RNA-based antiviral immunity

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Abstract | In eukaryotic RNA-based antiviral immunity, viral double-stranded RNA is recognized as a pathogen-associated molecular pattern and processed into small interfering RNAs (siRNAs) by the host ribonuclease Dicer. After amplification by host RNA-dependent RNA polymerases in some cases, these virus-derived siRNAs guide specific antiviral immunity through RNA interference and related RNA silencing effector mechanisms. Here, I review recent studies on the features of viral siRNAs and other virus-derived small RNAs from virus-infected fungi, plants, insects, nematodes and vertebrates and discuss the innate and adaptive properties of RNA-based antiviral immunity.

Innate immune responses are triggered immediately after pathogen attack by mechanisms that have been evolutionarily conserved across a wide range of eukaryotes[1]. In innate immunity, a limited number of germline-encoded immune receptors recognize broadly conserved pathogen-associated molecular patterns (PAMPs) to activate multiple signalling cascades and the transcription of nonspecific immune effector genes. Families of well-characterized pattern recognition receptors (PRRs) include the Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), which were first cloned in *Drosophila melanogaster*, plant and mammalian systems, respectively[1–3].

In the past decade, it has been discovered that virus infection in diverse eukaryotic hosts also induces the production of virus-derived small RNAs. These virus-derived small RNAs share features with host endogenous small interfering RNAs (siRNAs), microRNAs (miRNAs) or Piwi-interacting RNAs (piRNAs) and so, similar to host small RNAs, they can potentially mediate RNA interference (RNAi) and related RNA silencing pathways (as shown for *D. melanogaster* in FIG. 1), resulting in specific antiviral immunity.

Key challenges in the field of RNA-based antiviral immunity are therefore to characterize the molecular and biochemical features of virus-derived small RNAs that are produced after infection, to identify the genetic pathway(s) for their biogenesis and to determine their effector mechanisms in antiviral immunity. In this Review, I focus on the relative abundance, properties, precursors, biogenesis, effector mechanisms and host amplification of one type of virus-derived small RNA — viral siRNAs. This is because fungi, plants and invertebrate animals clearly produce viral siRNAs to guide specific antiviral immunity and virus-derived small RNAs recently sequenced from mammalian cells share features with siRNAs. Research findings used for discussion and comparison are mostly from host species in the Plantae (*Arabidopsis thaliana* and *Nicotiana benthamiana*), Fungi (*Cryphonectria parasitica;* the chestnut blight fungus) and Animalia (*D. melanogaster* and *Caenorhabditis elegans*) kingdoms. In addition, some mammalian viruses are known to encode their own miRNAs or to be regulated by host-encoded miRNAs, and a recent study reported the detection of virus-derived piRNAs in *D. melanogaster* cells[4,5]. Unlike the well-characterized role for siRNAs, the roles of miRNAs and piRNAs in antiviral immunity have still to be clearly defined; these roles are briefly discussed in Supplementary information S1 (box) because of space limitations.

RNA silencing pathways

The canonical RNAi pathway has been characterized most extensively in *D. melanogaster* (FIG. 1b), in which it was found that long double-stranded RNA (dsRNA) is diced progressively from the termini into a pool of 21-nucleotide siRNA duplexes by the dsRNA-specific endoribonuclease (RNase) Dicer[6,7]. These siRNAs then select and destroy their mRNA targets by base-pairing with and guiding the endoribonucleic cleavage (or slicing) of the target mRNA by an Argonaute (AGO) protein in the middle of the siRNA–mRNA duplex[6,7]. Therefore, siRNAs determine the target specificity of AGO-mediated slicing in RNAi.

AGO proteins bind small RNAs with high affinity, contain a Piwi domain that is structurally similar to RNase H and are divided into AGO and Piwi subfamilies based on the sequence similarity of the Piwi domain. siRNAs and miRNAs bind to members of the AGO subfamily in an RNA-induced silencing complex (RISC). By contrast, piRNAs bind to AGO proteins from the Piwi subfamily, which are found in animals but not in plants. siRNAs and miRNAs are 21–24 nucleotides in length

Pathogen-associated molecular patterns (PAMPs). Molecular patterns that are found in pathogens but not mammalian cells. Examples include various microbial products, such as bacterial lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNA, which bind to Toll-like receptors.

Pattern recognition receptors (PRRs). Host receptors (such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) or RIG-I-like receptors (RLRs)) that can sense pathogen-associated molecular patterns and initiate signalling cascades that lead to an innate immune response. These can be membrane bound (such as TLRs) or soluble cytoplasmic receptors (such as RIG-I, melanoma differentiation-associated gene 5 (MDA5) and NLRs).

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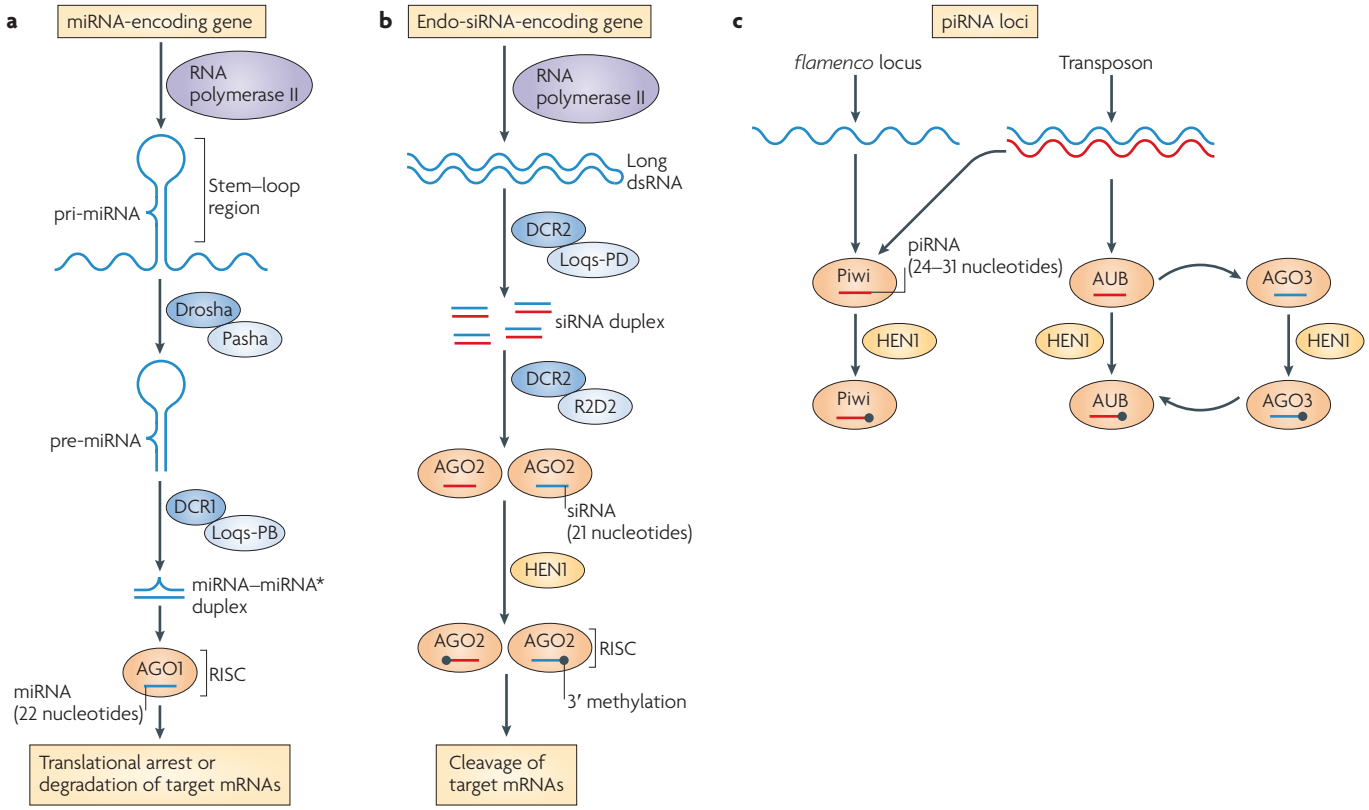

Figure 1 | ***Drosophila melanogaster* encodes three small RNA pathways that are highly conserved in mammals.** **a** | After transcription by RNA polymerase II from microRNA (miRNA)-encoding genes, primary miRNA (pri-miRNA) transcripts are processed sequentially by the type III ribonucleases (RNases) Drosha, in the nucleus, and Dicer1 (DCR1), in the cytoplasm — which form heterodimers with the double-stranded RNA (dsRNA)-binding proteins Pasha and Loquacious-isoform PB (Loqs-PB), respectively. This processing forms precursor miRNAs (pre-miRNAs) and then 22-nucleotide miRNAs. Pasha and Loqs-PB correspond to the mammalian proteins microprocessor complex subunit DGCR8 and interferon-inducible dsRNA-dependent protein kinase activator A (also known as PACT), respectively. miRNAs bind to Argonaute 1 (AGO1) in an RNA-induced silencing complex (RISC) to mediate translational arrest or the degradation of target mRNAs. **b** | Production of small interfering RNAs (siRNAs) from long dsRNA precursors involves one type III RNase (DCR2) and possibly Loqs-PD, although another dsRNA-binding protein, R2D2, is required for siRNA binding to AGO2 and loading into the RISC. **c** | The biogenesis of Piwi-interacting RNAs (piRNAs) may not require a type III RNase. piRNAs are mostly antisense (shown in red) to transposon transcripts and bind to Piwi or Aubergine (AUB), but AGO3 binds to the low abundant sense piRNAs (shown in blue) and collaborates with AUB to amplify piRNAs. The methyltransferase HEN1 adds the 2′-*O*-methyl modification (depicted by a black circle) at the 3′-end of siRNAs and piRNAs after binding to AGO proteins. Additional components that interact with AGO proteins in the RISC and related effector complexes are not shown.

Small interfering RNAs
(siRNAs). 21–24-nucleotide double-stranded RNAs with two-nucleotide 3′ overhangs and 5′-monophosphate and 3′-hydroxyl termini. They are processed from long double-stranded RNA precursors by Dicer. Plant and animal genomes encode many siRNAs with complete or extensive sequence complementarity to endogenous mRNA transcripts.

MicroRNAs
(miRNAs). 21–25-nucleotide single-stranded RNAs with 5′-monophosphate and 3′-hydroxyl termini. They are processed by Dicer from a structured region of single-stranded nuclear transcripts. The seed region of miRNAs corresponds to nucleotides 2–8 and seed pairing has a crucial role in the recognition of the target mRNA.

and are products of Dicer[6–8]. Perfectly base-paired long dsRNA is the precursor of siRNAs, whereas a single miRNA is typically processed from a primary miRNA (pri-miRNA, which is transcribed from a miRNA-encoding gene) that contains an imperfectly base-paired stem–loop region known as precursor miRNA (pre-miRNA). piRNAs from vertebrates and *D. melanogaster* are larger in size than siRNAs and miRNAs, ranging from 24 to 31 nucleotides, and are Dicer independent[6,7]. Similar to siRNAs, both miRNAs and piRNAs can guide target mRNA slicing when they are bound to a catalytically active AGO protein and have near perfect sequence complementarity to the target RNA. However, animal miRNAs mostly function to repress translation of their mRNA targets instead of inducing slicing. This occurs as a result of mismatches with target mRNAs outside the seed region (nucleotides 2–8) of miRNAs. In addition to the post-transcriptional effector mechanisms of slicing and translational repression, siRNAs and piRNAs can inhibit the transcription of their target genes by guiding the specific methylation of DNA or histones[6–8]. RNAi is often used to refer to gene silencing by siRNAs and/or long dsRNA, whereas RNA silencing is a broader term describing AGO-mediated silencing mechanisms that are programmed by siRNAs, miRNAs or piRNAs.

Virus-derived small interfering RNAs

Discovery and diversity. Virus-derived small RNAs have been detected in diverse eukaryotic host species since their initial detection in plants[9] and animals[10]. Indeed, the discovery of potato virus X (PVX)-derived small RNAs in 1999 (REF. 9) occurred before siRNAs had been experimentally defined for the first time in *D. melanogaster*[11,12]. However, it was known at the time that virus infection induces virus-specific, homology-dependent RNA degradation in the infected plants[13,14].

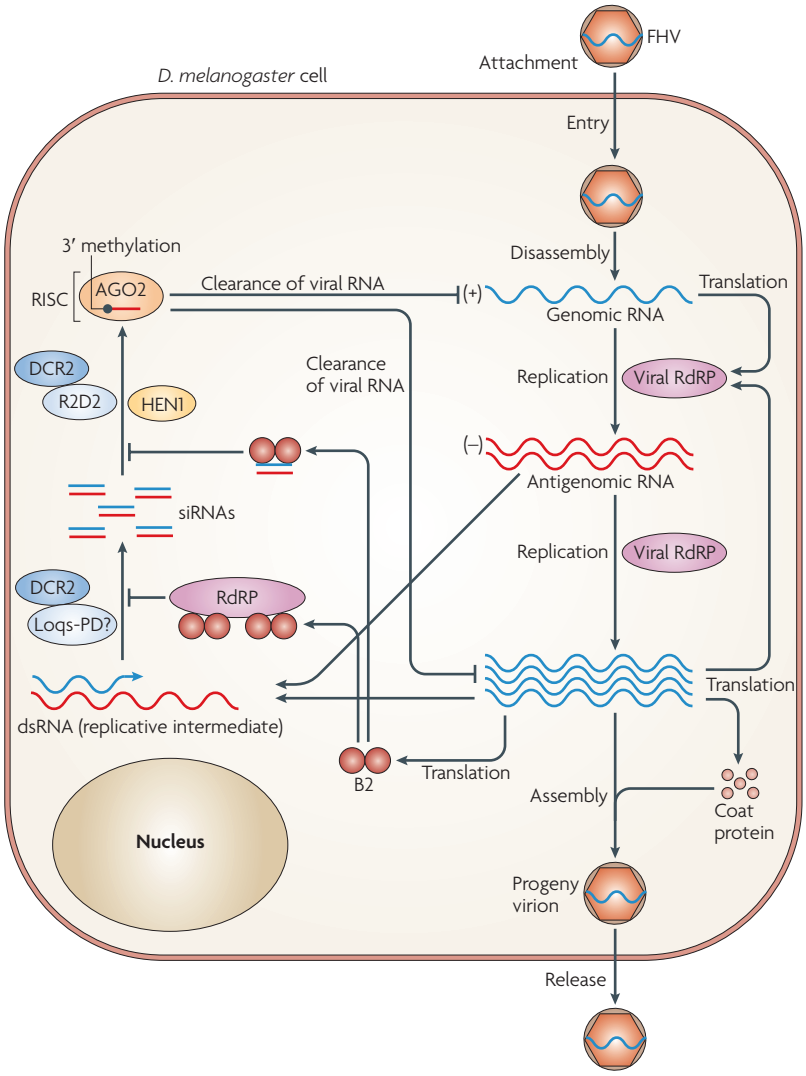

Figure 2 | **Key steps in RNA-based antiviral immunity induced in *Drosophila melanogaster* by infection of positive-strand RNA viruses such as flock house virus.** Following entry and uncoating of flock house virus (FHV) virions, the genomic positive-strand RNA ((+)RNA) serves as both mRNA for the translation of viral RNA-dependent RNA polymerase (RdRP) and as a template for the synthesis of antigenomic negative-strand RNA ((–)RNA). Preferential production of (+)RNA by viral RdRP is achieved by multiple rounds of initiation of RNA synthesis from the 3′ end of the low abundant (–)RNA. The resulting double-stranded RNA (dsRNA) formed between the 5′-terminal nascent progeny (+)RNA and the (–)RNA template is recognized by Dicer 2 (DCR2) and cleaved into small interfering RNAs (siRNAs), thereby triggering RNA-based antiviral immunity. The viral siRNAs are assembled with Argonaute 2 (AGO2) into the RNA-induced silencing complex (RISC), methylated at the 3′ end (depicted by a black circle) by HEN1 and used to guide specific clearance of FHV RNAs. As a counter-defence, FHV encodes a viral suppressor of RNA silencing (BOX 1), the B2 protein, which targets two steps in this immune pathway: inhibition of viral siRNA production by binding to viral RdRP and the viral dsRNA precursor, and sequestration of viral siRNAs by binding duplex siRNAs. Loqs-PD, loquacious-isoform PD.

Therefore, the detection of abundant antisense small RNAs in plants infected with the positive-strand RNA virus ((+) RNA virus) PVX that are similar in length to the small RNAs associated with post-transcriptionally silenced experimental transgenes supported the hypothesis that these virus-derived small RNAs are the specificity determinants of RNA-based antiviral immunity[9].

Accumulation of Flock house virus (FHV) siRNAs in infected *D. melanogaster* cells provided the first experimental evidence for the induction of RNA silencing that targets virus infection in an animal host[10]. The FHV-derived small RNAs were classified as siRNAs because, in addition to being similar in length to siRNAs processed from synthetic long dsRNA, they hybridized to probes specific for any region and either polarity of the viral genome (which is in line with the sequence-independent nature of the dsRNA from which siRNAs are derived). These FHV siRNAs are distinct from the subsequently discovered virus-derived miRNAs in invertebrates and vertebrates (Supplementary information S1 (box)), which are processed from specific sites and polarity of the viral genome[4]. The FHV-derived siRNAs are likely to have an antiviral role during infection of *D. melanogaster* because clearance of FHV genomic RNA and subgenomic RNA from infected cells requires AGO2 (REF. 10), which has been shown to load siRNAs derived from synthetic dsRNA into the RISC[15] (FIG. 2). Consistent with this finding, the FHV B2 protein, which is a viral suppressor of RNA silencing (BOX 1), is essential for FHV infection but becomes dispensable after depletion of AGO2 in *D. melanogaster* cells[10].

Subsequent studies have detected virus-derived small RNAs and RNA-based antiviral immunity in fungi, plants, *D. melanogaster*, mosquitoes, silkworms and *C. elegans*. The target viruses have many different types of genome, including (+)RNA, negative-strand RNA ((–)RNA), dsRNA, single-stranded DNA (ssDNA) and dsDNA (TABLE 1). An early study failed to detect siRNAs derived from several (+)RNA viruses in infected mammalian cells by standard small RNA sequencing protocols[16]. However, a recent survey in a wide range of mammalian host systems by deep sequencing has identified virus-derived small RNAs from four (+)RNA viruses and one (–)RNA virus[17], which contain a subpopulation of small RNAs with similar features to the viral siRNAs that are detected in plants and invertebrates (see below).

Abundance. The potency of RNA-based antiviral immunity is positively correlated with the abundance of viral siRNAs in the infected cells (in the absence of viral interference of their antiviral activity)[18,19]. Viral siRNAs can be the most dominant species in the population of small RNAs found in an infected host cell[5,17,18,20–30]. For example, approximately 20% of the total small RNAs sequenced from *D. melanogaster* cells infected with an FHV mutant that does not express the viral suppressor of RNA silencing protein B2, which would otherwise suppress viral siRNA biogenesis, were siRNAs that were a perfect match for FHV, compared with 2.7% of *D. melanogaster* miRNAs[18]. However, the abundance of viral siRNAs is low in adult *C. elegans* cells that carry an FHV replicon, ranging from 0.1% to 0.5% of total small RNAs[5,17]. Perfect-match viral siRNAs vary from 3% to 64% of the total small RNAs sequenced from virus-infected plants[24,28,29]. Viral siRNAs constitute 0.1% and 14% of total sequenced small RNAs in female adult mosquitoes infected with West Nile virus (WNV) by blood-meal feeding and

Box 1 | Viral suppressors of RNA silencing

Diverse RNA and DNA viruses of plants and animals encode proteins that are suppressors of RNA silencing (for recent comprehensive reviews, see REFS 92–94,117). Many viral suppressors of RNA silencing target the RNA components of the RNA silencing pathway, as represented by the following five suppressors.

- Animal nodaviral B2 protein binds both viral double-stranded RNA (dsRNA) replicative intermediates and the viral RNA-dependent RNA polymerase (RdRP) to inhibit the Dicer 2 (DCR2)-dependent production of viral small interfering RNAs (siRNAs) inside the viral replication complex in infected *Drosophila melanogaster* cells[18].
- Plant geminiviral V2 protein may compete for binding to 5′ overhang-containing dsRNA with an *Arabidopsis thaliana* protein, SGS3, that is essential for secondary siRNA synthesis[95–97].
- Plant tombusviral P19 protein selectively binds to siRNAs and prevents their incorporation into silencing effector complexes[98].
- Plant tobamoviral P126 protein, which is also a structural component of the viral replication complex as FHV B2, binds to siRNAs and suppresses their 3′ methylation[99], which might destabilize viral siRNAs.
- Plant crinivirus-encoded RNase3 may block RNA silencing by degrading siRNAs[100].

Similarly, many viral suppressors of RNA silencing target the protein components of the RNA silencing pathway.

- Plant cucumoviral 2b protein binds to both Argonaute 1 (AGO1) and siRNAs[69,101], which might explain the inhibition of host RdRP-dependent synthesis of viral secondary siRNAs by this protein[29,51].
- Plant caulimoviral P6 protein binds to dsRNA-binding protein 4 (DRB4) to inhibit the biogenesis of 21-nucleotide siRNAs[102].
- Plant poleroviral P0 protein, which contains an F-box-like domain, may bind AGO1 and promotes its degradation[103,104].
- Plant geminiviral AL2 and L2 proteins interact with and inactivate adenosine kinase and suppress the cytosine methylation of DNA involved in transcriptional gene silencing[74,75].
- Insect dicistroviral 1A protein binds to *D. melanogaster* AGO2 and inhibits its activity[44,105].

The activity of viral suppressors of RNA silencing is essential to ensure productive virus replication in plant, fungal and invertebrate hosts, which indicates the importance of RNA-based antiviral immunity as a defence mechanism in these hosts; however, it is not yet clear whether the same is true in vertebrates.

Piwi-interacting RNAs
(piRNAs). 24–31-nucleotide single-stranded RNAs with 5′-monophosphate and 3′-hydroxyl termini. They are independent of Dicer for biogenesis, bind to the Piwi subfamily of Argonaute (AGO) proteins for function and are found in animals but not in plants, possibly because plants do not encode any AGO protein in the Piwi subfamily.

RNA interference
(RNAi). Specific gene silencing that is induced by long double-stranded RNA or small interfering RNAs. It is used widely to knock down gene expression in plants and animals.

RNA silencing
Specific gene silencing guided by all classes of small silencing RNAs such as siRNAs, miRNAs and piRNAs.

Piwi domain
The highly conserved carboxy-terminal domain of Argonaute (AGO) proteins, which contains an RNase H motif. The catalytic centre consists of a DDH triad that functions as a metal coordinating site. AGO binding to a target RNA that is highly complementary to the loaded small interfering RNA brings the scissile phosphate, opposite nucleotides 10 and 11 of the small RNA guide, into the enzyme active site, allowing cleavage of the target RNA to leave 5′-monophosphate and 3′-hydroxyl termini.

RNA-induced silencing complex
(RISC). The effector complex of RNA silencing that contains at least two components: a single-stranded small interfering RNA or microRNA and an AGO protein.

Positive-strand RNA virus
((+)RNA virus). These viruses use RNA as genetic material. Their virions contain single-stranded genomic RNA that functions directly as mRNA and is sufficient to initiate viral infection after entry into a host cell. Tobacco mosaic virus, poliovirus and hepatitis C virus are examples.

with Sindbis virus by thorax injection, respectively[25,26]. In addition to viral suppression of siRNA biogenesis, viral siRNA abundance might be influenced by the method of virus acquisition and the presence or absence of a host mechanism that amplifies viral siRNAs (see later).

Properties. Host siRNAs and miRNAs that are produced by Dicer contain 5′-monophosphate and 3′-hydroxyl termini. Host miRNAs and siRNAs from plants and siRNAs and piRNAs from *D. melanogaster* have a 2′-*O*-methyl group at their 3′ ends introduced by the RNA methyltransferase HEN1 that protects the small silencing RNAs from degradation[6–8]. Virus-derived small RNAs isolated from plants and *D. melanogaster* also have a monophosphate group at the 5′ end, but include populations with methylated and unmethylated 3′ ends[18,29–33]. A recent study[18] shows that in *D. melanogaster* cells acutely infected with FHV, viral siRNAs bound to AGO2 are methylated at the 3′ ends (FIG. 2). The unmethylated subpopulation of viral siRNAs might correspond to those that are not bound to AGO2, because methylation of *D. melanogaster* small silencing RNAs occurs after binding to AGO proteins[6–8]. In *D. melanogaster* cells persistently infected with a virus similar to FHV, however, bulk viral siRNAs are not methylated at the 3′ end[27] and thus may not be bound to AGO2, which may explain in part the lack of viral siRNA-directed RNA silencing in persistently infected cells. Viral siRNAs in *N. benthamiana* plants infected with a potyvirus are also unmethylated[34]. However, lack of 3′ methylation in this example is attributed to potyvirus-mediated suppression of RNA silencing. Therefore, in most cases, virus-derived small RNAs have similar terminal properties to host endogenous small silencing RNAs, in contrast to most RNase hydrolysis products, which have 5′-hydroxyl and 2′,3′ cyclic phosphate, or 2′ or 3′ monophosphate, termini.

Biogenesis of virus-derived siRNAs

Precursors. Standard cloning and sequencing of 228 virus-derived small RNAs from *N. benthamiana* plants infected with Cymbidium ringspot virus (CymRSV) revealed a strong bias for (+)-strands (80%) and either the presence of hot spots or the absence of viral small RNAs corresponding to specific regions of the viral genomic and antigenomic RNA[22]. A similar (+)-strand bias of virus-derived small RNAs was also detected by standard and deep sequencing in plants infected with several additional (+)RNA viruses[22–24,28] and in mosquitoes infected with WNV, which is also a (+)RNA virus[26]. As several viral small RNA hot spots of CymRSV correspond to regions of viral genomic RNA that can be folded into stem–loop structures and as (+)RNA viruses produce 10–100-fold more (+)RNA than (–)RNA in an infected cell (which would be expected to result in a (+)-strand bias of viral small RNAs), it has been proposed that pre-miRNA-like structural regions present in viral ssRNAs are the precursors of virus-derived small RNAs that trigger RNA-based antiviral immunity[22,30].

However, replication of several (+)RNA viruses in plants, nematodes or mosquitoes does not induce a strong bias for (+)-strands in virus-derived small RNA populations[21,23,24,29] and approximately equal ratios of (+)- and (–)-strand virus-derived small RNAs have been cloned from infected *D. melanogaster* cells for all of the

Table 1 | **Virus-derived small RNAs targeting representative viruses in fungi, plants, insects, nematodes and mammals**

Viral genome	Virus name	Host	Type of vsRNA	Biogenesis	VSR	Refs
(+)RNA	Cucumber mosaic virus	Plants (for example, *Arabidopsis thaliana*)	siRNAs	DCL2–DCL4 and RDR1 and RDR6	2b	29,51
	Cymbidium ringspot virus	Plants	siRNAs	ND	P19	30,106
	Potato virus X	Plants	siRNAs	ND	P25	9
	Tobacco rattle virus	Plants (for example, *A. thaliana*)	siRNAs	DCL2–DCL4 and RDR1, RDR2 and RDR6	16K	24,50, 89
	Turnip crinkle virus	Plants (for example, *A. thaliana*)	siRNAs	DCL2 and DCL4	P38	50
	Turnip mosaic virus	Plants (for example, *A. thaliana*)	siRNAs	DCL2 and DCL4, and RDR1, RDR2 and RDR6	HC-Pro	40
	Hypovirus CHV1-EP713	Fungi	siRNAs	DCR2	P29	20
	Flock house virus	Insects (for example, *Drosophila melanogaster*)	siRNAs	DCR2	B2	10,18
		Caenorhabditis elegans‡	siRNAs	DCR-1?	B2	17,55
	Drosophila A virus	*D. melanogaster*	siRNAs	DCR2?	ND	5
	American nodavirus	*D. melanogaster*	siRNAs and piRNAs	ND	B2	5
	Drosophila C virus	*D. melanogaster*	siRNAs and piRNAs	DCR2?	1A	5
	Nora virus	*D. melanogaster*	siRNAs	DCR2?	ND	5
	Sindbis virus§	Mosquitoes	siRNAs	ND	ND	25
	West Nile virus§	Mosquitoes	siRNAs	ND	ND	26
	Poliovirus	*Homo sapiens*	siRNAs?	ND	ND	17
	Hepatitis C virus	*Homo sapiens*	siRNAs?	ND	ND	17
(–)RNA	Vesicular stomatitis virus	*C. elegans*‡	siRNAs	DCR-1?	ND	17
(–)RNA: ambisense	Tomato yellow ring virus	Plants	siRNAs	ND	NS(s)	107
dsRNA	*Drosophila* X virus	*D. melanogaster*	siRNAs	DCR2?	ND	5
	Drosophila toivirus	*D. melanogaster*	siRNAs	ND	ND	5
	Drosophila biranvirus	*D. melanogaster*	siRNAs	ND	ND	5
ssDNA: circular	African cassava mosaic virus	Plants	siRNAs	ND	AC2	108
	Cabbage leaf curl virus	Plants (for example, *A. thaliana*)	siRNAs	DCL2–DCL4	AL2	31
	Pepper golden mosaic virus	Plants	siRNAs	ND	ND	78
dsDNA: pararetro	Cauliflower mosaic virus	Plants (for example, *A. thaliana*)	siRNAs (and miRNAs?)	DCL1–DCL4	P6	31,53
dsDNA	Herpes simplex virus 1	*Homo sapiens*	miRNAs (16)	ND	ND	109,110
	Mouse cytomegalovirus	*Mus musculus*	miRNAs (18)	ND	ND	111,112
	Epstein–Barr virus	*Homo sapiens*	miRNAs (25)	ND	ND	4,113
	Simian virus 40	Monkeys	miRNA (1)	ND	ND	114
	Mouse polyomavirus	*Mus musculus*	miRNA (1)	ND	ND	115
	Heliothis virescens ascovirus	Lepidoptera	miRNA (1)	ND	ND	116

*The number of cloned miRNA species for each animal DNA virus is given in parentheses. ‡Replication of flock house virus in nematode animals or infection of primary nematode cells by vesicular stomatitis virus. §Mosquito-borne human viruses. DCL, Dicer-like protein; DCR, Dicer; dsDNA, double-stranded DNA; dsRNA, double-stranded RNA; miRNA, microRNA; (–)RNA, negative-strand RNA; ND, not determined; piRNA, Piwi-interacting RNA; (+)RNA, positive-strand RNA; siRNA, small interfering RNA; ssDNA, single-stranded DNA; VSR, viral suppressor of RNA silencing; vsRNA, virus-derived small RNA.

Subgenomic RNA
RNA transcripts of the viral RNA genome that contain only part of the sequence present in the entire genome and usually function as mRNA.

Deep sequencing
Also referred to as next-generation sequencing. This includes the Roche 454, Illumina and other high-throughput DNA sequencing platforms.

(+)RNA and dsRNA viruses examined[5,18,27]. These findings support a different model in which virus-derived small RNAs are siRNAs and are processed from a dsRNA precursor[35] (FIG. 2). This model is consistent with genetic studies that clearly show that RNA-based antiviral immunity in *D. melanogaster* and *C. elegans* is controlled by the dsRNA–siRNA pathway and that production of virus-derived small RNAs in *D. melanogaster* and *A. thaliana* is dependent on the Dicer protein (or proteins) that produces the host endogenous siRNAs, but is independent of the miRNA-producing Dicer.

In addition, deep sequencing has revealed that virus-derived small RNAs provide continuous coverage of every genomic position of many (+)RNA and dsRNA viruses in plant, insect and nematode cells, including those viruses that induce a (+)-strand bias of virus-derived small RNAs[18,24,28–30]. Viral siRNAs produced by the RNA-based antiviral immune system in plants, mosquitoes, *D. melanogaster* and *C. elegans* overlap in sequence[5,17,18,24–29]. As a result, cloned viral siRNAs are readily assembled back into large contigs covering the entire length of infecting viral genomes[5,36]. This property has allowed the development of a novel approach known as virus discovery by deep sequencing and assembly of total small RNAs (vdSAR) isolated from a host organism of interest[5,36]. Therefore, these virus-derived small RNAs are distinct from miRNAs, which are excised from one stem of a stem–loop precursor as a single, discrete species[37].

As reported for endogenous siRNAs, virus-derived small RNAs in *D. melanogaster* are predominantly 21 nucleotides in length, bind to AGO2 and are methylated at the 3′ ends. In addition, structure prediction programmes fail to place most of the virus-derived small RNA hot spots identified from tobacco mosaic virus (TMV) genomic RNA in stable stem–loop structures that would indicate a pre-miRNA-like precursor[28]. Last, many (+)RNA viruses, such as alphavirus, TMV and CymRSV, produce one or more 3′ co-terminal subgenomic RNAs that are much more abundant than genomic RNA. However, the densities of virus-derived small RNAs targeting inside and outside of the subgenomic RNA-coding region of the viral RNA genome are either insignificantly different or not in proportion to the ratio of subgenomic RNA to genomic RNA[5,24,25,28]. These findings show that virus-derived small RNAs are processed as siRNAs from a viral dsRNA precursor and that the generation of any strand bias or hot spots, both of which were detected for siRNAs sequenced from transgenic plants expressing a green fluorescent protein (GFP)-specific dsRNA[38], may occur after dicing by unknown mechanisms. It should be pointed out that specific hot spots detected by deep sequencing platforms are not always reproducible and require verification either by sequencing biological replicates or by independent approaches, such as gel blot hybridization[18,29,30].

Are viral siRNAs derived from viral dsRNA produced in a specific step of the (+)RNA virus replication cycle (FIG. 3)? A recent study investigated the genomic distribution pattern of FHV (+)- and (–)-strand siRNAs produced in *D. melanogaster* cells infected with an FHV mutant that lacks the viral suppressor of RNA silencing protein B2, which inhibits the dicing of long dsRNA into siRNAs[18,39]. The results showed that most of the sequenced viral siRNA (+)- and (–)-strands map to the 5′-terminal region (~400 nucleotides) of the viral genomic RNA1 (FIG. 3). Therefore, the presence of abundant 5′-terminal (+)- and (–)-strand viral siRNAs in the infected cells strongly supports a model in which the viral dsRNA replicative intermediates formed between the 5′-terminal nascent progeny (+)RNA and the (–)RNA template are the main precursor of viral siRNAs and, therefore, have a key role in the induction of RNA-based antiviral immunity in *D. melanogaster*. A similar 5′-terminal bias of viral siRNAs was also detected for two of the five RNA viruses that infect *D. melanogaster* S2 cells[5]. A greater abundance of 5′-terminal viral siRNAs was also detected in *A. thaliana* plants infected with a viral suppressor of RNA silencing-deficient cucumber mosaic virus (CMV) mutant. However, infection of *A. thaliana* with a viral suppressor of RNA silencing-deficient turnip mosaic virus (TuMV), or infection of *N. benthamiana* with a viral suppressor of RNA silencing-deficient CymRSV or with several other plant (+)RNA viruses, does not induce a 5′-terminal bias of viral siRNAs[24,29,30,40]. Therefore, the induction of RNA-based antiviral immunity by different RNA viruses might involve distinct types of dsRNA precursor.

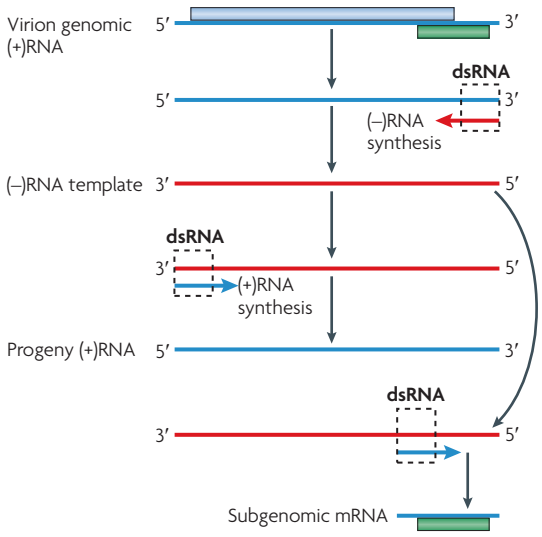

Figure 3 | **The replication cycle of a positive-strand RNA virus includes multiple steps that yield double-stranded RNA.** Nascent double-stranded RNA (dsRNA) (as indicated by the dashed boxes) may be produced during the synthesis of: negative-strand RNA ((–)RNA) from the 3′ end of the genomic positive-strand RNA ((+)RNA); (+)RNA from the 3′ end of the antigenomic (–)RNA; or subgenomic mRNA internally from the antigenomic (–)RNA.

Role of host Dicer family proteins. Dicer family proteins typically contain two RNase III domains, a canonical dsRNA-binding domain, a novel RNA-binding PAZ domain and a DEAD/H box RNA helicase domain. Dicer proteins initiate RNA silencing by recognizing dsRNA substrates and processing them into 21–24-nucleotide fragments with characteristic terminal structures[6,7,35]. The known terminal properties of virus-derived small

RNAs and their dsRNA precursors are consistent with the hypothesis[35] that, analogous to the PRRs of the innate immune system, the host Dicer proteins function as a distinct family of PRRs, which detect viral dsRNA as a PAMP and then process it into siRNAs to function as the specificity determinants of the immune effector complex in RNA-based antiviral immunity.

D. melanogaster encodes Dicer 1 (DCR1) and DCR2, which are required for the biogenesis of miRNAs and siRNAs of predominantly 22 and 21 nucleotides in length, respectively[6,7,41] (FIG. 1). Compared with wild-type flies, *dcr2*-mutant flies have increased disease susceptibility to the following (+)RNA viruses from three virus families: FHV, Sindbis virus, *Drosophila* C virus (DCV) and cricket paralysis virus[42–44]. Each of these viruses accumulated to higher levels and was more virulent in *dcr2*-mutant flies than in wild-type flies, which shows that DCR2 provides protection against diverse (+)RNA viruses in *D. melanogaster*. Genetic analyses[18,27,44] in cell culture by dsRNA depletion and in embryos and adult flies carrying loss-of-function mutations in key genes of the RNAi pathway show that FHV siRNAs are produced by DCR2, but that downstream components of the host siRNA pathway have no detectable effects on the viral siRNA biogenesis[18,27,44] (FIG. 2). These components include AGO2 and the dsRNA-binding protein R2D2, which forms a heterodimer with DCR2 to facilitate loading of host siRNA into the RISC. By contrast, the abundance of endogenous siRNAs in *D. melanogaster* depends on loading into AGO2-containing complexes[6,7], which indicates that viral siRNAs occur mainly in an AGO2-free form. Several new components of *D. melanogaster* antiviral immunity have recently been identified, including *Ars2* and a dsRNA uptake pathway[45,46], but it is not known whether any of these genes are involved in the biogenesis of viral siRNAs.

In terms of fungi, both *Neurospora crassa* and *C. parasitica* encode two Dicer proteins, which differ by the presence of a dsRNA-binding domain in DCR2 but not in DCR1 (REFS 20,47). Although the two *N. crassa* Dicer proteins act redundantly in transgene-induced RNA silencing[47], only DCR2 of *C. parasitica* is required for the biogenesis of viral siRNAs and defence against a (+)RNA virus member of the family *Hypoviridae*[20].

A. thaliana, which is a model organism in plant biology, encodes four Dicer-like (DCL) proteins. miRNAs are predominantly made by DCL1, whereas DCL4, DCL2 and DCL3 produce three size classes of endogenous siRNAs that are 21, 22 and 24 nucleotides in length, respectively[8,48] (FIG. 4). Plant defences against (+)RNA viruses are controlled by two siRNA-producing DCL proteins in a hierarchical manner[49–52] (FIG. 5). DCL4-dependent 21-nucleotide viral siRNAs are the most abundant species of viral siRNA in wild-type plants infected with (+)RNA viruses, but DCL2 alone can initiate equally potent RNA-based antiviral immunity in mutant plants that do not express DCL4[49–52]. Therefore, only *DCL4* and *DCL2* double-knockout plants are defective in viral RNA silencing and have increased disease susceptibility to infection with diverse (+)RNA viruses, including tobacco rattle virus, turnip crinkle virus, CMV and oilseed rape mosaic virus[49–52]. By contrast, DCL3-dependent 24-nucleotide viral siRNAs alone are insufficient to confer virus resistance, but they might enhance antiviral RNA silencing mediated by DCL4 and/or DCL2 in certain conditions, such as systemic silencing[49–52]. Plant viruses encode diverse viral suppressors of RNA silencing (BOX 1) that can suppress either the production or the antiviral activity of viral siRNAs and can interfere with the interpretation of the antiviral function of viral siRNAs from experimental results. For example, absence of an impact of the detected viral siRNAs on virus infection might be caused by expression of a viral suppressor of RNA silencing by the infecting virus that inhibits the antiviral activity, but not the production, of the viral siRNAs.

The most abundant species of viral siRNAs in *A. thaliana* cells infected with DNA viruses are 24 nucleotides in length and are produced by DCL3, although DCL4- and DCL2-dependent viral siRNAs of 21 and 22 nucleotides in length, respectively, can also be detected[31,53,54]. Interestingly, a significant decrease in accumulation of viral siRNAs that target the DNA virus cauliflower mosaic virus (CaMV) was detected in partial loss-of-function *DCL1* mutants for all three siRNA species (21, 22 and 24 nucleotides), but not those targeting DNA geminiviruses[31,53], indicating that DCL1 facilitates the production of viral siRNAs by DCL2, DCL3 and, in some cases, DCL4. Silencing of CaMV and geminivirus mRNAs is significantly inhibited in *DCL2*, *DCL3* and *DCL4* triple-knockout *A. thaliana* plants, but neither virus replicates to higher DNA titres or causes more severe disease symptoms[31,53]. Low levels of 21-nucleotide viral siRNAs, possibly produced by DCL1, were detected in the triple-knockout plants infected with both (+)RNA and DNA viruses[31,49–53]. The observation that the triple-knockout plants are hypersusceptible to (+)RNA viruses but remain resistant to DNA viruses suggests that viral siRNAs might guide distinct effector mechanisms against the two groups of viruses in *A. thaliana* (see below).

It is known that the single Dicer protein of *C. elegans*, which makes both miRNAs and siRNAs, is required for RNA-based antiviral immunity, but its specific activity in the production of viral siRNAs has yet to be demonstrated[55–57]. It is currently unknown how distinct viruses are detected by a specific Dicer protein in host species that encode multiple Dicer proteins. *D. melanogaster* DCR2 might function as a common sensor and producer of siRNAs for both (+)RNA and dsRNA viruses because cloned and sequenced siRNAs targeting these viruses are predominantly 21 nucleotides in length[5,18,25–27]. However, neither *Drosophila* X virus (DXV) (a dsRNA virus) nor Nora virus (a recently identified (+)RNA virus) was more virulent in homozygous *dcr2*-mutant flies than in wild-type flies[58,59], in contrast to FHV and other (+)RNA viruses. So, in the absence of the dominant antiviral DCR2, these viruses may be recognized by DCR1 or an alternative small RNA biogenesis pathway in a manner analogous to the hierarchical antiviral action of *A. thaliana* DCL4 and DCL2. Alternatively, DXV and Nora virus may encode a yet-to-be-identified viral suppressor of RNA silencing (BOX 1) that is as effective as the *dcr2* loss-of-function mutation in the inhibition of RNA-based antiviral immunity. Moreover, RDE-4 of

dsRNA uptake pathway
The endocytic pathway that mediates cell entry of double-stranded RNA in insect cells.

Systemic silencing
RNA silencing that occurs in tissues distant from the site where RNA silencing is initially induced, as a result of non-cell autonomous spread of RNA silencing in plants and *Caenorhabditis elegans*.

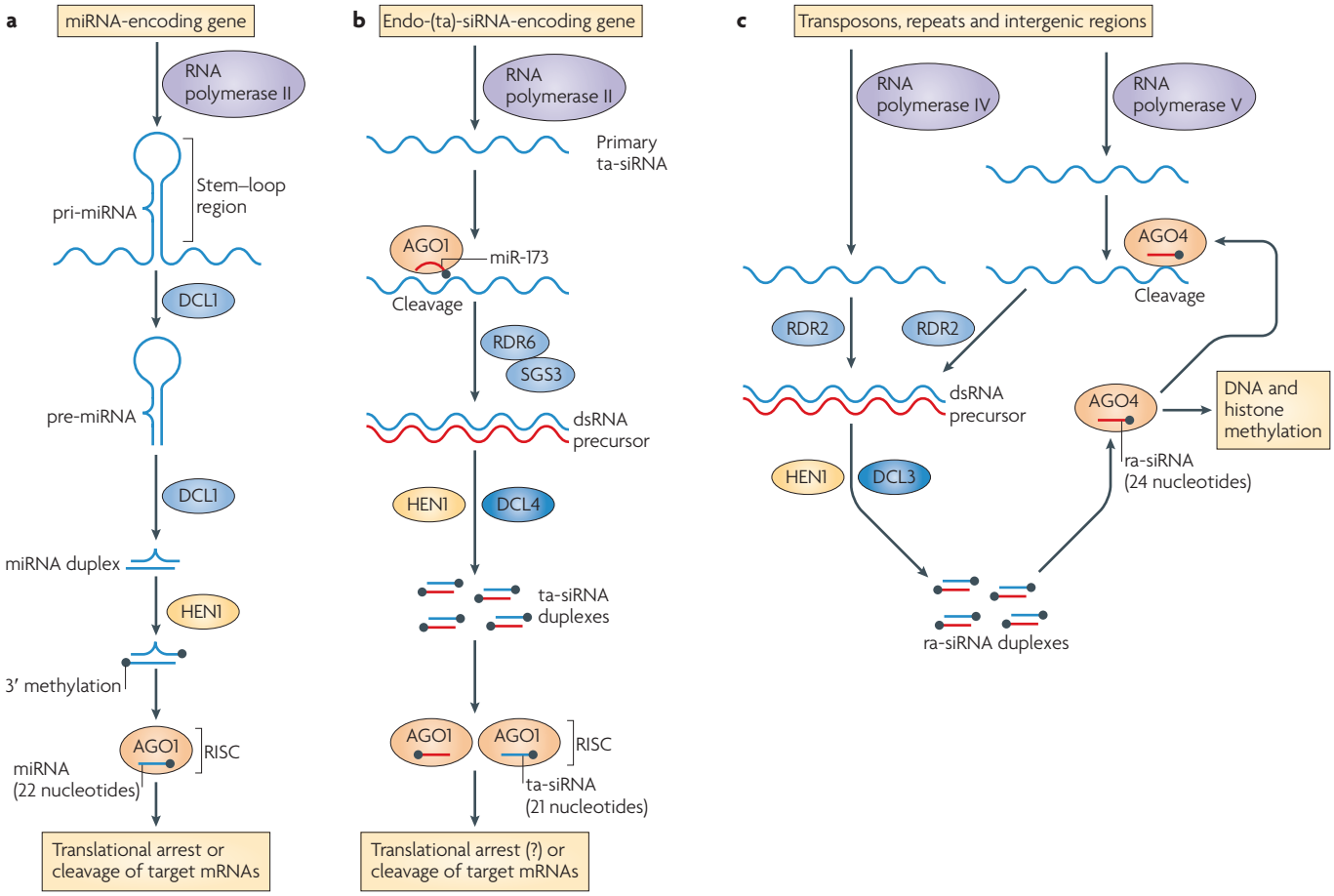

Figure 4 | **Plant small RNA pathways.** In *Arabidopsis thaliana*, endogenous microRNAs (miRNAs) (**a**) and *trans*-acting small-interfering RNAs ((ta)-siRNAs) (**b**) are produced by Dicer-like 1 (DCL1) and DCL4, respectively. Both bind to Argonaute 1 (AGO1), which is able to slice and inhibit the translation of the target mRNA. The double-stranded RNA (dsRNA) precursor of ta-siRNAs is synthesized by one of the six cellular RNA-dependent RNA polymerases (RdRPs) — RDR6 together with suppressor of gene silencing 3 (SGS3) — after AGO1-mediated cleavage of the primary ta-siRNA transcripts guided by miRNA-173 (miR-173). Similar to ta-siRNA biogenesis, the overexpression of mRNA-producing transgenes in plants triggers RDR6-dependent dsRNA synthesis and DCL4-mediated production of siRNAs in a pathway that requires SGS3 protein, which binds to 5′-overhang-containing dsRNA. By contrast, the 24-nucelotide repeat-associated (ra)-siRNAs (**c**) are produced by DCL3 after transcripts produced by the plant-specific RNA polymerase IV are converted to dsRNA by RDR2. AGO4-bound ra-siRNAs can induce methylation of DNA and histones or cleavage of transcripts produced by the plant-specific RNA polymerase V and subsequent ra-siRNA biogenesis by the RDR2-dependent pathway. *A. thaliana* HEN1 directly methylates the 3′ end of the Dicer-produced small RNA duplexes (depicted by a black circle) possibly because it contains a dsRNA-binding domain that is absent in the *Drosophila melanogaster* HEN1 homologue. pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex.

C. elegans, which is similar to the *D. melanogaster* R2D2-like dsRNA-binding protein that is found in a complex with Dicer[6,7], is essential for viral siRNA biogenesis[55,57]. Therefore, virus sensing and viral siRNA production by Dicer may require a cofactor (or cofactors) that might have a role in the specific recognition of distinct viruses by RNA-based antiviral immunity.

Dicer proteins contain a DEAD/H box RNA helicase domain that is highly homologous to that of the mammalian RLRs. A recent study shows that virus-induced expression of the *D. melanogaster* gene *Vago*, which controls virus load in flies, is abolished in *dcr2*-null mutants and flies with a missense mutation in the helicase domain of *dcr2* but not in *AGO2*- and *r2d2*-null flies[60]. So, DCR2 may be the virus sensor that induces *Vago* expression in an RNAi-independent pathway. However, further work will be necessary to determine whether *Vago* induction depends on the siRNA-producing activity of DCR2. Unlike *D. melanogaster*, *C. elegans* encodes three Dicer-related helicases (DRH-1–DRH-3), which are orthologous to the mammalian RLRs[61]. A recent genetic analysis has identified *drh-1* as an essential component of RNA-based antiviral immunity in *C. elegans*, whereas *drh-2*, which does not encode the amino-terminal domain conserved between DRH-1 and DRH-2, might be a negative regulator[55]. However, DRH-1 functions downstream of virus sensing as *drh-1*-mutant worms can produce viral siRNAs. These data indicate that this evolutionarily conserved set of host Dicer proteins participate in antiviral immunity by distinct mechanisms.

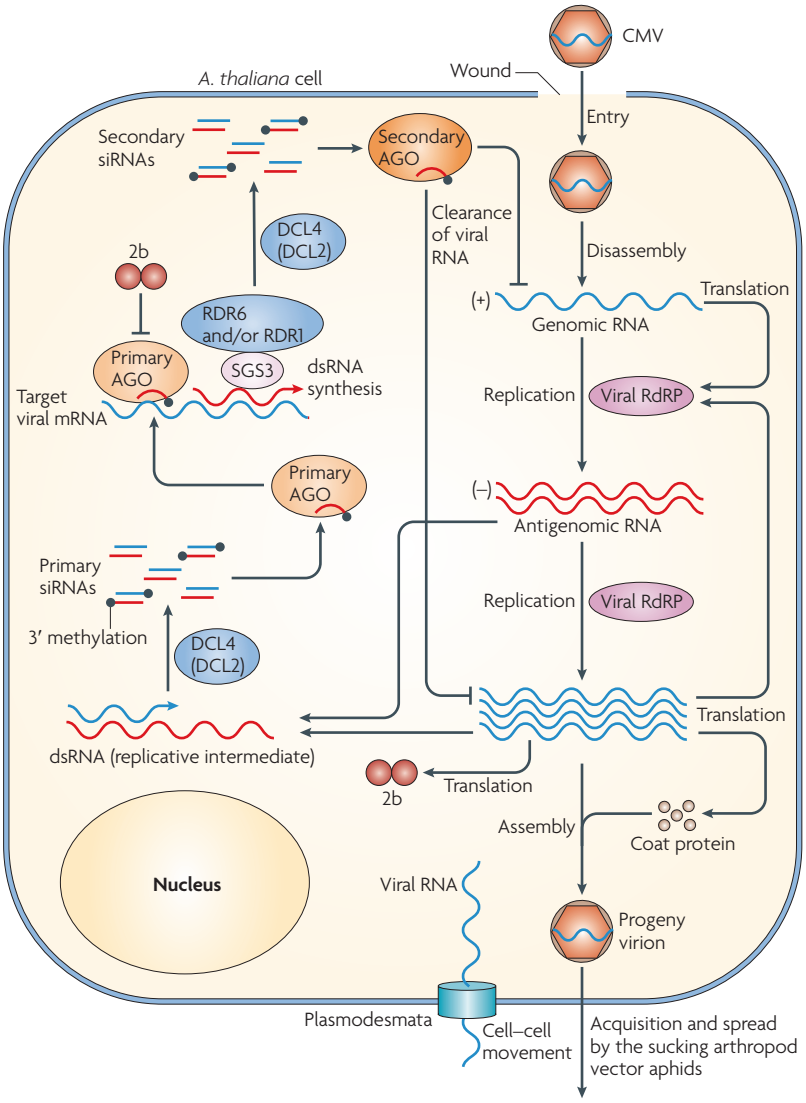

Figure 5 | **A model for RNA-based antiviral immunity induced in *Arabidopsis thaliana* by infection of positive-strand RNA viruses such as cucumber mosaic virus.** Plant viruses enter cells through a wound and spread cell to cell via plasmodesmata that connect adjacent cells. Dicer-like 4 (DCL4), DCL3 and DCL2 all have the potential to produce viral small interfering RNAs (siRNAs). However, viral siRNAs targeting positive-strand RNA ((+)RNA) viruses are predominantly made by DCL4 in wild-type plants and either DCL4 or DCL2 alone, but not DCL3, is sufficient to confer virus resistance. RNA-based antiviral immunity in plants depends on the amplification of viral siRNAs by the cellular RNA-dependent RNA polymerases (RdRPs) RDR1 or RDR6. Available data indicate that primary viral siRNAs processed from viral double-stranded RNA (dsRNA) replicative intermediates may be loaded in an Argonaute (AGO) protein (primary AGO) to guide the initial viral mRNA cleavages that trigger *de novo* dsRNA synthesis by RDR1- and RDR6-dependent pathways. Viral secondary siRNAs processed from the new dsRNA by one or more DCL proteins may be loaded in a secondary AGO to guide more potent slicing of cucumber mosaic virus (CMV) RNAs. CMV encodes a viral suppressor of RNA silencing (BOX 1), the 2b protein, which inhibits the production of viral secondary siRNAs, possibly by binding to AGO1 and dsRNA. SGS3, suppressor of gene silencing 3.

Immune effector mechanisms

All three classes of small silencing RNA direct silencing of target genes after binding to an AGO protein in a RISC-like effector complex. Some AGO proteins slice the target RNA using the RNase activity of the Piwi domain, whereas others recruit additional proteins into related RISC complexes to mediate either translational repression of the target mRNA or transcriptional silencing of the target DNA. In principle, the dicing of viral dsRNA replicative intermediates by DCR2, which would prevent replication of the viral genome, as revealed by deep sequencing of viral siRNAs from infected *D. melanogaster* cells[26,27], might be an effector mechanism of RNA-based antiviral immunity. However, RNA-based antiviral immunity is abolished in many species by genetic inactivation of a single AGO protein, including AGO2 of *D. melanogaster*[10,43,59], AGO1 and AGO7 of *A. thaliana*[62,63], RDE-1 and C04F12.1 of *C. elegans*[39,55–57] and AGO2 of *C. parasitica*[64]. These findings indicate that dicing of viral genomic material alone is insufficient and that AGO-mediated silencing activity is essential for small RNA-based antiviral immunity in fungi, plants and invertebrates[35,65].

In RNA silencing, AGO proteins are the effector molecules of specific gene silencing, the specificity of which is determined by the AGO-bound siRNAs. The AGO gene family is larger than the Dicer gene family and perhaps as a consequence much less is known about the role of AGO proteins in RNA-based antiviral immunity. Both AGO1 and AGO2 in the *D. melanogaster* AGO subfamily can slice target mRNAs that have extensive sequence complementarity to the bound siRNA or miRNA. However, miRNAs and siRNAs bind preferentially to AGO1 and AGO2, respectively, and miRNAs of *D. melanogaster* (and other invertebrates and vertebrates) have mismatches with their target mRNAs outside the seed region. Therefore, only AGO2 is involved in the canonical RNAi pathway, whereas AGO1 silences gene expression by inhibiting mRNA translation without slicing[6,7] (FIG. 1). It has been shown that RNA-based antiviral immunity is efficiently inhibited by knockdown of AGO2 in *D. melanogaster* cell culture and in adult flies carrying a loss-of-function mutation in the *AGO2* gene[10,43,59]. Co-immunoprecipitation also shows that binding of viral siRNAs to AGO2 is strongly preferred to AGO1 in persistently and acutely infected *D. melanogaster* cells[18,66] (FIG. 2). As viral siRNAs are perfectly complementary to the viral RNA genome from which they are derived, it is expected that AGO2 directs specific slicing of the target viral RNA as guided by the loaded viral siRNAs. However, AGO2-mediated slicing of viral RNAs in *D. melanogaster* cells has yet to be shown experimentally.

All of the ten AGO proteins of *A. thaliana* belong to the AGO subfamily[8,67]. AGO1 binds most miRNAs and also has a role in siRNA pathways. Many *A. thaliana* miRNAs have near-perfect complementary target mRNAs and therefore may direct both slicing and translational arrest of their mRNA targets[8,67]. AGO1 and AGO7 have been shown to have an antiviral role in genetic studies[62,63]. In addition, AGO1, AGO2 and AGO5 of *A. thaliana* bind to viral siRNAs in infected cells[68,69]. However, many questions about how AGO proteins regulate RNA-based antiviral immunity in plants remain to be addressed. For example, it is not known whether any of these *A. thaliana* AGO proteins have virus-specific slicing activity or function to inhibit viral mRNA translation[65,70,71]. RISC-like complexes that contain viral siRNAs and that slice specific viral RNAs have

been isolated from infected *N. benthamiana* plants[72,73], but further analysis is necessary to determine whether these complexes contain an AGO protein homologous to those seen in *A. thaliana*.

In the nucleus, AGO4 and AGO6 are required for the methylation of DNA and histones that inhibits gene transcription, and both AGO proteins are guided by DCL3-dependent 24-nucleotide siRNAs (FIG. 4). Recent studies[74,75] indicate that compared with RNA viruses, DNA viruses are targeted in *A. thaliana* by an additional AGO4-mediated transcriptional gene silencing effector mechanism, as suggested by previous studies[76,77]. AGO4-deficient mutant plants have increased susceptibility to two geminiviruses, cabbage leaf curl virus and beet curly top virus (BCTV). Unlike wild-type plants, AGO4-deficient mutants are unable to maintain resistance to a BCTV mutant that does not express the viral suppressor of RNA silencing protein L2 (BCTV-ΔL2). Viral siRNAs detected in geminivirus-infected plants mainly consist of 24 nucleotides, most of which target the intergenic region of the geminivirus genome[78], which contains oppositely oriented promoters[54]. Notably, whereas nearly all cytosines in the intergenic region of BCTV-ΔL2 are methylated in wild-type plants, the same region of BCTV-ΔL2 is only lightly methylated in *ago4*-mutant plants[75]. Therefore, viral siRNAs may bind AGO4 to guide viral DNA methylation and transcriptional gene silencing in a similar pathway to that which silences transposons and repeat elements in the nucleus[8]. It is possible that resistance of *DCL2*, *DCL3* and *DCL4* triple-knockout plants to DNA viruses[31,53] is mediated by the AGO4 pathway, which may be less dosage-sensitive to viral siRNAs than the RNA-based antiviral immune effector mechanisms that target RNA viruses.

Therefore, RNA-based antiviral immunity has highly specific effector mechanisms that target only the inducing virus, which is analogous to the specificity of mammalian adaptive immunity. In RNA-based antiviral immunity, virus clearance is mediated by AGO proteins with the specificity determined by an AGO-bound viral siRNA present in the effector complex. It is of interest to note that viral siRNAs, as the specificity determinants of this form of immunity, are processed directly from the RNA of the inducing virus, which is analogous to the short peptide epitopes of pathogenic origin that act as the specificity determinants in vertebrate adaptive immunity.

In both plants and *C. elegans*, the induction of RNA silencing generates a silencing signal that spreads both from cell to cell and over long distances to direct RNA silencing with the same specificity in neighbouring cells and distant tissues[79]. Recent studies in *A. thaliana* have implicated siRNAs as the mobile signal[79–81]. As reviewed previously[65], the available data suggest that the non-cell autonomous nature of RNA silencing has the potential to immunize cells and tissues ahead of the viral infection in plants, similar to adaptive immunity in vertebrates. A recent study indicates that RNA-based antiviral immunity is also non-cell autonomous in *D. melanogaster* and that it requires both the RNAi core machinery and the recently described dsRNA uptake pathway[46].

Secondary siRNAs
Small interfering RNAs (siRNAs) produced by processes that require a cellular RNA-dependent RNA polymerase, in contrast to primary siRNAs that are diced from exogenous double-stranded RNA (dsRNA) or dsRNA synthesized by viral RNA-dependent RNA polymerase.

Amplification of viral siRNAs

Vertebrate immune responses require processes to amplify immune effector mechanisms, such as the induction of transcription factors that activate expression of immune effector genes and the clonal expansion of antibody-producing plasma cells. Recent studies[29,40] have shown that effective RNA-based antiviral immunity in *A. thaliana* also depends on the amplification of viral siRNAs that are processed initially from viral dsRNA replicative intermediates. In *C. elegans*, the potency of RNAi triggered by long dsRNA depends on the production of secondary siRNAs, which require *de novo* synthesis of complementary RNA on target mRNAs by RNA-dependent RNA polymerase (RdRP) activity[6,7]. Fission yeast, plants (FIG. 4), nematodes, insects and mammals encode endogenous siRNAs with perfect complementarity to host mRNA transcripts and the biogenesis of several classes of endogenous siRNAs requires RdRP activity[6–8]. Genes homologous to the RdRP family identified by genetic screens from plants, fungi and *C. elegans* are not found in *D. melanogaster* and vertebrates[6,7]. However, elongator subunit 1 of the *D. melanogaster* polymerase II core elongator complex, D-elp1, which is conserved in all eukaryotes, has recently been shown to have RdRP activity and to have a role in RNAi[82].

It is known that two of the six putative RdRPs in *A. thaliana*, RDR1 and RDR6, and one of the four putative RdRPs in *C. elegans*, RRF-1, participate in antiviral immunity because mutants defective for these proteins have increased susceptibility to some of the RNA viruses examined[55–57,83–87]. However, until recently it was not known whether host RdRPs regulate virus resistance indirectly through the activity of RdRP-dependent siRNAs of host origin or directly through the amplification of viral siRNAs. Early studies found no significant differences in the accumulation of viral siRNAs between wild-type and single RdRP loss-of-function mutants of *A. thaliana* infected with wild-type viruses[51,88]. It is now clear that the detection of RdRP-dependent production of viral siRNAs requires the use of either viral suppressor of RNA silencing-deficient virus mutants or multiple RdRP knockout *A. thaliana* plants[29,40,51,89]. A mutant form of CMV that does not express the viral suppressor of RNA silencing protein 2b (CMV-Δ2b) is non-pathogenic in wild-type and single-RDR-knockout plants, but becomes highly virulent and accumulates to high levels in *RDR1* and *RDR6* double-knockout plants and *RDR1*, *RDR6* and *RDR2* triple-knockout plants[29]. Low levels of primary viral siRNAs detected in the double- and triple-knockout plants might mediate a basal level of RNA-based antiviral immunity because the CMV-Δ2b titre is lower in these plants than in *DCL2* and *DCL4* double-knockout and *DCL2*, *DCL3* and *DCL4* triple-knockout plants (which do not produce primary or secondary viral siRNAs). Consistently, viral siRNAs produced in *RDR6*-knockdown *N. benthamiana* plants are ineffective at directing RNA silencing of homologous host transcripts[90]. These findings suggest a model in which new viral dsRNA is synthesized by host RDR1 or RDR6 for processing by the antiviral Dicer-like proteins into viral secondary siRNAs to amplify RNA-based antiviral immunity (FIG. 5). Notably, approximately equal ratios of sense and antisense viral

siRNAs have been cloned from both the wild-type and triple-RDR-knockout plants infected with CMV-Δ2b[29]. Therefore, both primary and secondary viral siRNAs are processed from dsRNA precursors, which are most probably synthesized by the viral RNA replicase and host RdRP, respectively. Use of a TuMV mutant that is deficient for its encoded viral suppressor of RNA silencing protein HC-Pro has also shown that viral secondary siRNAs are produced and are active in antiviral silencing in *A. thaliana*[40]. However, the TuMV mutant remained non-pathogenic in the triple-RDR-knockout plants as in wild-type plants[40], which might be a result of insertion of the long GFP-coding sequence into the viral genome during generation of the mutant virus. Alternatively, resistance of the triple-RDR-knockout mutant to the TuMV mutant, but not to CMV-Δ2b, might indicate that primary viral siRNAs have a more important role in RNA-based antiviral immunity against some viruses compared with others.

Deep sequencing further shows that although either RDR1 or RDR6 alone is sufficient to confer resistance to CMV in *A. thaliana*, each protein targets specific regions of the CMV RNA genome for siRNA amplification[29], suggesting that they are involved in distinct pathways for the production of viral secondary siRNAs. As both RDR1- and RDR6-dependent viral siRNAs are products of Dicer and are structurally indistinguishable, future genetic characterization of the two RdRP pathways may have to rely on mapping specific regions of the CMV-Δ2b genome that are targeted by these two proteins. CMV infection is naturally associated with a non-homologous 336-nucleotide single-stranded satellite RNA, which depends on CMV for replication and packaging. Although siRNAs from both the satellite RNA and CMV are produced by the same set of Dicer-like proteins, the satellite RNA is not targeted by RDR1 or RDR6 for siRNA amplification[29], further showing the target specificity of the antiviral RDR proteins.

Future studies will be necessary to determine why secondary siRNAs are essential for RNA-based antiviral immunity in plants (FIG. 5) and whether the same process also occurs in animals (FIG. 2). Accumulation of viral secondary siRNAs may simply increase the total abundance of viral siRNAs in an infected cell. In *C. elegans*, primary and secondary siRNAs from exogenous dsRNA bind to specific AGO effector proteins[91]. Therefore, an alternative hypothesis is that the production of secondary siRNAs may be coupled with binding to a specific AGO protein that is different from and more potent in mediating RNA silencing than the AGO protein that binds primary siRNAs. In this regard, it will be important to determine whether viral primary and RDR1- or RDR6-dependent secondary siRNAs bind to distinct AGO proteins.

Mammalian virus-derived small RNAs

The above data clearly show that plants and invertebrates produce viral siRNAs to guide specific antiviral immunity that is mediated by AGO proteins. Virus-derived small RNAs cloned recently from mammalian cells contain a subpopulation with features of viral siRNAs derived from a dsRNA precursor[17]. For example, (+)-strand/(–)-strand ratios are approximately 1/1 for small RNAs derived from hepatitis C virus (HCV), WNV, poliovirus and vesicular stomatitis virus (VSV), despite the fact that the full-length genomic RNA of these ssRNA viruses accumulates to much higher levels than the antigenomic RNA during infection. The cloned population of virus-derived small RNAs in HCV and polio infection contains pairs of siRNA duplexes with one or two unpaired nucleotides at the 3′ ends, suggesting that they are excised from a dsRNA precursor by a type III RNase. Moreover, there is *in vivo* association of AGO proteins with a population of virus-derived small RNAs exhibiting de-enrichment of paired siRNA-like virus-derived small RNAs. However, the more abundant viral small RNAs do not correspond to predicted secondary structures, thereby ruling out biogenesis through the miRNA pathway[17]. The abundance of virus-derived small RNAs is generally low in infected mammalian cells. In poliovirus and WNV infections, the absence of a functional interferon-α/β (IFNα/β) receptor in the host increases the abundance of dsRNA-derived virus-derived small RNAs, indicating the possibility of crosstalk between the dsRNA-induced RNAi and type I IFN pathways[17].

However, it remains to be determined whether virus-derived small RNAs mediate specific silencing of viral RNAs in mammalian host cells, as found in plants and invertebrates, or modulate virus infection by RNAi-independent mechanisms. Several mammalian viruses encode essential pathogenic proteins that have RNAi suppressor activity in experimental induced-RNAi assays in mammalian and/or heterologous systems[92]. The identification of virus-derived small RNAs in mammalian cells[17] will now make it possible to determine whether any of the RNAi suppressor proteins expressed by mammalian viruses interfere with either the biogenesis or function of viral small RNAs, thus indicating that virus-derived small RNAs have an important role in mammalian immunity.

Conclusions

RNA silencing by an AGO effector protein with specificity determined by small RNAs is a widely conserved mechanism in eukaryotes. It is clear that RNA silencing functions as an antiviral defence mechanism in fungi, plants and invertebrate animals. In these hosts, dsRNA replicative intermediates of RNA viruses are recognized as a PAMP by the host PRR Dicer, which leads to processing of viral dsRNA into siRNAs to be incorporated in an AGO effector complex. Viral siRNAs have the same terminal structures and in *D. melanogaster* and *A. thaliana* are produced by the same Dicer protein and bind to the same AGO protein, as do the endogenous siRNAs. It is therefore likely that virus clearance in RNA-based antiviral immunity involves specific AGO-mediated slicing of the invading viral genomic RNA and mRNAs as guided by the cognate viral siRNAs. Interestingly, it also seems that viral siRNA-guided methylation of viral DNA is sufficient to confer resistance in plants against DNA viruses in the absence of any cleavage of viral transcripts. In *A. thaliana*, viral primary siRNAs processed from viral RNA and replication products are further amplified by host RdRPs, but it is unclear why these viral secondary siRNAs have an essential role in RNA-based antiviral immunity against (+)RNA viruses.

Satellite RNA
Non-coding linear or circular RNA molecules of a few hundred nucleotides in length that are replicated and packaged into virions by a 'helper virus', but have no significant sequence homology with the 'helper virus' genome. Different strains of satellite RNA may attenuate or intensify the disease symptoms induced by the helper virus.

Infection of (+)RNA viruses in mammalian cells also induces the production of virus-derived small RNAs that share features with the viral siRNAs that are detected in plants and invertebrates, but the possibility of RNA-based antiviral immunity in mammals requires further investigation. It is also unclear whether DNA viruses are targeted by the siRNA pathway in invertebrates and vertebrates as in plants. However, several families of nucleus-replicating mammalian DNA viruses encode discrete species of miRNAs to modulate viral pathogenesis and immunity. Notably, the piRNA pathway (Supplementary information S1 (box)), which is highly conserved between invertebrates and vertebrates, has the potential to recognize infection in *D. melanogaster* and produce viral piRNAs from (+)RNA viruses, suggesting a new role for piRNAs in antiviral defence.

In summary, RNA-based antiviral immunity involves recognition of viral dsRNA by the host immune receptor Dicer, which shares features for the detection of various forms of viral nucleic acids with several TLRs and RLRs in mammalian innate immunity. However, RNA-based antiviral immunity, as programmed by the viral siRNAs, is highly specific and has the potential to spread non-cell autonomously; it therefore also has strong parallels with epitope-specific adaptive immunity in mammals. Future challenges are to investigate the biogenesis and activity of viral secondary siRNAs and to determine just how much of what we have learnt in plant and invertebrate systems is applicable to mammals.

1. Takeuchi, O. & Akira, S. Innate immunity to virus infection. *Immunol. Rev.* **227**, 75–81 (2009).
2. Franchi, L., Warner, N., Viani, K. & Nunez, G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol. Rev.* **227**, 106–128 (2009).
3. Yoneyama, M. & Fujita, T. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol. Rev.* **227**, 54–65 (2009).
4. Pfeffer, S. *et al.* Identification of virus-encoded microRNAs. *Science* **304**, 734–736 (2004).
This article reported the first evidence for virus-derived miRNAs.
5. Wu, Q. *et al.* Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proc. Natl Acad. Sci. USA* **107**, 1606–1611 (2010).
This paper reported the first evidence for virus-derived piRNAs in animals.
6. Kim, V. N., Han, J. & Siomi, M. C. Biogenesis of small RNAs in animals. *Nature Rev. Mol. Cell Biol.* **10**, 126–139 (2009).
7. Carthew, R. W. & Sontheimer, E. J. Origins and mechanisms of miRNAs and siRNAs. *Cell* **136**, 642–655 (2009).
8. Chen, X. Small RNAs and their roles in plant development. *Annu. Rev. Cell Dev. Biol.* **25**, 21–44 (2009).
9. Hamilton, A. J. & Baulcombe, D. C. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952 (1999).
References 9, 10 and 17 reported the first evidence for virus-derived siRNAs in plants, invertebrates and mammals, respectively.
10. Li, H. W., Li, W. X. & Ding, S. W. Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319–1321 (2002).
11. 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).
12. Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296 (2000).
13. 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).
14. Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. A similarity between viral defense and gene silencing in plants. *Science* **276**, 1558–1560 (1997).
15. Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146–1150 (2001).
16. Pfeffer, S. *et al.* Identification of microRNAs of the herpesvirus family. *Nature Methods* **2**, 269–276 (2005).
17. Parameswaran, P. *et al.* Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog.* **6**, e1000764 (2010).
18. Aliyari, R. *et al.* Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in *Drosophila*. *Cell Host Microbe* **4**, 387–397 (2008).
Use of deep sequencing in this study led to identification of the first viral dsRNA precursor of virus-derived siRNAs.
19. Vanitharani, R., Chellappan, P. & Fauquet, C. M. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proc. Natl Acad. Sci. USA* **100**, 9632–9636 (2003).
20. Zhang, X., Segers, G. C., Sun, Q., Deng, F. & Nuss, D. L. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. *J. Virol.* **82**, 2613–2619 (2008).
21. Yoo, B. C. *et al.* A systemic small RNA signaling system in plants. *Plant Cell* **16**, 1979–2000 (2004).
22. Molnar, A. *et al.* Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J. Virol.* **79**, 7812–7818 (2005).
23. Ho, T., Pallett, D., Rusholme, R., Dalmay, T. & Wang, H. A simplified method for cloning of short interfering RNAs from *Brassica juncea* infected with Turnip mosaic potyvirus and Turnip crinkle carmovirus. *J. Virol. Methods* **136**, 217–223 (2006).
24. Donaire, L. *et al.* Deep-sequencing of plant viral small RNAs reveals effective and widespread targeting of viral genomes. *Virology* **392**, 203–214 (2009).
25. Myles, K. M., Wiley, M. R., Morazzani, E. M. & Adelman, Z. N. Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes. *Proc. Natl Acad. Sci. USA* **105**, 19938–19943 (2008).
26. Brackney, D. E., Beane, J. E. & Ebel, G. D. RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. *PLoS Pathog.* **5**, e1000502 (2009).
27. Flynt, A., Liu, N., Martin, R. & Lai, E. C. Dicing of viral replication intermediates during silencing of latent *Drosophila* viruses. *Proc. Natl Acad. Sci. USA* **106**, 5270–5275 (2009).
28. Qi, X., Bao, F. S. & Xie, Z. Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. *PLoS ONE* **4**, e4971 (2009).
29. Wang, X. B. *et al.* RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **107**, 484–489 (2010).
References 29 and 40 established the role of viral secondary siRNAs in RNA-based antiviral immunity.
30. Szittya, G. *et al.* Structural and functional analysis of viral siRNAs. *PLoS Pathog.* **6**, e1000838 (2010).
31. Blevins, T. *et al.* Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res.* **34**, 6233–6246 (2006).
32. Akbergenov, R. *et al.* Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Res.* **34**, 462–471 (2006).
33. Ebhardt, H. A., Thi, E. P., Wang, M. B. & Unrau, P. J. Extensive 3′ modification of plant small RNAs is modulated by helper component-proteinase expression. *Proc. Natl Acad. Sci. USA* **102**, 13398–13403 (2005).
34. Lozsa, R., Csorba, T., Lakatos, L. & Burgyan, J. Inhibition of 3′ modification of small RNAs in virus-infected plants require spatial and temporal co-expression of small RNAs and viral silencing-suppressor proteins. *Nucleic Acids Res.* **36**, 4099–4107 (2008).
35. Aliyari, R. & Ding, S. W. RNA-based viral immunity initiated by the Dicer family of host immune receptors. *Immunol. Rev.* **227**, 176–188 (2009).
36. Kreuze, J. F. *et al.* 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**, 1–7 (2009).
37. Meyers, B. C. *et al.* Criteria for annotation of plant microRNAs. *Plant Cell* **20**, 3186–3190 (2008).
38. Molnar, A. *et al.* Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**, 872–875 (2010).
39. Lu, R. *et al.* Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature* **436**, 1040–1043 (2005).
References 39, 56 and 57 established an antiviral role for RNAi in *C. elegans*.
40. Garcia-Ruiz, H. *et al. 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**, 481–496 (2010).
41. 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).
42. Galiana-Arnoux, D., Dostert, C., Schneemann, A., Hoffmann, J. A. & Imler, J. L. Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in *Drosophila*. *Nature Immunol.* **7**, 590–597 (2006).
43. van Rij, R. P. *et al.* The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in *Drosophila melanogaster*. *Genes Dev.* **20**, 2985–2995 (2006).
44. Wang, X. H. *et al.* RNA interference directs innate immunity against viruses in adult *Drosophila*. *Science* **312**, 452–454 (2006).
References 42, 43, 44 and 59 identified a key antiviral role for the dsRNA–siRNA pathway initiated by Dicer-2 in adult *D. melanogaster*.
45. Sabin, L. R. *et al. Ars2* regulates both miRNA- and siRNA-dependent silencing and suppresses RNA virus infection in *Drosophila*. *Cell* **138**, 340–351 (2009).
46. Saleh, M. C. *et al.* Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature* **458**, 346–350 (2009).
47. Catalanotto, C. *et al.* Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Mol. Cell. Biol.* **24**, 2536–2545 (2004).
48. Voinnet, O. Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**, 669–687 (2009).
49. Bouche, N., Lauressergues, D., Gasciolli, V. & Vaucheret, H. An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.* **25**, 3347–3356 (2006).
50. Deleris, A. *et al.* Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. *Science* **313**, 68–71 (2006).
51. Diaz-Pendon, J. A., Li, F., Li, W. X. & Ding, S. W. Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *Plant Cell* **19**, 2053–2063 (2007).

52. Fusaro, A. F. *et al.* RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep.* **7**, 1168–1175 (2006).
References 49–52 identified a key antiviral role for the RNA silencing pathway initiated by DCL4 and DCL2 in *A. thaliana* plants.
53. Moissiard, G. & Voinnet, O. RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* Dicer-like proteins. *Proc. Natl Acad. Sci. USA* **103**, 19593–19598 (2006).
54. Raja, P., Wolf, J. N. & Bisaro, D. M. RNA silencing directed against geminiviruses: Post-transcriptional and epigenetic components. *Biochim. Biophys. Acta* **1799**, 337–351 (2010).
55. Lu, R., Yigit, E., Li, W. X. & Ding, S. W. An RIG-I-like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in *Caenorhabditis elegans*. *PLoS Pathog.* **5**, e1000286 (2009).
56. Schott, D. H., Cureton, D. K., Whelan, S. P. & Hunter, C. P. An antiviral role for the RNA interference machinery in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA* **102**, 18420–18424 (2005).
57. Wilkins, C. *et al.* RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans*. *Nature* **436**, 1044–1047 (2005).
58. Habayeb, M. S., Ekstrom, J. O. & Hultmark, D. Nora virus persistent infections are not affected by the RNAi machinery. *PLoS ONE* **4**, e5731 (2009).
59. Zambon, R. A., Vakharia, V. N. & Wu, L. P. RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*. *Cell. Microbiol.* **8**, 880–889 (2006).
60. Deddouche, S. *et al.* The DExD/H-box helicase Dicer-2 mediates the induction of antiviral activity in *Drosophila*. *Nature Immunol.* **9**, 1425–1432 (2008).
61. Tabara, H., Yigit, E., Siomi, H. & Mello, C. C. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExX-box helicase to direct RNAi in *C. elegans*. *Cell* **109**, 861–871 (2002).
62. Morel, J. B. *et al.* Fertile hypomorphic *argonaute* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**, 629–639 (2002).
63. Qu, F., Ye, X. & Morris, T. J. *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc. Natl Acad. Sci. USA* **105**, 14732–14737 (2008).
64. Sun, Q., Choi, G. H. & Nuss, D. L. A single Argonaute gene is required for induction of RNA silencing antiviral defense and promotes viral RNA recombination. *Proc. Natl Acad. Sci. USA* **106**, 17927–17932 (2009).
65. Ding, S. W. & Voinnet, O. Antiviral immunity directed by small RNAs. *Cell* **130**, 413–426 (2007).
66. Czech, B. *et al.* An endogenous small interfering RNA pathway in *Drosophila*. *Nature* **453**, 798–802 (2008).
67. Vaucheret, H. Plant ARGONAUTES. *Trends Plant Sci.* **13**, 350–358 (2008).
68. Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M. & Watanabe, Y. The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol.* **49**, 493–500 (2008).
69. Zhang, X. *et al.* Cucumber mosaic virus-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* **20**, 3255–3268 (2006).
70. Chen, X. A microRNA as a translational repressor of *apetala 2* in *Arabidopsis* flower development. *Science* **303**, 2022–2025 (2004).
71. Brodersen, P. *et al.* Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**, 1185–1190 (2008).
72. Omarov, R. T., Ciomperlik, J. J. & Scholthof, H. B. RNAi-associated ssRNA-specific ribonucleases in Tombusvirus P19 mutant-infected plants and evidence for a discrete siRNA-containing effector complex. *Proc. Natl Acad. Sci. USA* **104**, 1714–1719 (2007).
73. Pantaleo, V., Szittya, G. & Burgyan, J. Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *J. Virol.* **81**, 3797–3806 (2007).
74. Raja, P., Sanville, B. C., Buchmann, R. C. & Bisaro, D. M. Viral genome methylation as an epigenetic defense against geminiviruses. *J. Virol.* **82**, 8997–9007 (2008).
75. Buchmann, R. C., Asad, S., Wolf, J. N., Mohannath, G. & Bisaro, D. M. Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation. *J. Virol.* **83**, 5005–5013 (2009).
76. Seemanpillai, M., Dry, I., Randles, J. & Rezaian, A. Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. *Mol. Plant Microbe Interact.* **16**, 429–438 (2003).
77. Al Kaff, N. S. *et al.* Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* **279**, 2113–2115 (1998).
78. Rodriguez-Negrete, E. A., Carrillo-Tripp, J. & Rivera-Bustamante, R. F. RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J. Virol.* **83**, 1332–1340 (2009).
79. Voinnet, O. Non-cell autonomous RNA silencing. *FEBS Lett.* **579**, 5858–5871 (2005).
80. Molnar, A. *et al.* Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**, 872–875 (2010).
81. Dunoyer, P. *et al.* Small RNA duplexes function as mobile silencing signals between plant cells. *Science* **328**, 912–916 (2010).
References 80 and 81 demonstrated short- and long-distance transport of 21–24-nucleotide siRNAs in plant cells.
82. Lipardi, C. & Paterson, B. M. Identification of an RNA-dependent RNA polymerase in *Drosophila* involved in RNAi and transposon suppression. *Proc. Natl Acad. Sci. USA* **106**, 15645–15650 (2009).
83. Mourrain, P. *et al. Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533–542 (2000).
84. Xie, Z., Fan, B., Chen, C. & Chen, Z. An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. *Proc. Natl Acad. Sci. USA* **98**, 6516–6521 (2001).
85. Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. C. 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**, 1842–1852 (2005).
86. 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).
87. Qu, F. *et al.* RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. *J. Virol.* **79**, 15209–15217 (2005).
88. Xie, Z. *et al.* Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2**, e104 (2004).
89. Donaire, L. *et al.* Structural and genetic requirements for the biogenesis of tobacco rattle virus-derived small interfering RNAs. *J. Virol.* **82**, 5167–5177 (2008).
90. Vaistij, F. E. & Jones, L. Compromised virus-induced gene silencing in *RDR6*-deficient plants. *Plant Physiol.* **149**, 1399–1407 (2009).
91. Yigit, E. *et al.* Analysis of the *C. elegans* Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell* **127**, 747–757 (2006).
92. Li, F. & Ding, S. W. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annu. Rev. Microbiol.* **60**, 503–531 (2006).
93. Diaz-Pendon, J. A. & Ding, S. W. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol.* **46**, 303–326 (2008).
94. Ruiz-Ferrer, V. & Voinnet, O. Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* **60**, 485–510 (2009).
95. Fukunaga, R. & Doudna, J. A. dsRNA with 5′ overhangs contributes to endogenous and antiviral RNA silencing pathways in plants. *EMBO J.* **28**, 545–555 (2009).
96. Elkashef, S. & Ding, S. W. Possible new RNA intermediate in RNA silencing. *Nature Chem. Biol.* **5**, 278–279 (2009).
97. Glick, E. *et al.* Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc. Natl Acad. Sci. USA* **105**, 157–161 (2008).
98. 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).
99. Vogler, H. *et al.* Modification of small RNAs associated with suppression of RNA silencing by tobamovirus replicase protein. *J. Virol.* **81**, 10379–10388 (2007).
100. Cuellar, W. J. *et al.* Elimination of antiviral defense by viral RNase III. *Proc. Natl Acad. Sci. USA* **106**, 10354–10358 (2009).
101. Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T. & Masuta, C. Characterization of silencing suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiol.* **48**, 1050–1060 (2007).
102. Haas, G. *et al.* Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *EMBO J.* **27**, 2102–2112 (2008).
103. Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P. & Ziegler-Graff, V. The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. *Curr. Biol.* **17**, 1615–1621 (2007).
104. Baumberger, N., Tsai, C. H., Lie, M., Havecker, E. & Baulcombe, D. C. The Polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Curr. Biol.* **17**, 1609–1614 (2007).
105. Nayak, A. *et al.* Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in *Drosophila*. *Nature Struct. Mol. Biol.* **17**, 547–554 (2010).
106. 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).
107. Hassani-Mehraban, A., Brenkman, A. B., van den Broek, N. J., Goldbach, R. & Kormelink, R. RNAi-mediated transgenic Tospovirus resistance broken by intraspecies silencing suppressor protein complementation. *Mol. Plant Microbe Interact.* **22**, 1250–1257 (2009).
108. Chellappan, P., Vanitharani, R., Pita, J. & 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).
109. Umbach, J. L. *et al.* MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780–783 (2008).
110. Jurak, I. *et al.* Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. *J. Virol.* **84**, 4659–4672 (2010).
111. Buck, A. H. *et al.* Discrete clusters of virus-encoded microRNAs are associated with complementary strands of the genome and the 7.2-kilobase stable intron in murine cytomegalovirus. *J. Virol.* **81**, 13761–13770 (2007).
112. Dolken, L. *et al.* Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation. *J. Virol.* **81**, 13771–13782 (2007).
113. Umbach, J. L. & Cullen, B. R. The role of RNAi and microRNAs in animal virus replication and antiviral immunity. *Genes Dev.* **23**, 1151–1164 (2009).
114. Sullivan, C. S., Grundhoff, A. T., Tevethia, S., Pipas, J. M. & Ganem, D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* **435**, 682–686 (2005).
115. Sullivan, C. S. *et al.* Murine Polyomavirus encodes a microRNA that cleaves early RNA transcripts but is not essential for experimental infection. *Virology* **387**, 157–167 (2009).
116. Hussain, M., Taft, R. J. & Asgari, S. An insect virus-encoded microRNA regulates viral replication. *J. Virol.* **82**, 9164–9170 (2008).
117. Wu, Q. F. Wang, X. B. and Ding, S. W. Viral suppressors of RNA-based viral immunity: host targets. *Cell Host Microbe* **8**, 12–15 (2010).

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