ANNUAL REVIEWS

Annual Review of Virology

The Role of Viruses in Identifying and Analyzing RNA Silencing

David C. Baulcombe

Department of Plant Sciences, University of Cambridge, Cambridge, United Kingdom; email: dcb40@cam.ac.uk

Annu. Rev. Virol. 2022. 9:353–73

First published as a Review in Advance on June 2, 2022

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-091919-064218

Keywords

adaptive immunity, RNA mobility, RNA-dependent RNA polymerase, DNA methylation, viral suppressors of RNA silencing, innate immunity

Abstract

Adaptive antiviral immunity in plants is an RNA-based mechanism in which small RNAs derived from both strands of the viral RNA are guides for an Argonaute (AGO) nuclease. The primed AGO specifically targets and silences the viral RNA. In plants this system has diversified to involve mobile small interfering RNAs (siRNAs), an amplification system involving secondary siRNAs and targeting mechanisms involving DNA methylation. Most, if not all, plant viruses encode multifunctional proteins that are suppressors of RNA silencing that may also influence the innate immune system and fine-tune the virus-host interaction. Animal viruses similarly trigger RNA silencing, although it may be masked in differentiated cells by the interferon system and by the action of the virus-encoded suppressor proteins. There is huge potential for RNA silencing to combat viral disease in crops, farm animals, and people, although there are complications associated with the various strategies for siRNA delivery including transgenesis. Alternative approaches could include using breeding or small molecule treatment to enhance the inherent antiviral capacity of infected cells.

INTRODUCTION

RNA silencing refers to a family of related mechanisms in which Piwi/Argonaute (AGO) family proteins are effectors that are guided by small RNA molecules to their RNA or DNA targets (1). The outcome is normally silencing through epigenetic mechanisms if the target is DNA or various post-translational mechanisms for RNA. The different RNA silencing pathways are normally defined by the type of small interfering RNA (siRNA) so that, in animals, there are three variants featuring microRNAs (miRNAs), siRNAs, and Piwi-associated RNAs (piRNAs). In plants there are multiple siRNA pathways with overlapping characteristics in addition to miRNAs but no piRNAs (2).

The elucidation of these various pathways followed from a series of unexpected observations involving transgenes and exogenous RNAs including, most notably, the Nobel Prize–winning findings about double-stranded (ds) RNA interference (RNAi) in worms (3). A connection of RNA silencing with virus immunity emerged in plants because viral transgenes had activated a previously uncharacterized defense system against virus disease (4).

In this review I first describe how the connections were made between RNA silencing and virus immunity. I then summarize the more recent developments in our understanding of this process, its role in antiviral immunity, and the implications for RNA biology. The recent developments involve epigenetics, mobile RNA, and the suppressors of silencing that connect RNA silencing and other immune systems. I compare the antiviral RNA silencing systems in plants and animals and, finally, reflect on the potential for RNA silencing to provide solutions to viral disease in agriculture and medicine.

ADAPTIVE ANTIVIRAL IMMUNITY IN PLANTS

The concept of adaptive immunity has been reinforced by animal studies over the past 70 years (5). It is acquired following exposure to a pathogen, is specific for that pathogen or close relatives, and, in mammals, is associated with immunoglobulin or T cell receptor gene rearrangement, clonal selection, and expansion of B and T lymphocytes. Innate immunity, in contrast, is hardwired into the genome of the infected organism. In plants there is no equivalent of immunoglobulin or T cell receptor gene rearrangement and no mechanism for clonal selection of resistant cells. Most of the well-characterized immune systems are genetically encoded (6) and are, therefore, inherently innate. They might involve physical barriers, antimicrobial compounds, or induced responses, including cell death, that reduce the pathogen's rate of reproduction or systemic spread.

Even before the development of transgenic plants, there were, however, indications of antiviral immunity in plants with adaptive characteristics. In nepovirus-infected plants, for example, the later-emerging leaves post inoculation were recovered: They were symptom-free and resistant to secondary infection (7, 8). Similarly, on plants infected with Tobacco mosaic virus (TMV; *Tobamovirus*) and other viruses, there were green islands that were free of the mosaic symptoms and recalcitrant to secondary infection (9, 10). Other viruses induced cross protection in which secondary infection is prevented. The cross protection is adaptive in the sense that it is specific and was acquired following a primary infection. Proposed mechanisms invoked coat protein (11, 12) or antisense RNA (13) from the primary virus that would prevent uncoating or gene expression from a second virus.

At first it seemed likely that similar coat protein or antisense RNA-based mechanisms could be triggered by viral transgenes to produce the type of parasite-derived resistance that had been demonstrated previously with Qß phage in *Escherichia coli* (14). Consistent with this idea, there were many reports of virus-resistant transgenic plants carrying viral transgenes (15). In some of these lines with coat protein transgenes, the underlying resistance mechanism is likely to

be protein mediated, as anticipated. In TMV-resistant transgenic plants, for example, the excess of transgenic TMV coat protein blocked disassembly of an inoculated virus and thereby inhibited translation and replication of its RNA genome (16, 17).

In other transgenic lines, however, the antiviral phenotype did not correlate with the level of transgene expression (15). The resistance did not necessarily require expression of protein from the transgene, and it was sometimes more strain specific than would be expected from simple protein-based mechanisms (18).

Additional insight followed from the characterization of gene silencing in plants with nonviral transgenes and from RNAi worms. In plants with sense transgenes corresponding to flower pigmentation (19, 20), fruit ripening (21), and phenylalanine ammonia lyase (22) genes, there was co-ordinate suppression—cosuppression—of the transgene and the corresponding endogenous gene. These findings aligned with the characterization of RNAi in worms in which endogenous gene expression was suppressed by sense RNA introduced by injection or feeding (3, 23).

These various findings were reconciled in the context of virus immunity by four key discoveries. First, RNAi in worms was caused by contaminating dsRNA in the sense RNA preparations (3). Second, the sense RNA transgenes in plants produced small RNAs corresponding to both the sense and the antisense strands of the silencing target (24). Third, small RNAs of similar size to those in transgenic plants were produced from the Potato virus X (PVX; *Potexvirus*) genome in infected plants (24). And fourth, silencing of a virus in transgenic or cross-protection experiments depended on sequence similarity between the transgene or viral inducer and the targeted viral genome (18, 25, 26).

The connection was reinforced by genetic and biochemical analyses that uncovered a common core pathway of RNA silencing systems in plants and some animals. An RNA-dependent RNA polymerase (RDR) produces dsRNA from a single-stranded precursor. Dicer (Dcr) or Dicer-like (DCL) in plants converts this dsRNA into siRNAs by cleavage, and a second nuclease, AGO (reviewed in 27), is the effector of silencing. Other proteins bound to this effector form an RNA-induced silencing complex (RISC) that can target viral RNA for degradation or translational arrest. The degradation mechanism is referred to as slicing, and the core mechanism is post-transcriptional RNA or gene silencing.

In RNA virus-infected plants, the viral dsRNA precursor of siRNAs is produced directly as a replication intermediate of the viral genome or the viral single-stranded RNA is rendered double stranded by the host-encoded RDR (**Figure 1*a*,*b***). DNA viruses may be transcribed convergently on opposite DNA strands so that overlapping transcripts may base-pair to form dsRNA (**Figure 1*c***). In some systems there is a positive-strand bias in the viral siRNA populations, suggesting that there may be cryptic inverted repeats in viral genomes forming a foldback RNA substrate of Dcr or DCL (28, 29) (**Figure 1*d***). Much of this strand bias, however, is likely due to sequestration of the negative-strand siRNA during extraction and purification due to a large excess of viral positive-strand RNA in infected cells. It is likely therefore that processing of foldback structures is a minor contribution to viral siRNA populations (30).

There has been functional diversification of this core silencing pathway and, correspondingly, multiplication of genes in RNA silencing pathways (2). *Arabidopsis*, for example, has four *DCL*s, six *RDR*s, and ten *AGO*s and corresponding functional diversification of the RNA silencing pathways targeted to genes, transposons, and other chromosomal features. Some of these diversifications are associated with endogenous rather than viral RNAs. One diversification, for example, involves an inverted repeat RNA that is processed by DCL to release a single dominant miRNA species without the involvement of RDRs. In plants the target messenger RNAs (mRNAs) of miRNAs are normally sliced by the AGO nuclease (31).

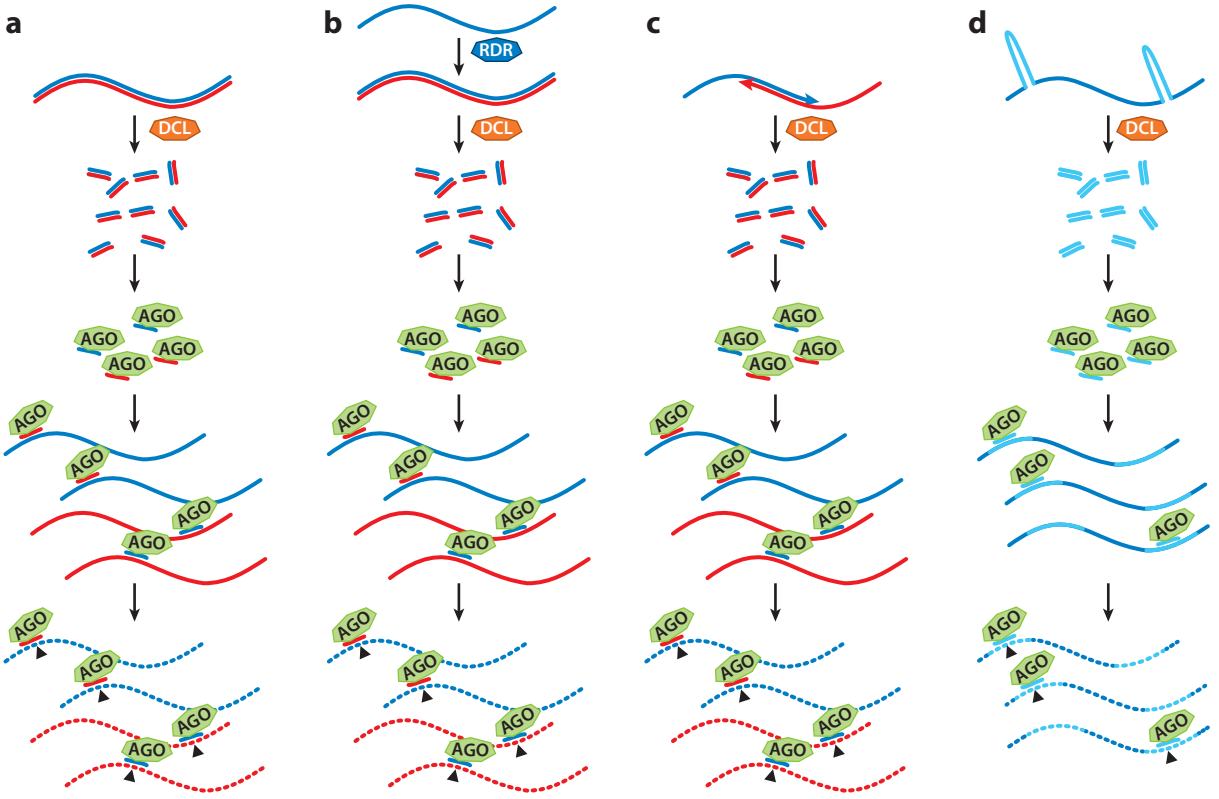


Figure 1

Viral RNA silencing. The core process in RNA silencing involves conversion of dsRNA or base-paired RNA into siRNAs through the action of DCL nucleases. One of the two siRNA strands binds to and serves as a guide in the targeting of AGO nucleases to their target RNAs that are normally cleaved opposite position 10 of the siRNA. The cleaved RNAs are often degraded by non-sequence-specific nucleases in the plant cell. The DCL substrate may be the viral replication intermediate (*a*), dsRNA produced by the action of RDR proteins on single-stranded viral RNA (*b*), converging transcripts from DNA viruses (*c*), or foldback regions in the viral single-stranded RNA (*d*). Blue and red lines indicate opposite strands of viral RNA, and dotted lines indicate RNA in the process of degradation. The foldback regions in panel *d* are shown in light blue. In panels *a–c*, the siRNA targets could be either positive- or negative-strand RNA of the opposite polarity. In panel *d*, they would be on same strand as the siRNA precursor but on the opposite side of a foldback hairpin loop. Abbreviations: AGO, Argonaute; DCL, Dicer-like; dsRNA, double-stranded RNA; RDR, RNA-dependent RNA polymerase; siRNA, small interfering RNA.

Other diversified RNA silencing pathways do, however, have a connection with viruses. All four types of DCL have been associated with virus RNA silencing (32), two of the RDRs (RDR1, RDR6) (33, 34), and at least five of the AGO proteins (AGO1, AGO2, AGO4, AGO5, AGO7) (35–39). The diversification of viral RNA silencing is discussed further in the sections titled Transitive and Relay Amplification of RNA Silencing in Antiviral Defense and RNA Silencing and Epigenetics.

The likelihood that all or most plant viruses are targeted by RNA silencing has been exploited in a recently developed metagenomic strategy of virus identification through shotgun sequencing of siRNAs from plant (and animal) extracts (40, 41). The siRNA sequences are then assembled computationally, and the contiguous sequences can then be aligned to databases of viral genomes (42). This approach has already led to the discovery of many novel viruses and will revolutionize

virus diagnostics and disease monitoring in crops, farm animals, and invertebrate vectors of disease.

In principle, the simple core mechanism of RNA silencing could reduce virus accumulation in an initially infected cell. In practice, however, the process is more complicated due to the diversification of RNA silencing pathways, the involvement of a mobile siRNA signal, and the amplification of initially established silencing. There are also epigenetic effects in addition to the post-transcriptional mechanisms described above. A further complication is because antiviral effectiveness is moderated by virus-encoded suppressors of RNA silencing (VSRs) that are discussed in more detail in the section titled Viral Suppressors of RNA Silencing: Multifunctional Proteins and reviewed elsewhere (43). There can also be interactions of RNA silencing with the innate immune systems. The following sections discuss these various aspects of antiviral RNA silencing.

MOBILE SMALL INTERFERING RNA

Analyses of transgene silencing in plants and of RNAi in worms show clearly that RNA silencing is not a cell-autonomous effect (23, 44, 45). In animals the dsRNA precursor of siRNAs is transported across cell membranes (46). In plants, in contrast, the siRNA rather than dsRNA is the likely mobile species, and it can be transported, without having to cross membranes, through the symplasm—the network of connections between cells involving plasmodesmatal channels between cells (47). The symplasm joins adjacent cells but also extends long distances in the plant through the phloem of the vascular system that connects the growing points in the shoot through to regions close to the apices of the roots.

By extrapolation from these findings it is an attractive hypothesis that mobile siRNA might also influence the spread of virus disease in an infected plant (47). If the viral siRNA could move with or ahead of the spreading virus, it could prime the RNA silencing-based defense systems to block a virus as it entered a cell in distal parts of the plant away from the initially infected cells.

Consistent with that hypothesis, the meristem exclusion of PVX was lost in a knockdown *Nicotiana benthamiana* for RDR6, although this gene is active in the nonmeristematic regions of the plant (48). A likely scenario is that, in a wild-type plant, RDR6 synthesized viral siRNA that is transported into the meristem where it would silence any viral RNA and prevent viral genome replication. Other analyses are consistent with mobile siRNA preventing a *Tombusvirus* from moving out of the vascular system in an infected plant (49) or affecting translation of viral RNA and symptoms of *Tobamovirus* disease in cells at the infection front in *Arabidopsis* (50). There is, however, no direct evidence to support the role of mobile siRNA in virus infection.

Recently developed tools have been informative about the mobility of siRNAs in transgenic plants, and they will fill the evidence gap to test the role of mobile siRNA in virus-infected plants when they are used in the context of virus infection. These tools involved expression of DCL and an siRNA-binding VSR—P19—in specific cell types (51), and they revealed, in the context of transgene RNA silencing, that DCLs are required in the incipient rather than recipient cells of mobile siRNA. They also showed that the siRNAs move in a duplex structure between cells and that one of the two siRNA strands binds to AGO proteins in the recipient cells to form a silencing-competent RISC.

In the context of endogenous genes there are mobile miRNAs and siRNAs that form developmental gradients (52) from the abaxial to the adaxial sides of leaves (53), and they transmit physiological signals between the root and the shoot (31). Mobile miRNAs, for example, contribute to the phosphate homeostasis in the plant associated with the changes in nutrient status (54). The

possible role of mobile siRNA in virus disease will now be testable using the cell type–specific expression of P19 in virus-infected plants.

TRANSITIVE AND RELAY AMPLIFICATION OF RNA SILENCING IN ANTIVIRAL DEFENSE

Transgenic silencing of green fluorescent protein in *N. benthamiana* could be triggered by transient transgene expression on one leaf of a plant and the mobile signal—likely, as discussed above, to be siRNA—spread through the plant following a photosynthetic source-sink gradient (44). The degree of silencing did not fade at greater distances from the leaf in which the silencing was triggered and persisted after detachment of this leaf (55). The initiation of silencing from one part of the transgene in the source leaf led later to siRNAs in recipient leaves targeted to other regions on the 5′ or 3′ side of the initiator (55). A similar transitive process was later rediscovered in *Caenorhabditis elegans* (56).

Initially there was no direct evidence to connect transitive silencing with antiviral defense, although, with the implied potential for amplification of a silencing effect, it fit nicely with the mobile siRNA hypothesis described above (47). More recent analysis of the mechanism of transitivity, however, provides a compelling direct connection with some if not all viruses.

The mechanism of transitive silencing has been most thoroughly characterized in connection with clusters of host siRNAs derived either from protein-coding mRNAs or from noncoding RNAs, and they are RDR dependent (57). The biogenesis pathway of these endogenous siRNAs requires that the precursor single-stranded RNA is first targeted by an miRNA. An RDR is then recruited to produce dsRNA that is cleaved by DCL at many sites along the length to produce the siRNA clusters.

This pathway involves a primary miRNA and the multiple siRNAs from the RDR product that are, therefore, secondary. The pattern of secondary siRNA production is often phased with the first position of the phasing pattern corresponding to the miRNA cleavage site (57). In the context of host RNA silencing, these RNAs are referred to as phased or *trans*-acting siRNAs. The process accounts for transitivity in plants because the secondary siRNAs are adjacent to the primary siRNA.

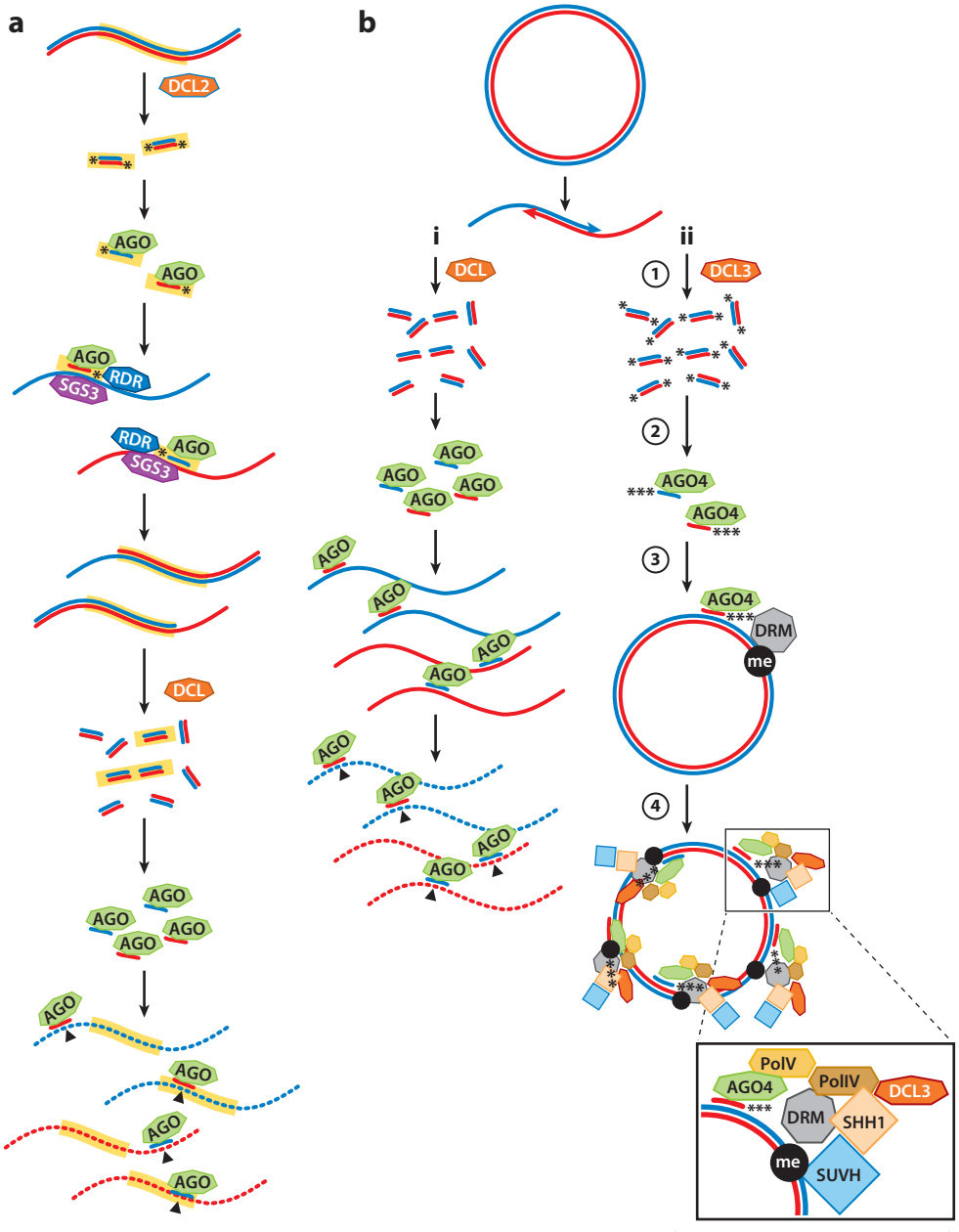
A distinct feature of the miRNAs or siRNAs that trigger secondary siRNA production is that they are 22 nucleotides long rather than the 21-nucleotide species associated with the canonical RNA silencing (58, 59). There are two routes to production of 22-nucleotide siRNAs. One involves an miRNA precursor with a secondary structure such that the DCL cleavage sites are separated by 22 bp (58, 59). The other involves the DCL2 isoform that releases 22-nucleotide siRNAs from fully base-paired dsRNA (60–62).

The preferential role of 22-nucleotide rather than 21-nucleotide siRNAs in transitive silencing is because the twenty-second nucleotide protrudes from the AGO nucleoprotein and allows recruitment of a dsRNA binding protein, SGS3 (63). The AGO-SGS3 complex binds to the target mRNA, stalls the ribosomes if it is an mRNA, and promotes recruitment of the RDR and secondary siRNA production.

The connection between viruses and transitive RNA silencing arises because DCL2 features prominently in antiviral RNA silencing (60, 61, 64–66) (**Figure 2*a***). It is likely, therefore, that RDR and SGS3 are associated with waves of primary and secondary viral siRNA production. The involvement of 22-nucleotide siRNAs with these DCL2-dependent pathways is also consistent with the mobile siRNA hypothesis described above because 22-nucleotide siRNAs are implicated in the traffic of RNA between parasite plants and their hosts (67) that, like mobile silencing, is a symplastic process.

RNA SILENCING AND EPIGENETICS

In the early analyses of nonviral transgene silencing in plants there was a link between the RNA silencing mechanism and DNA methylation (68) of silencer transgenes. Some investigators interpreted this correlation as an indicator that RNA silencing had an epigenetic basis (69). We now know, however, that the core mechanism described above is unequivocally genetic. A transgene or viral gene is the genetic determinant of siRNA production, and the target gene is silenced only



(Caption appears on following page)

Figure 2 (*Figure appears on preceding page*)

Transitivity and amplification in viral RNA silencing. Transitive silencing at the RNA level (*a*) involves 22-nucleotide siRNA rather than the more normal 21-nucleotide size class produced by DCL1 and DCL4. DCL2 is often involved in 22-nucleotide siRNA biogenesis. The presence of the additional single nucleotide (indicated by a *single asterisk*) promotes binding of SGS3 when the AGO/siRNA complex targets a viral RNA so that an RDR can be recruited to synthesize a dsRNA precursor of secondary siRNAs. These secondary siRNAs silence their target as the primary siRNAs in the canonical pathway. They are more abundant than the primary siRNAs and, if the primary siRNA is from a localized region of the viral genome (*yellow shaded*), the secondary siRNAs may extend beyond that region. Silencing of DNA viruses (*b*) is likely to be at the RNA level in the early stages of infection (*b*, *i*) through any of the routes shown in **Figure 1**. Illustrated here is the pathway shown in **Figure 1*c*** involving converging transcripts. The dsRNAs and siRNAs are likely to increase in abundance as the level of viral DNA increases and the excess then spills over into the RdDM pathway (*b*, *ii*) in which the siRNAs include those of 24 nucleotides (indicated by *three asterisks*) (*b*, *ii*, ①) are bound by AGO4 and other similar members of the AGO family of proteins. Establishment of RdDM is not well understood, but it may involve direct binding of the AGO siRNA complex to the DNA (*b*, *ii*, ②) and recruitment of a DRM and ultimately methylation of the target DNA (*b*, *ii*, ③). This pioneer round of DNA methylation may then lead to histone modifications (not shown), binding of SHH1 and SUVH proteins and recruitment of PolIV and PolV to this DNA, further rounds of RdDM (*b*, *ii*, ④), and ultimately transcriptional silencing of the viral genome. The end result of this process, as with the transitive post-transcriptional silencing (*a*), is amplification of the silencing pathway and effects that that may extend beyond the region targeted by the primary siRNA. Abbreviations: AGO, Argonaute; DCL, Dicer-like; DRM, domains rearranged methyl transferase; dsRNA, Dicer-substrate small interfering RNA; me, methyl deoxycytidine; RdDM, RNA-directed DNA methylation; RDR, RNA-dependent RNA polymerase; siRNA, small interfering RNA.

when these genes are active. The link with DNA methylation arises because, in certain situations, it is a secondary consequence of RNA silencing alongside the post-transcriptional mechanisms affecting RNA accumulation or translation.

The first indication that RNA could direct DNA methylation was from transgenic plants carrying transgenes based on viroids—circular noncoding RNA pathogens of plants. The viroid transgenes were methylated only if the plant contained replicating viroid RNA (70). Of course, the replicating viroid RNA would have a dsRNA intermediate, and we can now understand these findings as indications of a variation on the canonical RNA silencing pathway leading to RNA-directed DNA methylation (RdDM) (2). A similar process targets DNA viruses in plants including geminiviruses, pararetroviruses, and retrotransposons—transposons with retrovirus-like characteristics (71).

A likely scenario in RNA-directed silencing of DNA viruses is that, in the initially infected cell, the viral or retro-element DNA is transcribed and recruited into the core post-transcriptional RNA silencing pathway, as with RNA viruses (**Figure 2*b*,*i***). If the RNA silencing is weak, however, and there is continued viral RNA accumulation, then there would be a transition to RdDM (**Figure 2*b*,*ii***) (71).

The post-transcriptional and RdDM mechanisms involve different isoforms of the main RDR, DCL, and AGO proteins (72) (**Figure 2*b***). Associated with the RdDM in *Arabidopsis*, for example, the pathway involves RDR2, DCL3, and AGO4, although in some situations other DCLs, RDR6, AGO2, and AGO6 may also play a role (73–75). The target RNAs in RdDM are nuclear rather than cytoplasmic (76), and the AGO proteins recruit DNA methyl transferases to the target DNA locus rather than acting as ribonucleases or structures to block translation (72). Other key components of the RdDM pathway are the atypical forms of DNA-dependent RNA polymerase II known as PolIV and PolV (72). PolIV produces the precursor of the siRNAs in this pathway (77, 78), and PolV generates chromatin-associated scaffold RNAs (79) that are targets of the siRNA.

RdDM is effective as protection against viruses because DNA methylation is normally associated with repression of transcription (72): Viral gene expression would be reduced once the viral

genomes are methylated. The antiviral effectiveness of this process is also likely to be reinforced by a feedback characteristic of the RdDM pathway. There are SUVH2/9 (80, 81) and SHH1 proteins (82) that interact with PolV or PolIV, respectively, and they bind directly or indirectly to methylated DNA. Once the RdDM is established on the viral DNA, these interactions lead to additional recruitment of PolV and PolIV and accelerated RdDM. This feedback reinforces RdDM, and it ensures that viral DNA replication did not overtake RNA silencing-based resistance in the infected cells.

VIRAL SMALL INTERFERING RNAs AS TOOLS IN BIOTECHNOLOGY AND AS VIRULENCE FACTORS

Host mRNAs are silenced by viral vectors carrying fragments of nuclear genes (83, 84). Inserts derived from transcribed regions mediate post-transcriptional mechanisms, whereas those from promoters trigger RdDM and transcriptional silencing (85). These virus-induced gene silencing (VIGS) approaches have provided useful technology in functional genomics and as experimental tools for the analysis of RdDM.

Tobacco rattle virus (*Tobravirus*) has been particularly useful in VIGS-based functional genomics in *N. benthamiana* (86), and many other viruses including Foxtail mosaic virus (*Potexvirus*) and Geminiviruses have been adapted for crop species (87). Discoveries made using these tools include key components of the innate immune system in plants including BAK1, a kinase partner of receptor-like kinases involved in recognition of pathogen-associated molecular patterns (88). A forward VIGS screen revealed a pioneer member of the RNL class of Nucleotide oligomerization domain (NOD)-like receptors (NLRs) associated with intracellular recognition of pathogen-derived effectors in disease resistance (86).

In the analysis of RdDM, the use of VIGS allowed the demonstration that, once established, the epigenetic mark could be maintained into the next generation in the absence of the virus (85). These experiments are some of the clearest demonstrations that establishment and maintenance are separate stages in epigenetics and that newly established epigenetic marks can be inherited between generations. The RNA-mediated establishment phase leads to methylation of cytosines in all possible CHH contexts (where H is any base except G). The RNA-independent maintenance phase, in contrast, involves DNA methyl transferases that act exclusively at CHG and CG contexts, and it is stable across generations (89).

It is likely that there is VIGS of host mRNAs if there is sufficient sequence similarity of host and viral genomes. The Y satellite RNA of Cucumber mosaic virus (CMV; *Cucuomovirus*), for example, is the template for siRNAs that target an mRNA required for chlorophyll biosynthesis (CHL1) (90, 91). Host RNA silencing also occurs with a viroid siRNA that targets callose synthase mRNA (92) and, in Turnip mosaic virus (TuMV; *Potyvirus*)-infected rape, the NBR1 mRNA (93) is silenced by a viral siRNA. TMV and grapevine-infecting virus (Grapevine fleck virus and Grapevine rupestris stem pitting-associated virus; *Tymovirus*) siRNAs (94, 95) also target host mRNAs, and Tomato yellow leaf curl virus (TYLCV; *Geminivirus*) caused stunting and leaf curling because it induced silencing of a long noncoding RNA (designated as SlLNR1) (96).

In some of these examples the host gene silencing would promote virus spread within or between plants. Yellow mosaic resulting from CHL1 silencing, for example, would make the plant attractive to aphid vectors (97), and callose synthase and NBR1 are cofactors of host immunity and their silencing would allow for increased virus accumulation.

These examples illustrate how viral siRNAs could be virulence factors of the virus, although we cannot rule out that these effects are due to chance similarity of the host and viral genomes. Further confirmatory work is needed through, for example, functional analysis of target site mutations in

the host genes and their effects on the virus. Investigation of target genes in hosts and nonhosts of the virus to test for coadaptation in the interacting genomes would also be helpful. Evidence for coadaptation would detect positive selection of viral genomes to target immunity cofactor mRNAs in the host but not the nonhosts. Correspondingly, the target sequences in the host would be under negative or diversifying selection to evade targeting by the viral siRNAs.

VIRAL SUPPRESSORS OF RNA SILENCING: MULTIFUNCTIONAL PROTEINS

Infection of plants with two or more viruses often results in disease that is more severe than that caused by the individual viruses or than an additive effect (98). This synergism is often lethal for the infected plant and a major cause of crop loss in agriculture. Maize lethal necrosis disease, for example, is a major problem in Central and East Africa, leading in some instances to complete loss of the crop due to co-infection by Maize chlorotic mottle virus (*Tombusvirus)* and Sugarcane mosaic or other members of the *Potyvirus* group (99, 100). Many other crops are affected by established examples of synergism, and metagenomic and next-generation sequencing indicate that double infection is the rule rather than an exception (101). Synergism may turn out to be an underappreciated factor in the management of virus disease in crops.

An insightful hypothesis of synergism invoked suppressors of host defense encoded by the interacting viruses (98) and specifically the idea that these proteins would block the antiviral RNA silencing pathways. The first tests of this hypothesis involved the synergism between PVX, Tobacco etch virus (TEV; *Potyvirus*), and the TEV helper component-proteinase (HC-Pro) (102, 103). This protein had been previously implicated in aphid transmission of the virus and proteolytic processing of the virus-encoded polyprotein (104), but its involvement in synergism is because it is a viral suppressor of RNA silencing.

There is no single explanation for the mechanism whereby HC-Pro suppresses silencing. It blocks binding of siRNAs into the AGO effector complex, blocks methylation of the siRNA 3′ end by blocking S-adenosyl methione production or by binding to the methyl transferase, downregulates AGO1 production, binds directly to AGO1, downregulates expression of RDR6, or blocks siRNA mobility (reviewed in 104). Adding to the complexity of the HC-Pro story, there is the potential for it to bind and cooperate with a calmodulin-like host-encoded suppressor of RNA silencing (105, 106).

The VSR mechanisms associated with the small CMV-encoded 2b protein (107) are also complicated. This protein is located in both the nucleus and cytoplasm, although the silencing suppression is cytoplasmic (108). It binds directly to siRNA duplexes, and this activity is required for silencing suppression at least for the CMV subgroup 1 and Tomato aspermy virus (*Cucumovirus*) orthologs (108, 109). These 2b proteins also bind to and block the ribonuclease activity of host-encoded AGO proteins (110–112), and they block the long-distance movement of mobile siRNA (113).

Further mechanistic diversity is revealed by other VSRs (reviewed in 43) that, in different examples, target almost every step of the post-transcriptional and transcriptional RNA silencing pathways (**Figure 3①**). In many examples the VSR may bind directly to the RNAs or host-encoded proteins involved in the core pathways (43), but there are other examples where other host proteins are involved. The *Tombusvirus* P19 suppressor of RNA silencing, for example, binds to 21-nucleotide siRNA duplexes, but it also interacts with plasmodesmatal kinases (BAM1 and BAM2) and may block movement of siRNA between cells consistent with the mobile siRNA hypothesis (114). The C4 VSR from TYLCV also interacts with BAM kinases (115). A second VSR from the same virus—the V2 protein—affects the Cajal body localization of the AGO4 protein

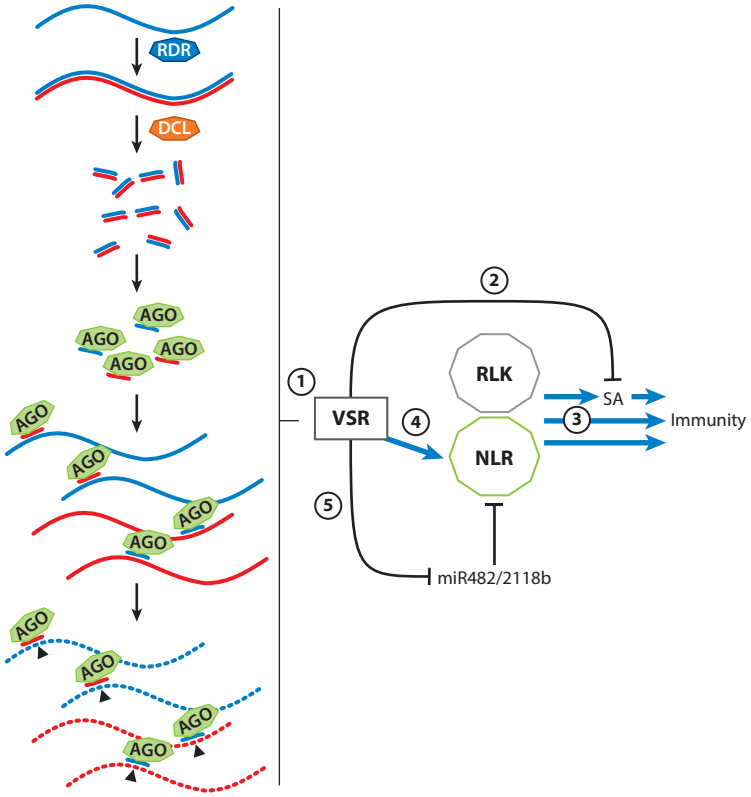


Figure 3

Viral suppressors of RNA silencing as multifunctional proteins. VSRs from different viruses may target any of the stages of RNA silencing by binding directly to them, by promoting their degradation, or by stimulating an endogenous suppressor of RNA silencing (①). They can also block the SA or other pathways (②) in the innate immune system of plants that are downstream of RLK or NLR receptors in the innate immune system (③). VSRs can also have opposing effects by stimulating NLR receptors (④) or by blocking the suppression of the NLR mRNAs by miR482/2118b (⑤). Abbreviations: AGO, Argonaute; DCL, Dicer-like; mRNA, messenger RNA; NLR, Nucleotide oligomerization domain (NOD)-like receptor; RDR, RNA-dependent RNA polymerase; RLK, receptor-like kinase; SA, salicylic acid; VSR, virus-encoded suppressor of RNA silencing.

in the RdDM pathways (116, 117). The P0 protein (Turnip yellows virus; *Polerovirus*) binds to autophagy-related proteins and thereby targets the intracellular degradation of AGO proteins so that antiviral silencing is suppressed.

Many of the VSRs also suppress RNA silencing-independent defense systems (**Figure 3**②,③). The CMV (2b) (118), Cauliflower mosaic virus (P6) (119), and *Potyvirus* (HC-Pro) (119) VSRs, for example, block the effects of salicylic acid—a defense hormone in the innate immune systems of plants (120)—and the V2 geminiviral VSR blocks a calmodulin-binding transcription activator-3 that activates defense genes when insect vectors of the virus feed on plants (121).

Other examples of VSR multifunctionality, however, act oppositely in that they activate the RNA silencing-independent defense systems (**Figure 3**④). Thus, the VSRs P25 of PVX (122, 123), NSs of Tomato spotted wilt virus (*Tospovirus*) (124), and P38 of Turnip crinkle virus (*Carmovirus*) (125) interact directly or indirectly with intracellular receptors, and they thereby activate the innate immune systems of plants.

A similar outcome occurs because many of the intracellular receptors (NLRs) in the innate immune system are regulated by miRNAs (miR472/482/2118b) in *Solanaceae* and other species (126–129) (**Figure 3⑤**). The miRNA silencing pathways are mechanistically similar to the viral siRNA pathways, and they are suppressed by VSRs. The NLRs are upregulated in virus-infected plants because the miRNA pathway, being similar to the mechanism of siRNA silencing, is blocked by VSRs (126) and would be a consequent activation of innate immunity (130).

The opposing action of VSRs on RNA silencing and innate immune systems is seemingly contradictory in terms of a defense and counter-defense model of virus-host interactions in which viral fitness corresponds to the effectiveness of its counter-defense strategy. However, the fitness of the virus is unlikely to have a simple relationship to counter-defense and, similarly, the fitness of the host may not always correspond to the most effective defense systems (131–133). It could be, for example, that viruses spread within and are transmitted between plants more effectively if the disease symptoms are moderated by the host's immune systems. Correspondingly, the host plant may benefit if the costs of disease resistance are moderated by miRNAs or VSRs. In this light it is probably useful to move beyond a simple defense and counter-defense or arms race perspective on the interaction of hosts and their viruses (6). A more useful framework would take into account the possibility that fitness of both hosts and their viruses can be optimized by fine-tuning of defense systems under natural section, as suggested in Reference 43. The VSRs, therefore, are instruments of fine-tuning as well as classical counter-defense systems.

COMPARISON OF ANTIVIRAL RNA SILENCING IN PLANTS AND ANIMALS

Ironically, given its role in revealing the mechanism of RNAi, it was difficult to find evidence for an antiviral role of this process in *C. elegans*. The question was resolved eventually, however, with the discovery of Nodaviruses (Orsay and Santeuil viruses) in a naturally RNAi-deficient *C. elegans* and *Caenorhabditis briggsae* (134, 135). These viruses do not encode suppressors of RNA silencing (135). In silencing-competent worms, an RNA binding protein—DRH1—recruits Dcr-1 perhaps by binding to the viral RNA replication intermediate, and an AGO (RDE-1)-containing RISC is the effector responsible for degradation of viral RNA (136). Other RNA binding proteins play a role and, like in plants, there is an RDR (RRF-1) to amplify the effect. Unlike in plants, however, the amplification mechanism may be Dcr independent because the secondary siRNAs have 5′ triphosphate (136, 137). Also, unlike RNA silencing in plants or with RNAi from exogenous dsRNA, there is no evidence for a non-cell-autonomous effect or transgenerational persistence for the antiviral silencing (138).

Antiviral RNA silencing is well documented in insects including *Drosophila*, mosquitoes, and other insects infected with various RNA and DNA viruses (139). The dsRNA trigger is likely to be the viral replication intermediate with RNA viruses and, with DNA viruses, converging and overlapping transcripts or RNAs with foldback regions. Proteins involved are the usual suspects including Dcr-2, AGO-2, and various RNA binding proteins including R2D2. There is no RDR homolog in insect systems.

In mammalian cells, an antiviral RNA silencing pathway is also very similar to the core pathway in plants but without an RDR (140, 141). In cell culture assays, a Dcr isoform from an alternatively spliced mRNA lacking an internal helicase domain is the antiviral Dcr, and the dsRNA replication intermediate is its likely substrate, as demonstrated with Sindbis, Zika, and Severe acute respiratory syndrome coronavirus 2 RNA viruses but not vaccinia and Herpes simplex DNA viruses (142). The viral siRNAs are predominantly 22 nucleotides, and the effector of silencing is the ribonuclease-competent AGO2, indicating that the viral RNA is targeted by slicing, as in plants (143, 144).

Mammalian RNA viruses generally encode VSRs that bind to dsRNA or Dcr (140, 141). Knock out of the VSR gene in Nodamura virus (144), Influenza A virus (145), Human Enterovirus 71 (145), and Dengue virus 2 (146) results in viral siRNAs that are more abundant than with wild-type viruses. This effect of the VSRs is likely to explain why, in many mammalian cell types, it is difficult to detect the viral siRNAs.

An additional complication is antagonism of interferon responses and antiviral silencing in somatic mammalian cells. This effect is likely due, in part, to induction of dsRNA binding protein LGP2 in the interferon response that also interacts with and inhibits Dcr2 (146, 147). The finding that RNA silencing provides better protection against viruses in stem cells and other cells that are hyporesponsive to interferons is likely a consequence of this antagonism in somatic cells.

The common function of RNA silencing as an antiviral mechanism in divergent organisms indicates that this process had a similar role in a eukaryotic ancestor of plants and cells. It would have persisted because it is adaptive and it turns the pathogen against itself. It also has an amplification property because, for each molecule of viral RNA that is the substrate of DCL proteins, there would be multiple siRNAs. This amplification effect would be reinforced because the siRNAs are part of a RISC that is catalytic and with the potential to sequentially target and inactivate many different viral RNAs. The involvement of RDRs in plants and worms would also contribute to this amplification property of antiviral RNA silencing.

PROSPECTS FOR RNA SILENCING IN THE CONTROL OF VIRUS DISEASES IN PLANTS AND ANIMALS

The most obvious application of RNA silencing to prevent virus disease would use RNA, and the simplest applications would be transgenic, as in the experiments with parasite-derived resistance and plants. A few examples of parasite-derived resistance have been tested in the field, but the underlying mechanism has not been explored. It is likely, however, that RNA silencing features in at least some of the examples (148). They include resistance in papaya to Papaya mosaic virus (*Potyvirus*) that has been hugely successful in Hawaii. Other examples include cucurbits, sweet pepper, tomato (CMV), and potato [Potato virus Y (*Potyvirus*) and Potato leafroll virus (*Luteovirus*)]. Similar transgenic approaches might also be appropriate with farm animals including pigs, cattle, and chickens in which virus diseases are major problems. A recent example describes protection of pigs against Foot-and-mouth disease virus with short hairpin transgenes targeted to the viral genome (149).

Alternatively, the silencer RNA—either dsRNA or siRNA—could be delivered to the host where it would be processed and incorporated into RISC and target silencing of any viral RNA. The silencing mechanisms would be post-transcriptional or epigenetic depending on the nature of the virus. In the most favorable scenarios, there would be amplification and transitivity as described above so that the effect would be strong and persistent so that protection against disease would last for long enough. Such environmental RNA approaches have been successful in plants and animals (A. Voloudakis, unpublished article). In plants the RNA delivered as a nanoparticle complex provided protection against CMV (150). Mice could also be protected against Herpes simplex virus (151).

There are, however, disadvantages to these approaches. In part there is a regulatory complication due to the cumbersome regulation of genetically modified plants and animals in many parts of the world (152, 153). Ideally the approval of these products could be streamlined because the RNA silencing transgenes would not encode proteins and the hazard of immunogenicity would be nonexistent.

With the environmental RNA approach there are also the complications of delivery. The RNA would need to be included in a formulation, for example, that would be stable and ensure that the

RNA would be taken up in the appropriate cells. Nanoparticle or other conjugates are potential components of this formulation (150) but, until now, there are very few therapeutic RNAs for use in people. The challenge of an appropriate delivery formulation is likely to be one of the limiting factors with therapeutic or prophylactic RNA (154).

A further complication is breakdown of resistance. If there are just one or two silencer small RNAs, there would be strong selection for mutant viruses with reduced complementarity between the siRNA and the viral genome. Such selection was evident within five to ten passages on plants with artificial miRNAs targeted at TuMV (155). The likelihood of selection for resistance-breaking mutants can be reduced if there are multiple antiviral siRNAs delivered.

A second reason for breakdown of resistance could be if the protected organism is infected with a nontargeted virus. This virus would cause disease unaffected by the silencer RNA, and its VSR would reduce the protection against the targeted virus. Similarly, even in the absence of a second virus, there could be selection for mutant versions of the target virus with potent VSRs that could prevent the action of a silencer RNA.

There are, however, alternative approaches to RNA silencing in protection against viruses that do not require RNA delivery. In crops, for example, selection for natural variants or mutants with enhanced expression of RNA silencing genes could lead to enhanced resistance against viruses. Mutations in the miR402 or miR6026 (35, 60), for example, would increase the level of their target mRNAs including those encoding antiviral AGO2 or DCL2. Enhanced DCL2, in particular, might have an enhanced antiviral effect because this DCL isoform generates the 22-nucleotide siRNAs that trigger secondary siRNA production on their target RNAs that would amplify the activity of any antiviral RNA silencing (60, 61). Similarly, loss of function in miR482 and related miRNAs would increase the level of NLR proteins and the level of innate immunity in plants (130).

If genetic enhancement of antiviral RNA silencing is not possible, for example in humans or in trees with a long breeding cycle, such as citrus and cocoa, then an alternative approach might involve small molecule treatments. The aim of such treatments would be to influence the potential of the targeted cells to prevent virus accumulation using RNA silencing. In plant cells the aim would be to enhance the existing potential to use silencing, and in farm animals or humans it would be to release the latent potential of infected cells to use RNA silencing as an antiviral system.

The alternative approaches to RNA silencing may not provide full resistance against the targeted virus. However, a frequent lesson in disease resistance is that silver bullet approaches are often not durable (156): The pest or pathogen is under strong selection to overcome the resistance. In that light a partial resistance due to chemically or genetically modified RNA silencing may be useful as part of long-term strategies for virus resistance in plants and animals including humans.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The author is the Edward Penley Abraham Royal Society Research Professor. His research has been supported over the years by the Gatsby Charitable Foundation, the Biotechnology and Biological Sciences Research Council, the European Research Council, and the Balzan Foundation in addition to the Royal Society.

LITERATURE CITED

1. Kutter C, Svoboda P. 2008. miRNA, siRNA, piRNA: knowns of the unknown. *RNA Biol*. 5(4):181–88
2. Borges F, Martienssen RA. 2015. The expanding world of small RNAs in plants. *Nat. Rev. Mol. Cell Biol.* 16(12):727–41
3. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806–11
4. Baulcombe D. 2004. RNA silencing in plants. *Nature* 431:356–63
5. Vivier E, Malissen B. 2005. Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nat. Immunol.* 6(1):17–21
6. Jones JD, Dangl JL. 2006. The plant immune system. *Nature* 444(7117):323–29
7. Wingard SA. 1928. Hosts and symptoms of ring spot, a virus disease of plants. *J. Agric. Res.* 37:127–53
8. Ghoshal B, Sanfaçon H. 2015. Symptom recovery in virus-infected plants: revisiting the role of RNA silencing mechanisms. *Virology* 479–480:167–79
9. Sherwood JL. 1988. Mechanisms of cross protection between virus strains. In *Plant Resistance to Viruses*, ed. D Evered, S Harnett, pp. 144–57. Chichester, UK: Wiley & Sons
10. Moore CJ, Sutherland PW, Forster RLS, Gardner RC, MacDiarmid RM. 2001. Dark green islands in plant virus infection are the result of posttranscriptional gene silencing. *Mol. Plant-Microbe Interact.* 14(8):939–46
11. Sherwood JL, Fulton RW. 1982. The specific involvement of coat protein in tobacco mosaic virus cross protection. *Virology* 119:150–58
12. Sherwood JL. 1987. Demonstration of the specific involvement of coat protein in tobacco mosaic virus (TMV) cross protection using a TMV coat protein mutant. *Phytopathology* 118:358–62
13. Palukaitis P, Zaitlin M. 1984. A model to explain the "cross protection" phenomenon shown by plant viruses and viroids. *Plant-Microbe Interact.* 1:420–29
14. Sanford JC, Johnston SA. 1985. The concept of parasite-derived resistance: deriving resistance genes from the pathogen's own genome. *J. Theor. Biol.* 113(2):395–405
15. Baulcombe DC. 1996. Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8(10):1833–44
16. Powell PA, Sanders PR, Tumer NE, Fraley RT, Beachy RN. 1990. Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. *Virology* 175:531–38
17. Powell PA, Nelson RS, De B, Hoffmann N, Rogers SG, et al. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232:738–43
18. English JJ, Mueller E, Baulcombe DC. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* 8:179–88
19. Napoli C, Lemieux C, Jorgensen RA. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. *Plant Cell* 2:279–89
20. van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitji AR. 1990. Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2:291–99
21. Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, et al. 1988. Antisense RNA inhibition of polygalaturonase gene expression in transgenic tomatoes. *Nature* 334:724–26
22. Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, et al. 1990. Abnormal-plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. *PNAS* 87:9057–61
23. Timmons L, Fire A. 1998. Specific interference by ingested dsRNA. *Nature* 395(6705):854
24. Hamilton AJ, Baulcombe DC. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286(5441):950–52
25. Ratcliff F, Harrison BD, Baulcombe DC. 1997. A similarity between viral defense and gene silencing in plants. *Science* 276:1558–60
26. Covey SN, Al-Kaff NS, Langara A, Turner DS. 1997. Plants combat infection by gene silencing. *Nature* 385:781–82
27. Chen X. 2009. Small RNAs and their roles in plant development. *Annu. Rev. Cell Dev. Biol.* 25:21–44

28. Myles KM, Wiley MR, Morazzani EM, Adelman ZN. 2008. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *PNAS* 105(50):19938–43
29. Molnár A, Csorba T, Lakatos L, Várallyay É, Lacomme C, Burgyán J. 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J. Virol.* 79(12):7812–18
30. Harris CJ, Molnar A, Müller SY, Baulcombe DC. 2015. FDF-PAGE: a powerful technique revealing previously undetected small RNAs sequestered by complementary transcripts. *Nucleic Acids Res.* 43(15):7590–99
31. Song X, Li Y, Cao X, Qi Y. 2019. MicroRNAs and their regulatory roles in plant-environment interactions. *Annu. Rev. Plant Biol.* 70:489–525
32. Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 313(5783):68–71
33. Wang XB, Wu Q, Ito T, Cillo F, Li WX, et al. 2010. RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. *PNAS* 107(1):484–89
34. Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, et al. 2010. *Arabidopsis* RNA-dependent RNA polymerases and Dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip mosaic virus* infection. *Plant Cell* 22(2):481–96
35. Harvey JJW, Lewsey MG, Patel K, Westwood J, Heimstädt S, et al. 2011. An antiviral defense role of AGO2 in plants. *PLOS ONE* 6(1):e14639
36. Brosseau C, El Oirdi M, Adurogbangba A, Ma X, Moffett P. 2016. Antiviral defense involves AGO4 in an *Arabidopsis*–potexvirus interaction. *Mol. Plant-Microbe Interact.* 29(11):878–88
37. Qu F, Ye X, Morris TJ. 2008. *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *PNAS* 105(38):7–12
38. Carbonell A, Fahlgren N, Garcia-Ruiz H, Gilbert KB, Montgomery TA, et al. 2012. Functional analysis of three *Arabidopsis* ARGONAUTES using slicer-defective mutants. *Plant Cell* 24(9):3613–29
39. Wang XB, Jovel J, Udomporn P, Wang Y, Wu Q, et al. 2011. The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in *Arabidopsis thaliana*. *Plant Cell* 23(4):1625–38
40. Kreuze JF, Perez A, Untiveros M, Quispe D, Fuentes S, et al. 2009. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses. *Virology* 388(1):1–7
41. Wu Q, Luo Y, Lu R, Lau N, Lai EC, et al. 2010. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *PNAS* 107(4):1606–11
42. Zheng Y, Gao S, Padmanabhan C, Li R, Galvez M, et al. 2017. VirusDetect: an automated pipeline for efficient virus discovery using deep sequencing of small RNAs. *Virology* 500:130–38
43. Csorba T, Kontra L, Burgyán J. 2015. Viral silencing suppressors: tools forged to fine-tune host-pathogen coexistence. *Virology* 479–480:85–103
44. Voinnet O, Baulcombe DC. 1997. Systemic signalling in gene silencing. *Nature* 389:553
45. Palauqui J-C, Elmayan T, Pollien J-M, Vaucheret H. 1997. Systemic acquired silencing: Transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* 16:4738–45
46. Jose AM, Hunter CP. 2007. Transport of sequence-specific RNA interference information between cells. *Annu. Rev. Genet.* 41:305–30
47. Melnyk CW, Molnar A, Baulcombe DC. 2011. Intercellular and systemic movement of RNA silencing signals. *EMBO J.* 30(17):3553–63
48. Schwach F, Vaistij FE, Jones L, Baulcombe DC. 2005. An RNA-dependent RNA-polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol.* 138:1842–52
49. Havelda Z, Hornyik C, Crescenzi A, Burgyan J. 2003. In situ characterization of *Cymbidium Ringspot Tombusvirus* infection-induced posttranscriptional gene silencing in *Nicotiana benthamiana*. *J. Virol.* 77(10):6082–86
50. Kørner CJ, Pitzalis N, Peña EJ, Erhardt M, Vazquez F, Heinlein M. 2018. Crosstalk between PTGS and TGS pathways in natural antiviral immunity and disease recovery. *Nat. Plants* 4(3):157–64

51. Devers EA, Brosnan CA, Sarazin A, Albertini D, Amsler AC, et al. 2020. Movement and differential consumption of short interfering RNA duplexes underlie mobile RNA interference. *Nat. Plants* 6(7):789–99
52. D'Ario M, Griffiths-Jones S, Kim M. 2017. Small RNAs: big impact on plant development. *Trends Plant Sci*. 22(12):1056–68
53. Chitwood DM, Guo M, Nogueira FTS, Timmermans MCP. 2007. Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. *Development* 134:813–23
54. Pant BD, Buhtz A, Kehr J, Scheible WR. 2008. MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.* 53(5):731–38
55. Voinnet O, Vain P, Angell S, Baulcombe DC. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95(2):177–87
56. Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, et al. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* 107(4):465–76
57. Liu Y, Teng C, Xia R, Meyers BC. 2020. PhasiRNAs in plants: their biogenesis, genic sources, and roles in stress responses, development, and reproduction. *Plant Cell* 32(10):3059–80
58. Chen H-M, Chen L-T, Patel K, Li Y-H, Baulcombe DC, Wu S-H. 2010. 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. *PNAS* 107(34):15269–74
59. Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, et al. 2010. Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. *Nat. Struct. Mol. Biol.* 17(8):997–1003
60. Wang Z, Hardcastle TJ, Pastor AC, Yip WH, Tang S, Baulcombe DC. 2018. A novel DCL2-dependent miRNA pathway in tomato affects susceptibility to RNA viruses. *Genes Dev*. 32(17–18):1155–60
61. Wang T, Deng Z, Zhang X, Wang H, Wang Y, et al. 2018. Tomato *DCL2b* is required for the biosynthesis of 22-nt small RNAs, the resulting secondary siRNAs, and the host defense against ToMV. *Hortic. Res.* 5:62
62. Mlotshwa S, Pruss GJ, Peragine A, Endres MW, Li J, et al. 2008. *DICER-LIKE2* plays a primary role in transitive silencing of transgenes in *Arabidopsis*. *PLOS ONE* 3(3):e1755
63. Iwakawa H, Lam AYW, Mine A, Fujita T, Kiyokawa K, et al. 2021. Ribosome stalling caused by the Argonaute-microRNA-SGS3 complex regulates the production of secondary siRNAs in plants. *Cell Rep*. 35(13):109300
64. Parent JS, Bouteiller N, Elmayan T, Vaucheret H. 2015. Respective contributions of Arabidopsis DCL2 and DCL4 to RNA silencing. *Plant J.* 81(2):223–32
65. Bouché N, Lauressergues D, Gasciolli V, Vaucheret H, Bouche N. 2006. An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.* 25(14):3347–56
66. Gasciolli V, Mallory AC, Bartel DP, Vaucheret H. 2005. Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing *trans*-acting siRNAs. *Curr. Biol.* 15(16):1494–1500
67. Wafula E, DePamphilis CW, Johnson NR, Shahid S, Phifer T, et al. 2018. MicroRNAs from the parasitic plant *Cuscuta campestris* target host messenger RNAs. *Nature* 553(7686):82–85
68. VanHoudt H, Ingelbrecht I, VanMontagu M, Depicker A. 1997. Post-transcriptional silencing of a neomycin phosphotransferase II transgene correlates with the accumulation of unproductive RNAs and with increased cytosine methylation of 3′ flanking regions. *Plant J.* 12(2):379–92
69. Jorgensen RA. 1994. Developmental significance of epigenetic impositions on the plant genome—a paragenetic function for chromosomes. *Dev. Genet.* 15:523–32
70. Wassenegger M, Heimes S, Riedel L, Sanger HL. 1994. RNA-directed de novo methylation of genomic sequences in plants. *Cell* 76(3):567–76
71. Marí-Ordóñez A, Marchais A, Etcheverry M, Martin A, Colot V, Voinnet O. 2013. Reconstructing *de novo* silencing of an active plant retrotransposon. *Nat. Genet.* 45(9):1029–39
72. Matzke MA, Mosher RA. 2014. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nat. Rev. Genet.* 15(6):394–408

73. McCue AD, Nuthikattu S, Reeder SH, Slotkin RK. 2012. Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA. *PLOS Genet*. 8(2):e1002474
74. Pontier D, Picart C, Roudier F, Garcia D, Lahmy S, et al. 2012. NERD, a plant-specific GW protein, defines an additional RNAi-dependent chromatin-based pathway in *Arabidopsis*. *Mol. Cell* 48(1):121–32
75. McCue AD, Panda K, Nuthikattu S, Choudury SG, Thomas EN, Slotkin RK. 2014. ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation. *EMBO J*. 34:20–35
76. Wierzbicki AT, Ream TS, Haag JR, Pikaard CS. 2009. RNA polymerase V transcription guides ARGONAUTE4 to chromatin. *Nat. Genet*. 41(5):630–34
77. Herr AJ, Jensen MB, Dalmay T, Baulcombe DC. 2005. RNA polymerase IV directs silencing of endogenous DNA. *Science* 308(5718):118–20
78. Onodera Y, Haag JR, Ream T, Nunes PC, Pontes O, Pikaard CS. 2005. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* 120(5):613–22
79. Wierzbicki AT, Haag JR, Pikaard CS. 2008. Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. *Cell* 135(4):635–48
80. Johnson LM, Du J, Hale CJ, Bischof S, Feng S, et al. 2014. SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. *Nature* 507(7490):124–28
81. Liu ZW, Shao CR, Zhang CJ, Zhou JX, Zhang SW, et al. 2014. The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. *PLOS Genet*. 10(1):e1003948
82. Law JA, Du J, Hale CJ, Feng S, Krajewski K, et al. 2013. Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. *Nature* 498(7454):385–89
83. Kumagai MH, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill LK. 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *PNAS* 92:1679–83
84. Ruiz MT, Voinnet O, Baulcombe DC. 1998. Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10(6):937–46
85. Jones L, Ratcliff F, Baulcombe DC. 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Curr. Biol.* 11(10):747–57
86. Peart JR, Mestre P, Lu R, Malcuit I, Baulcombe DC. 2005. NRG1, a CC-NB-LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against tobacco mosaic virus. *Curr. Biol.* 15(10):968–73
87. Abrahamian P, Hammond RW, Hammond J. 2020. Plant virus-derived vectors: applications in agricultural and medical biotechnology. *Annu. Rev. Virol.* 7:513–35
88. Heese A, Hann DR, Gimenez-Ibanez S, Jones AME, He K, et al. 2007. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *PNAS* 104(29):12217–22
89. Gouil Q, Baulcombe DC. 2016. DNA methylation signatures of the plant chromomethyltransferases. *PLOS Genet*. 12(12):e1006526
90. Smith NA, Eamens AL, Wang MB. 2011. Viral small interfering RNAs target host genes to mediate disease symptoms in plants. *PLOS Pathog*. 7(5):e1002022
91. Shimura H, Pantaleo V, Ishihara T, Myojo N, Inaba J, et al. 2011. A viral satellite RNA induces yellow symptoms on tobacco by targeting a gene involved in chlorophyll biosynthesis using the RNA silencing machinery. *PLOS Pathog*. 7(5):e1002021
92. Adkar-Purushothama CR, Brosseau C, Giguère T, Sano T, Moffett P, Perreaulta JP. 2015. Small RNA derived from the virulence modulating region of the *Potato spindle tuber viroid* silences *callose synthase* genes of tomato plants. *Plant Cell* 27(8):2178–94
93. Pitzalis N, Amari K, Graindorge S, Pflieger D, Donaire L, et al. 2020. *Turnip mosaic virus* in oilseed rape activates networks of sRNA-mediated interactions between viral and host genomes. *Commun. Biol.* 3:702
94. Miozzi L, Gambino G, Burgyan J, Pantaleo V. 2013. Genome-wide identification of viral and host transcripts targeted by viral siRNAs in *Vitis vinifera*. *Mol. Plant Pathol.* 14(1):30–43
95. Qi X, Bao FS, Xie Z. 2009. Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. *PLOS ONE* 4(3):e4971
96. Yang Y, Liu T, Shen D, Wang J, Ling X, et al. 2019. *Tomato yellow leaf curl virus* intergenic siRNAs target a host long noncoding RNA to modulate disease symptoms. *PLOS Pathog*. 15(1):e1007534

97. Döring TF, Chittka L. 2007. Visual ecology of aphids—a critical review on the role of colours in host finding. *Arthropod-Plant Interact.* 1(1):3–16
98. Pruss G, Ge X, Shi XM, Carrington JC, Vance VB. 1997. Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9:859–68
99. Xia Z, Zhao Z, Chen L, Li M, Zhou T, et al. 2016. Synergistic infection of two viruses MCMV and SCMV increases the accumulations of both MCMV and MCMV-derived siRNAs in maize. *Sci. Rep.* 6:20520
100. Redinbaugh MG, Stewart LR. 2018. Maize lethal necrosis: an emerging, synergistic viral disease. *Annu. Rev. Virol.* 5:301–22
101. Mascia T, Gallitelli D. 2016. Synergies and antagonisms in virus interactions. *Plant Sci.* 252:176–92
102. Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, et al. 1998. A viral suppressor of gene silencing in plants. *PNAS* 95(22):13079–84
103. Kasschau KD, Carrington JC. 1998. A counterdefensive strategy of plant viruses: suppression of post-transcriptional gene silencing. *Cell* 95(4):461–70
104. Valli A, Gallo A, Rodamilans B, Lopez-Moya JJ, Garcia JA. 2017. The HCPro from the *Potyviridae* family: an enviable multitasking Helper Component that every virus would like to have. *Mol. Plant Pathol.* 19(3):744–63
105. Anandalakshmi R, Marathe R, Ge X, Herr JM, Mau C, et al. 2000. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* 290(5489):142–44
106. Nakahara KS, Masuta C, Yamada S, Shimura H, Kashihara Y, Wada TS. 2012. Tobacco calmodulin-like protein provides secondary defense by binding to and directing degradation of virus RNA silencing suppressors. *PNAS* 109(25):10113–18
107. Lucy AP, Guo HS, Li WX, Ding SW. 2000. Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J.* 19(7):1672–80
108. González I, Rakitina D, Semashko M, Taliansky M, Praveen S, et al. 2012. RNA binding is more critical to the suppression of silencing function of *Cucumber mosaic virus* 2b protein than nuclear localization. *RNA* 18(4):771–82
109. Chen HY, Yang J, Lin C, Yuan YA. 2008. Structural basis for RNA-silencing suppression by *Tomato aspermy virus* protein 2b. *EMBO Rep.* 9(8):754–60
110. Duan CG, Fang YY, Zhou BJ, Zhao JH, Hou WN, et al. 2012. Suppression of *Arabidopsis* ARGONAUTE1-mediated slicing, transgene-induced RNA silencing, and DNA methylation by distinct domains of the *Cucumber mosaic virus* 2b protein. *Plant Cell* 24(1):259–74
111. Hamera S, Song X, Su L, Chen X, Fang R. 2012. Cucumber mosaic virus suppressor 2b binds to AGO4-related small RNAs and impairs AGO4 activities. *Plant J.* 69(1):104–15
112. Zhang X, Yuan Y-R, Pei Y, Lin S-S, Tuschl T, et al. 2006. *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* 20(23):3255–68
113. Hamilton AJ, Voinnet O, Chappell L, Baulcombe DC. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO J.* 21(17):4671–79
114. Garnelo Gómez B, Rosas-Díaz T, Shi C, Fan P, Zhang D, et al. 2021. The viral silencing suppressor P19 interacts with the receptor-like kinases BAM1 and BAM2 and suppresses the cell-to-cell movement of RNA silencing independently of its ability to bind sRNA. *New Phytol.* 229(4):1840–43
115. Rosas-Diaz T, Zhang D, Fan P, Wang L, Ding X, et al. 2018. A virus-targeted plant receptor-like kinase promotes cell-to-cell spread of RNAi. *PNAS* 115(6):1388–93
116. Michaeli S, Clavel M, Lechner E, Viotti C, Wu J, et al. 2019. The viral F-box protein P0 induces an ER-derived autophagy degradation pathway for the clearance of membrane-bound AGO1. *PNAS* 116(45):22872–83
117. Wang L, Ding Y, He L, Zhang G, Zhu JK, Lozano-Duran R. 2020. A virus-encoded protein suppresses methylation of the viral genome through its interaction with AGO4 in the Cajal body. *eLife* 9:e55542
118. Ji LH, Ding SW. 2001. The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. *Mol. Plant-Microbe Interact.* 14(6):715–24
119. Love AJ, Geri C, Laird J, Carr C, Yun BW, et al. 2012. *Cauliflower mosaic virus* protein P6 inhibits signaling responses to salicylic acid and regulates innate immunity. *PLOS ONE* 7(10):e47535

120. Hunter LJR, Westwood JH, Heath G, Macaulay K, Smith AG, et al. 2013. Regulation of RNA-dependent RNA polymerase 1 and isochorismate synthase gene expression in *Arabidopsis*. *PLOS ONE* 8(6):e66530
121. Wang Y, Gong Q, Wu Y, Huang F, Ismayil A, et al. 2021. A calmodulin-binding transcription factor links calcium signaling to antiviral RNAi defense in plants. *Cell Host Microbe* 29(9):1393–406
122. Malcuit I, Marano MR, Kavanagh TA, De Jong W, Forsyth A, Baulcombe DC. 1999. The 25-kDa movement protein of PVX elicits *Nb*-mediated hypersensitive cell death in potato. *Mol. Plant-Microbe Interact.* 12(6):536–43
123. Voinnet O, Pinto YM, Baulcombe DC. 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA of plants. *PNAS* 96(24):14147–52
124. de Ronde D, Pasquier A, Ying S, Butterbach P, Lohuis D, Kormelink R. 2014. Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression. *Mol. Plant Pathol.* 15(2):185–95
125. Ren T, Qu F, Morris TJ. 2000. *HRT* gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. *Plant Cell* 12(10):1917–25
126. Shivaprasad PV, Chen H-M, Patel K, Bond DM, Santos BACM, Baulcombe DC. 2012. A microRNA superfamily regulates nucleotide binding site–leucine-rich repeats and other mRNAs. *Plant Cell* 24(3):859–74
127. De Vries S, De Vries J, Rose LE. 2019. The elaboration of miRNA regulation and gene regulatory networks in plant–microbe interactions. *Genes* 10(4):310
128. Zhang Y, Xia R, Kuang H, Meyers BC. 2016. The diversification of plant *NBS-LRR* defense genes directs the evolution of microRNAs that target them. *Mol. Biol. Evol.* 33(10):2692–705
129. Deng Y, Wang J, Tung J, Liu D, Zhou Y, et al. 2018. A role for small RNA in regulating innate immunity during plant growth. *PLOS Pathog.* 14(1):e1006756
130. Canto-Pastor A, Santos BAMC, Valli AA, Summers W, Schornack S, Baulcombe DC. 2019. Enhanced resistance to bacterial and oomycete pathogens by short tandem target mimic RNAs in tomato. *PNAS* 116(7):2755–60
131. Alizon S, Hurford A, Mideo N, Van Baalen M. 2009. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. *J. Evol. Biol.* 22(2):245–59
132. Doumayrou J, Avellan A, Froissart R, Michalakis Y. 2013. An experimental test of the transmission-virulence trade-off hypothesis in a plant virus. *Evolution* 67(2):477–86
133. González VM, Müller S, Baulcombe DC, Puigdomènech P. 2015. Evolution of NBS-LRR gene copies among dicot plants and its regulation by members of the miR482/2118 superfamily of miRNAs. *Mol. Plant* 8(2):329–31
134. Félix MA, Ashe A, Piffaretti J, Wu G, Nuez I, et al. 2011. Natural and experimental infection of *Caenorhabditis* nematodes by novel viruses related to nodaviruses. *PLOS Biol.* 9(1):e1000586
135. Félix MA, Wang D. 2019. Natural viruses of *Caenorhabditis* nematodes. *Annu. Rev. Genet.* 53:313–26
136. Ashe A, Bélicard T, Le Pen J, Sarkies P, Frézal L, et al. 2013. A deletion polymorphism in the *Caenorhabditis elegans* RIG-I homolog disables viral RNA dicing and antiviral immunity. *eLife* 2:e00994
137. Sijen T, Steiner FA, Thijssen KL, Plasterk RHA. 2007. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* 315(5809):244–47
138. Ashe A, Sarkies P, Le Pen J, Tanguy M, Miska EA. 2015. Antiviral RNA interference against Orsay virus is neither systemic nor transgenerational in *Caenorhabditis elegans*. *J. Virol.* 89(23):12035–46
139. Bronkhorst AW, Van Rij RP. 2014. The long and short of antiviral defense: small RNA-based immunity in insects. *Curr. Opin. Virol.* 7(1):19–28
140. Ding SW, Han Q, Wang J, Li WX. 2018. Antiviral RNA interference in mammals. *Curr. Opin. Immunol.* 54:109–14
141. Maillard PV, van der Veen AG, Poirier EZ, Reis e Sousa C. 2019. Slicing and dicing viruses: antiviral RNA interference in mammals. *EMBO J.* 38(8):e100941
142. Poirier EZ, Buck MD, Chakravarty P, Carvalho J, Frederico B, et al. 2021. An isoform of Dicer protects mammalian stem cells against multiple RNA viruses. *Science* 373(6551):231–36
143. Maillard PV, van der Veen AG, Deddouche-Grass S, Rogers NC, Merits A, Reis e Sousa C. 2016. Inactivation of the type I interferon pathway reveals long double-stranded RNA-mediated RNA interference in mammalian cells. *EMBO J.* 35(23):2505–18

144. Han Q, Chen G, Wang J, Jee D, Li WX, et al. 2020. Mechanism and function of antiviral RNA interference in mice. *mBio* 11(4):e03278-19
145. Li Y, Basavappa M, Lu J, Dong S, Cronkite DA, et al. 2016. Induction and suppression of antiviral RNA interference by influenza A virus in mammalian cells. *Nat. Microbiol.* 2:16250
146. Qiu Y, Xu YP, Wang M, Miao M, Zhou H, et al. 2020. Flavivirus induces and antagonizes antiviral RNA interference in both mammals and mosquitoes. *Sci. Adv.* 6(6):eaax7989
147. Zhang Y, Xu Y, Dai Y, Li Z, Wang J, et al. 2021. Efficient Dicer processing of virus-derived double-stranded RNAs and its modulation by RIG-I-like receptor LGP2. *PLOS Pathog.* 17(8):e1009790
148. Kreuze JF, Valkonen JP. 2017. Utilization of engineered resistance to viruses in crops of the developing world, with emphasis on sub-Saharan Africa. *Curr. Opin. Virol.* 26:90–97
149. Hu W, Zheng H, Li Q, Wang Y, Liu X, et al. 2021. shRNA transgenic swine display resistance to infection with the foot-and-mouth disease virus. *Sci. Rep.* 11:16377
150. Mitter N, Worrall EA, Robinson KE, Li P, Jain RG, et al. 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3(2):16207
151. Palliser D, Chowdhury D, Wang Q-Y, Lee SJ, Bronson RT, et al. 2006. An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 439:89–94
152. Zhang C, Wohlhueter R, Zhang H. 2016. Genetically modified foods: a critical review of their promise and problems. *Food Sci. Hum. Wellness* 5(3):116–23
153. Murray JD, Maga EA. 2016. A new paradigm for regulating genetically engineered animals that are used as food. *PNAS* 113(13):3410–13
154. Chakraborty C, Sharma AR, Sharma G, Doss CGP, Lee SS. 2017. Therapeutic miRNA and siRNA: moving from bench to clinic as next generation medicine. *Mol. Ther.-Nucleic Acids* 8:132–43
155. Lafforgue G, Martinez F, Sardanyes J, de la Iglesia F, Niu Q-W, et al. 2011. Tempo and mode of plant RNA virus escape from RNA interference-mediated resistance. *J. Virol.* 85(19):9686–95
156. Brown JKM. 2015. Durable resistance of crops to disease: a Darwinian perspective. *Annu. Rev. Phytopathol.* 53:513–39