in plants. *Plant Cell* **16**, 1979–2000 (2004).
63. Pruss, G., Ge, X., Shi, X. M., Carrington, J. C. & Vance, V. B. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**, 859–868 (1997).
64. Kasschau, K. D., Cronin, S. & Carrington, J. C. Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component-proteinase. *Virology* **228**, 251–262 (1997).
65. Anandalakshmi, R. *et al.* A viral suppressor of gene silencing in plants. *Proc. Natl Acad. Sci. USA* **95**, 13079–13084 (1998).
66. Kasschau, K. D. & Carrington, J. C. A counterdefensive strategy of plant viruses: suppression of post-transcriptional gene silencing. *Cell* **95**, 461–470 (1998).
67. Brigneti, G. *et al.* Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* **17**, 6739–6746 (1998).
68. Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses. *Proc. Natl Acad. Sci. USA* **96**, 14147–14152 (1999).
By showing that several viral pathogenicity determinants effectively inhibit RNA silencing, this work provides a rationale for the identification of many addtional RNA silencing suppressors that are encoded by plant and animal viruses.
69. Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C. & Voinnet, O. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* **16**, 1235–1250 (2004).
Together with references 70 and 75, this comparative study addresses the interplay between viral silencing suppression, the siRNA and miRNA pathways and the expression of symptoms in plants. It also provides clues about the mode of action of several silencing suppressors.
70. Chen, J., Li, W. X., Xie, D., Peng, J. R. & Ding, S. W. Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. *Plant Cell* **16**, 1302–1313 (2004).
71. Moissiard, G. & Voinnet, O. Viral suppression of RNA silencing. *Mol. Plant Pathol.* **1**, 71–82 (2004).
72. Roth, B. M., Pruss, G. J. & Vance, V. B. Plant viral suppressors of RNA silencing. *Virus Res.* **102**, 97–108 (2004).
73. Silhavy, D. *et al.* A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J.* **21**, 3070–3080 (2002).
74. Lakatos, L., Szittya, G., Silhavy, D. & Burgyan, J. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J.* **23**, 876–884 (2004).
75. Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. V. & Carrington, J. C. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev.* **18**, 1179–1186 (2004).
76. Vargason, J. M., Szittya, G., Burgyan, J. & Tanaka Hall, T. M. Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**, 799–811 (2003).
77. Ye, K., Malinina, L. & Patel, D. J. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* **426**, 874–878 (2003).
The structure of P19 homodimers that are directly bound to siRNAs is resolved in references 76 and 77.
78. Anandalakshmi, R. *et al.* A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* **290**, 142–144 (2000).
79. Kennedy, S., Wang, D. & Ruvkun, G. A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427**, 645–649 (2004).
This key paper clearly establishes the existence of negative regulatory pathways for RNAi in *C. elegans*, and probably in many other organisms. Such pathways might be recruited by some viral-encoded silencing suppressors, as suggested in reference 78.
80. Hartitz, M. D., Sunter, G. & Bisaro, D. M. The tomato golden mosaic virus transactivator (TrAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain. *Virology* **263**, 1–14 (1999).
81. van Wezel, W. R. *et al.* Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. *Mol. Plant Microbe Interact.* **15**, 203–208 (2002).
82. Van Wezel, R., Liu, H., Wu, Z., Stanley, J. & Hong, Y. Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. *J. Virol.* **77**, 696–700 (2003).
83. Trinks, D. *et al.* Suppression of silencing by geminivirus nuclear protein AC2 correlates with transactivation of host genes. *J. Virol.* **75**, 2517–2527 (2004).
84. Ketting, R., Haverkamp, T., van Luenen, H. & Plasterk, R. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**, 133–141 (1999).
85. Glazov, E. *et al.* A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*. *Plant J.* **35**, 342–349 (2003).
86. Samuel, C. E. Antiviral actions of interferons. *Clin. Microbiol. Rev.* **14**, 778–809 (2001).
87. Lu, S. & Cullen, B. R. Adenovirus VA1 non-coding RNA can inhibit small interfering RNA and microRNA biogenesis. *J. Virol.* **78**, 1957–1966 (2005).
88. Tuteja, J. H., Clough, S. J., Chan, W. C. & Vodkin, L. O. Tissue-specific gene silencing mediated by a naturally occurring chalcone synthase gene cluster in *Glycine max*. *Plant Cell* **16**, 819–835 (2004).
89. Senda, M. *et al.* Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *Plant Cell* **16**, 807–818 (2004).
A straightforward example of how viral suppression of RNA silencing can directly influence an agronomic trait in plants.
90. Kasschau, K. D. *et al.* P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA unction. *Dev. Cell* **4**, 205–217 (2003).
This important paper provides the first demonstration of a link between the expression of viral symptoms and inhibition of miRNA-guided functions.
91. Xie, Z., Kasschau, K. D. & Carrington, J. C. Negative feedback regulation of Dicer-like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.* **13**, 784–789 (2003).
92. Tijsterman, M. & Plasterk, R. H. Dicers at RISC; the mechanism of RNAi. *Cell* **117**, 1–3 (2004).
93. Schwartz, M. *et al.* A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* **9**, 505–514 (2002).
94. Tabler, M. & Tsagris, M. Viroids: petite RNA pathogens with distinguished talents. *Trends Plant Sci.* **9**, 339–348 (2004).
95. Ge, Q. *et al.* RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc. Natl Acad. Sci. USA* **100**, 2718–2723 (2003).
96. Boden, D., Pusch, O., Lee, F., Tucker, L. & Ramratnam, B. Human immunodeficiency virus type 1 escape from RNA interference. *J. Virol.* **77**, 11531–11535 (2003).
97. Das, A. T. *et al.* Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.* **78**, 2601–2605 (2004).
98. Dalmay, T., Szittya, G. & Burgyan, J. Generation of defective interfering RNA dimers of cymbidium ringspot tombusvirus. *Virology* **207**, 510–517 (1995).
99. Wang, M. B. *et al.* On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proc. Natl Acad. Sci. USA* **101**, 3275–3280 (2004).
The authors show how RNA silencing that is triggered by sub-viral parasites can result in symptom expression due to homology between the host and pathogen genomes.
100. Bitko, V. & Barik, S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol.* **1**, 34 (2001).
101. Angell, S. M. & Baulcombe, D. C. Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO J.* **16**, 3675–3684 (1997).
102. Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. A similarity between viral defense and gene silencing in plants. *Science* **276**, 1558–1560 (1997).
A classic paper that for the first time identifies RNA silencing as the causal agent of a naturally occuring antiviral response in plants.
103. Pfeffer, S. *et al.* P0 of beet Western yellows virus is a suppressor of posttranscriptional gene silencing. *J. Virol.* **13**, 6815–6824 (2002).

104. Saksela, K. Human viruses under attack by small inhibitory RNA. *Trends Microbiol.* **11**, 345–347 (2003).
105. Ge, Q. *et al.* Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl Acad. Sci. USA* **101**, 8676–8681 (2004).
106. Tompkins, S. M., Lo, C. Y., Tumpey, T. M. & Epstein, S. L. Protection against lethal influenza virus challenge by RNA interference *in vivo*. *Proc. Natl Acad. Sci. USA* **101**, 8682–8686 (2004).
107. Delgadillo, M. O., Saenz, P., Salvador, B., Garcia, J. A. & Simon-Mateo, C. Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J. Gen. Virol.* **85**, 993–999 (2004).
108. Bucher, E., Hemmes, H., de Haan, P., Goldbach, R. & Prins, M. The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J. Gen. Virol.* **85**, 983–991 (2004).
109. Lichner, Z., Silhavy, D. & Burgyan, J. Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences. *J. Gen. Virol.* **84**, 975–980 (2003).
110. Wianny, F. & Zernicka-Goetz, M. Specific interference with gene function by double-stranded RNA in early mouse development. *Nature Cell Biol.* **2**, 70–75 (2000).
111. Billy, E., Brondani, V., Zhang, H. D., Muller, U. & Filipowicz, W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl Acad. Sci. USA* **98**, 14428–14433 (2001).
112. Llave, C. MicroRNAs:more than a role in plant development? *Mol. Plant Pathol.* **5**, 361–366 (2004).
An insightful review, elaborating on the roles of endogenous small RNAs in plants, including the possibility that those molecules might confer antiviral immunity in some instances.
113. Seitz, H. *et al.* Imprinted microRNA genes transcribed antisense to a reciprocally imprinted retrotransposon-like gene. *Nature Genet.* **34**, 261–262 (2003).
114. Lin, S. P. *et al.* Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the *Dlk1–Gtl2* imprinted cluster on mouse chromosome 12. *Nature Genet.* **35**, 97–102 (2003).
115. Dangl, J. L. & Jones, J. D. Plant pathogens and integrated defence responses to infection. *Nature* **411**, 826–833 (2001).
116. Durrant, W. E. & Dong, X. Systemic acquired resistance. *Annu Rev. Phytopathol.* **42**, 185–209 (2004).
117. Li, H.-W. *et al.* Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *EMBO J.* **18**, 2683–2691 (1999).
118. Scholthof, H. B., Scholthof, K. B. G. & Jackson, A. O. Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from a potato virus X vector. *Plant Cell* **7**, 1157–1172 (1995).
119. Ji, L. H. & Ding, S. W. The suppressor of transgene RNA silencing encoded by *Cucumber mosaic virus* interferes with salicylic acid-mediated virus resistance. *Mol. Plant Microbe Interact.* **14**, 715–724 (2001).
120. Pruss, G. J. *et al.* The potyviral suppressor of RNA silencing confers enhanced resistance to multiple pathogens. *Virology* **320**, 107–120 (2004).
121. Stokes, T. L., Kunkel, B. N. & Richards, E. J. Epigenetic variation in *Arabidopsis* disease resistance. *Genes Dev.* **16**, 171–182 (2002).
122. Thomas, C. L., Leh, V., Lederer, C. & Maule, A. J. Turnip crinkle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana*. *Virology* **306**, 33–41 (2003).
123. Reed, J. C. *et al.* Suppressor of RNA silencing encoded by Beet yellows virus. *Virology* **306**, 203–209 (2003).
124. Lu, R. *et al.* Three distinct suppressors of RNA silencing encoded by a 20-kb viral genome. *Proc. Natl Acad. Sci. USA* **101**, 15742–15747 (2004).
125. Liu, L., Grainger, J., Canizares, M. C., Angell, S. M. & Lomonossoff, G. P. Cowpea mosaic virus RNA-1 acts as an amplicon whose effects can be counteracted by a RNA-2-encoded suppressor of silencing. *Virology* **323**, 37–48 (2004).
126. Yelina, N. E., Savenkov, E. I., Solovyev, A. G., Morozov, S. Y. & Valkonen, J. P. Long-distance movement, virulence, and RNA silencing suppression controlled by a single protein in hordei- and potyviruses: complementary functions between virus families. *J. Virol.* **76**, 12981–12991 (2002).
127. Dunoyer, P. *et al.* Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J.* **29**, 555–567 (2002).
128. Kubota, K., Tsuda, S., Tamai, A. & Meshi, T. Tomato mosaic virus replication protein suppresses virus-targeted posttranscriptional gene silencing. *J. Virol.* **77**, 11016–11026 (2003).
129. Bucher, E., Sijen, T., De Haan, P., Goldbach, R. & Prins, M. Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *J. Virol.* **77**, 1329–1336 (2003).

Acknowledgments

I thank all my laboratory colleagues for fruitful discussions. I am particularly grateful to S. Wei Ding, B. Cullen, T. Hohn and M. Poogin for providing access to data before their publication. Many thanks also to J. Carrington, E. Chapman, M.-B. Wang, P. Waterhouse, T. Hall, J. Vargason, L. Chaerle and C. Masuta for providing many of the items used for illustrations. Work in my laboratory is supported by the Centre National de la Recherche Scientifique, the Federation of European Biochemical Societies and the European Molecular Biological Organisation.

Competing interests statement

The author declares no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

Swiss-Prot: http://ca.expasy.org/sprot

DCR1 | AGO1 | AGO2 |

FURTHER INFORMATION

***Nature* web focus — RNA interference animation:**

http://www.nature.com/focus/rnai/animations/index.html

SUPPLEMENTARY INFORMATION

See online article: S1 (figure) | S2 (box)

Access to this links box is available online.

Author biography

Olivier Voinnet received his Ph.D. training in the laboratory of D. Baulcombe at the Sainsbury Laboratory, John Innes Centre, Norwich, UK. His thesis work contributed to the discovery that RNA silencing has essential antiviral functions in higher plants and was awarded the European Prize for Young Scientists by the journal *Science* in 2002. After a short period of post-doctoral training supported by the Royal Society, he was appointed by the Centre National de la Recherche Scientifique (CNRS) to establish his own laboratory at the Institut de Biology Moléculaire des Plantes du CNRS, Strasbourg, France. Research in his laboratory is aimed at understanding the mechanisms and roles of RNA silencing in plants and animals, and focuses particularly on the study of viral-encoded suppressors, the mechanisms of silencing movement in plants, the biology of microRNA molecules and the possible antiviral role of RNA silencing in vertebrates. More recently, his group has also become interested in the link between microRNA molecules and cancer in humans.

Online summary

- In plants and insects, virus-induced gene silencing (VIGS) is a mechanism whereby double-stranded features of viral genomes are recognized and processed by Dicer-like enzymes to generate antiviral small RNA molecules. The diversification and specialization of silencing components that are observed in both types of organisms might have arisen primarily as an adaptation to optimal recognition and processing of various double-stranded forms of pathogenic RNA molecules.
- No single RNA-silencing component has been broadly implicated in antiviral defence in plants so far. This probably reflects the functional redundancy and/or combinatorial interactions between individual members of large protein families that account for the remarkable diversity and complexity of plant RNA-silencing pathways.
- DNA viruses might be restricted by RNA silencing that acts at the transcriptional level. This occurs through small RNA pathways that direct chromatin modifications that are involved in epigenetic control of transposable elements and endogenous loci.
- The miRNA pathway might also restrict accumulation of viruses that produce nuclear transcripts that are folded in a way that mimics the structure of endogenous miRNA precursors. These are normally processed in the nucleus by Dicer-like enzymes to ensure regulatory functions in the cell. This phenomenon has been documented in human cells that are infected by the Epstein–Barr virus, transcripts of which are processed into at least five distinct miRNA molecules.
- In plants, nematodes and fungi, but not in *Drosophila melanogaster* or humans, primary VIGS reactions might be amplified through the activities of host-encoded RNA-dependent RNA polymerases (RdRPs). Amplification is also involved in the systemic spread of silencing, providing a form of genetic immune system that ensures clearance of viral and sub-viral pathogens.
- Plant and insect viruses have developed a range of counter-defensive measures against RNA silencing, one of which is the production of highly diverse suppressor proteins that inhibit distinct steps of the silencing pathway. Strategies for silencing suppression are varied and include the direct inhibition of silencing-effector molecules, recruitment of endogenous pathways that negatively control RNA silencing and modification of the host transcriptome.
- Viral suppression of RNA silencing often — although not always — has adverse effects on host biology, and forms the basis of some of the developmental and cytopathic symptoms that are associated with virus infections in plants, and probably other organisms. This is, at least partly, an incidental consequence of the primary suppression of VIGS at an intermediate step that is shared with the miRNA pathway.
- Viruses can also evade RNA silencing through a range of means that include sub-cellular compartmentalization and loss of silencing-target sequences due to high mutation rates. They might also deliberately hijack their host silencing pathways to establish optimal infection conditions.
- It remains unclear whether RNA silencing naturally limits viral infections in vertebrates as it does in plants and insects. Potent dsRNA-triggered defence pathways, such as the vertebrate interferon response, might mask or supplant the putative RNA-silencing response that is elicited by viruses in those organisms.
- It is possible that cellular microRNA molecules, rather than virus-derived siRNA molecules, might contribute to antiviral defence in vertebrates. This might be indirect, for example, by controlling the accumulation of basic compatibility factors, or direct, owing to the sequence complementarity of miRNA molecules to parasitic nucleic acids.

Online links

Swiss-Prot

DCR1
http://us.expasy.org/cgi-bin/niceprot.pl?Q9VCU9

AGO1
http://us.expasy.org/cgi-bin/niceprot.pl?O04379

AGO2
http://us.expasy.org/cgi-bin/niceprot.pl?Q9VUQ5

***Nature* web focus — RNA interference animation**
http://www.nature.com/focus/rnai/animations/index.html