

Antiviral Immunity Directed by Small RNAs

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Plants and invertebrates can protect themselves from viral infection through RNA silencing. This antiviral immunity involves production of virus-derived small interfering RNAs (viRNAs) and results in specific silencing of viruses by viRNA-guided effector complexes. The proteins required for viRNA production as well as several key downstream components of the antiviral immunity pathway have been identified in plants, flies, and worms. Meanwhile, viral mechanisms to suppress this small RNA-directed immunity by viruses are being elucidated, thereby illuminating an ongoing molecular arms race that likely impacts the evolution of both viral and host genomes.

Introduction

Two major lines of evidence indicate that several RNA silencing components form the core of an antiviral defense in plants and invertebrates. First, virus-derived siRNAs (viRNAs) targeting viral RNAs accumulate during infections. Second, to counteract this defense, viruses produce essential virulence factors called viral suppressors of RNA silencing (VSRs). Here, we review recent progress in understanding the mechanisms for production and function of viRNAs in plants and invertebrates. We emphasize how deciphering the genetics of antiviral RNA silencing in these organisms has been instrumental in elucidating the mode of action of VSRs. We also discuss emerging evidence that viruses often impinge on or subvert small RNA-directed functions, notably in mammals, and we underline the consequences of the intimate interactions between viruses and RNA silencing pathways for the evolution of both viral and host genomes.

Viruses and RNA Silencing

Viruses are obligate intracellular pathogens that infect all forms of life. Their genomes, packaged into virions, comprise DNA or RNA that is either single-stranded (ss) or double-stranded (ds). Both dsRNA and ssRNA viruses—the latter being further divided into positive-sense (+) and negative-sense (–) ssRNA viruses—encode their own RNA-dependent RNA polymerase (RdRP, or RDR for cellular RdRPs) for error-prone replication. RNA genomes can be replicated via DNA intermediates through the action of viral reverse transcriptases, as exemplified by retroviruses, or via RNA intermediates through the activity of host DNA-dependent RNA polymerases, exemplified by viroids. Viroids are composed of ~300 nucleotide circular ssRNAs and are pathogenic to many plant species.

Organisms have diverse mechanisms for combating viral infections. One mechanism—discovered first in plants and subsequently in invertebrates—is through RNA silencing. This mechanism of gene silencing depends

upon small RNAs that are 21–30 nucleotides in length and are divided into three main classes: small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-associated interfering RNAs (piRNAs). siRNAs and miRNAs are processed as duplexes from dsRNA precursors by an RNaseIII enzyme called Dicer (Hammond, 2005). Perfectly base-paired dsRNAs are the precursors of siRNA populations. In contrast, primary miRNA transcripts (pri-miRNA) contain imperfect intramolecular stem loops and are first processed within the nucleus. The resulting precursor-miRNAs (pre-miRNA) are then converted into a single mature miRNA species in the cytoplasm (Bartel, 2004). In plants, however, processing of miRNAs occurs entirely in the nucleus (Vaucheret, 2006). miRNAs regulate important biological processes, and, hence, plants and animals with compromised miRNA functions display severe developmental defects (Bartel, 2004; Vaucheret, 2006). In the fruit fly *Drosophila*, miRNAs and siRNAs are products of two distinct Dicers, Dicer-1 (Dcr1) and Dicer-2 (Dcr2), respectively (Hammond, 2005). On the other hand, worms and vertebrates only have one Dicer that produces both miRNAs and siRNAs. The model plant *Arabidopsis thaliana* encodes four Dicer-like proteins (DCL1 to DCL4): DCL1 primarily synthesizes miRNAs (Bartel, 2004), whereas DCL2, DCL3, and DCL4 process long dsRNA molecules of various cellular origins into siRNA populations that are 22, 24, and 21 nucleotides in length, respectively (Brodersen and Voinnet, 2006). In plants, fungi, and worms, siRNA production is amplified through de novo dsRNA synthesis by cellular RNA-dependent RNA polymerases, leading to secondary siRNA accumulation (Wassenegger and Krczal, 2006). In contrast to siRNAs and miRNAs, piRNAs—which are ~30 nucleotides in length and are found in the germline of flies and vertebrates—are Dicer independent (Zamore, 2007).

Effector complexes called RNA-induced silencing complexes (RISCs) are assembled upon loading of one selected small RNA strand into one member of the Argonaute (Ago) protein family (Tolia and Joshua-Tor, 2007). *Drosophila*

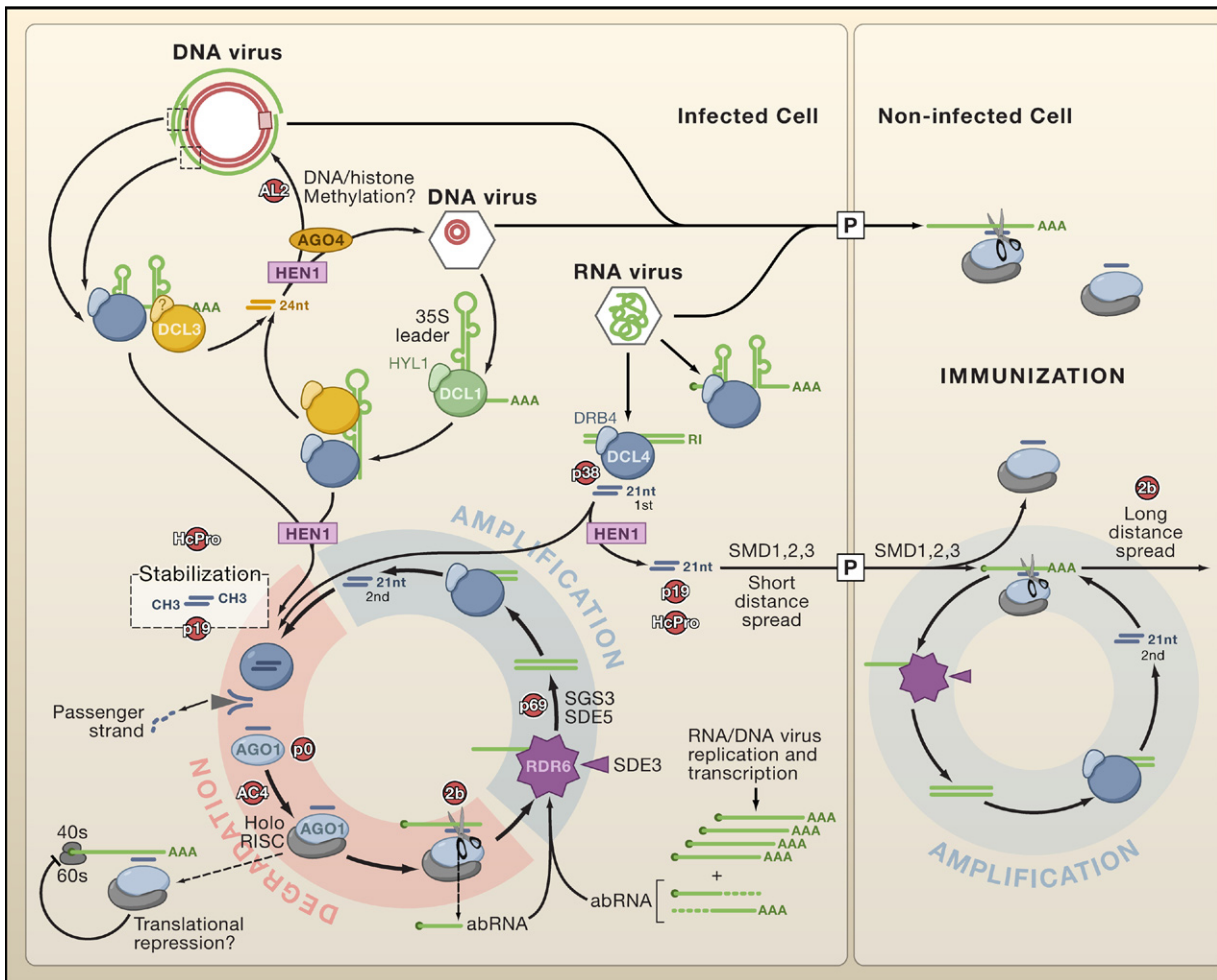


Figure 1. Antiviral Silencing in *Arabidopsis*

Dicer-like proteins (DCLs) are represented in association with known and unknown cognate double-stranded (ds)RNA-binding proteins (DRBs). Note the indirect implication of DCL1 in viRNA biogenesis from DNA viruses (e.g., *Cauliflower mosaic virus*) and the putative contribution of DCL3-dependent viRNAs to viral DNA/histone methylation. DCL4 is the primary Dicer to detect RNA viruses and is replaced by DCL2 if suppressed (for example by the VSR P38; see also Figure 3B). AGO1 is presented as a major antiviral slicer, but other AGO paralogs are likely to be involved, potentially also mediating translational repression. All viRNAs are stabilized through HEN1-dependent 2'-O-methylation. The figure shows how primary viRNAs (1st) are amplified into secondary viRNAs (2nd) in the RDR6-dependent pathway. A similar scheme is anticipated with the salicylic acid-induced RDR1 (not shown; Diaz-Pendon et al., 2007). Aberrant (ab) viral mRNAs lacking a cap or polyA tail (AAA) can enter RNA-dependent RNA polymerase pathways independently of 1st viRNA synthesis. A DCL4-dependent silencing signal (arbitrarily depicted as free 21 nucleotide viRNAs) moves through the plasmodesmata (P) to immunize neighboring cells. Movement may be enhanced through further rounds of amplification involving viral transcripts that enter immunized cells. VSRs and potential endogenous silencing suppressors (red) represent genetic rather than direct physical interactions with host silencing components.

Ago1 and Ago2 recruit miRNAs and siRNAs, respectively, whereas piRNAs interact with one of three related proteins, Piwi, Aubergine, and Ago3 (Zamore, 2007). Ago proteins are often named Slicer proteins because they cleave target ssRNAs at the duplex formed with the guide-strand small RNA (Tolia and Joshua-Tor, 2007). Nonetheless, miRNAs can guide gene silencing through translational arrest without slicing (Bartel, 2004), and siRNAs can mediate transcriptional nuclear gene silencing through DNA and/or histone methylation. For example, in *Arabidopsis*, DCL3-dependent 24 nucleotide siRNAs recruit AGO4 to transcriptionally silence transposons and DNA repeats through

chromatin modifications (Matzke and Birchler, 2005). Small RNAs also accumulate upon virus infection in plants and invertebrates, but their origin and the involvement of Dicer and Argonaute proteins in their processing and action were not well understood until recently.

**Small RNA-Directed Antiviral Immunity
Origin, Processing, and Stability of Virus-Derived siRNAs**

viRNAs from (+)ssRNA viruses were thought to be populations of siRNAs produced from long dsRNA replication intermediates (Figure 1). Accordingly, cloning and

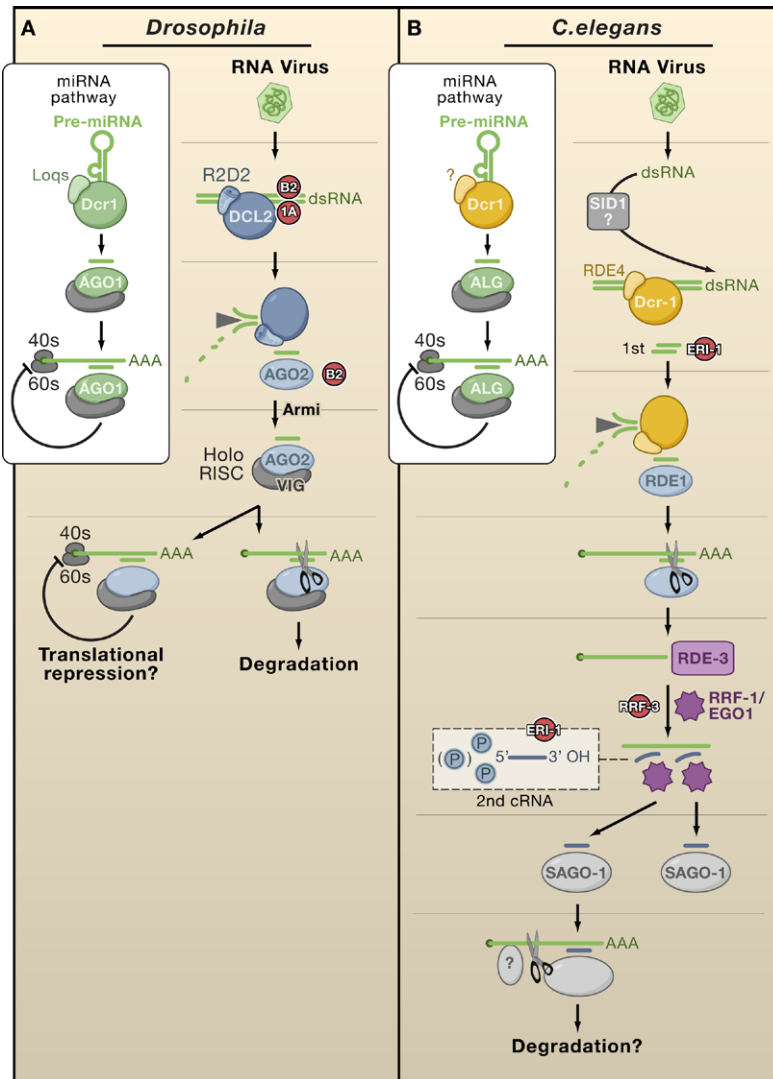


Figure 2. Antiviral Silencing in Flies and Worms

(A) The *Drosophila* pathway is conceptually similar to a linear antiviral silencing pathway in plants. Although R2D2 heterodimerizes with Dcr2, it is required for loading but not dicing of viRNA; the Armitage (Armi) protein allows assembly of the RISC holoenzyme. The box illustrates the involvement of Dcr1 and AGO1 in the miRNA pathway leading to translational repression. This pathway can be disrupted at multiple points by VSRs (red).

(B) Antiviral silencing in *C. elegans* has been inferred through studies of artificial infection systems. ALG, RDE-1, and SAGO are worm AGOs that recruit miRNAs, 1st siRNAs, and 2nd siRNAs, respectively. RRF-1 is thought to produce 2nd siRNAs or to copy RNAs (cRNAs) directly from RDE3-stabilized templates. SID-1 may possibly take up viral dsRNAs into cells. These pathways can be disrupted at multiple points by endogenous suppressors (red).

strand viRNAs accumulate much higher than antigenomic (replicating)-strand viRNAs, suggesting that they largely originate from hairpins formed by extensive folding of the covalently closed viroid RNA genome, necessary for virulence and spread (Itaya et al., 2007). Therefore, plant antiviral Dicers are probably stimulated by both long dsRNA replication intermediates and imperfect RNA hairpins required for the pathogen's biology. Other plant viRNA sources include dsRNA segments formed by overlapping sense-antisense transcripts produced by circular geminivirus ssDNA genomes (Figure 1). The origin of viRNAs from invertebrate viruses is yet to be characterized.

sequencing showed approximately equal ratios of (+) and (-) strand viRNAs derived from several (+)ssRNA plant viruses (Ho et al., 2006; Yoo et al., 2004). However, far more abundant (+)viRNAs than (-)viRNAs were detected in plants infected with tombusviruses and carmoviruses, and, notably, many (+)viRNAs mapped to discrete intramolecular hairpins within viral genomic RNA (Ho et al., 2006; Molnar et al., 2005; Figure 1). This finding was surprising, as those secondary structures should be strongly selected against by viruses, unless they are required for viral genome expression, replication, or encapsidation. For example, the 35S polycistronic transcript of the dsDNA plant virus *Cauliflower mosaic virus* contains an extensive secondary structure, the "35S leader" (Figure 1), which, in spite of being a major viRNA source, is preserved in all *Cauliflower mosaic virus* strains, possibly because it is crucial for ribosome shunting during translation (Moisiard and Voinnet, 2006). Similarly, viroid genomic-

In *Drosophila*, mutations in the two Dicer genes uncouple miRNA from siRNA biogenesis (Hammond, 2005). Because *dcr2* knockout adults develop normally, they were used to establish Dcr2 as a major immunity determinant against four distinct (+)ssRNA insect viruses: flock-house virus, *Drosophila C* virus, cricket paralysis virus, and Sindbis virus (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006). Flock-house virus titers are also increased in flies deprived of R2D2, a dsRNA-binding protein required downstream of dicing (Figure 2A), but, unlike in *dcr-2* mutants, viRNAs accumulate normally in *r2d2* mutants (Wang et al., 2006), implying that Dcr2 produces these molecules. In contrast, accumulation of *Drosophila X* virus (dsRNA genome) was unaffected in *dcr2* mutant flies (Zamboni et al., 2006), suggesting that Dcr-1 or perhaps the nuclease involved in piRNA biogenesis may be involved. Nonetheless, viRNAs remain to be detected in *Drosophila X* virus-infected flies.

Although natural *C. elegans* viruses have not been isolated, replication of flock-house virus and vesicular stomatitis virus—a (–)ssRNA virus that naturally infects biting flies and livestock—induces antiviral silencing in adult worms and embryonic cells, respectively, and detection of vesicular stomatitis virus-specific viRNAs was reported (Lu et al., 2005; Schott et al., 2005; Wilkins et al., 2005). Because vesicular stomatitis virus replication is enhanced in cells partially deprived of Dcr-1 (Schott et al., 2005), the sole Dicer of *C. elegans* likely has antiviral roles in worms (Figure 2B). However, Dcr-1 processing of viral RNA awaits biochemical demonstration.

Individual mutations in the four *Arabidopsis* DCLs had not been convincingly linked to changes in virus susceptibility, suggesting functional redundancy in antiviral immunity (Brodersen and Voinnet, 2006). Accordingly, recent studies demonstrate that inactivating both DCL4 and DCL2 is necessary and sufficient to elicit the highest susceptibility to several (+)ssRNA viruses and to dramatically decrease viRNA accumulation (Bouché et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Diaz-Pendon et al., 2007). DCL4 is the primary antiviral Dicer against these (+)ssRNA viruses and produces 21 nucleotide-long viRNAs. viRNA synthesis by DCL2 is hardly detectable when DCL4 is functional, but DCL2 rescues antiviral silencing if DCL4 is genetically inactivated or suppressed, producing 22 nucleotide-long viRNAs (Figure 1). DCL3-dependent synthesis of 24 nucleotide-long viRNAs has been only detected in wild-type plants infected with *Tobacco rattle virus* and *Cucumber mosaic virus* and in single or double *dcl2/dcl4* mutants infected with *Turnip crinkle virus*. Although loss of DCL3 activity in *dcl2/dcl4* mutants further increased virus titers, 24 nucleotide viRNAs were inactive in targeting homologous mRNA for degradation, presumably because DCL3 products normally guide chromatin modifications (Figure 3C). The contribution of the miRNA-specific DCL1 to immunity against (+)ssRNA viruses is negligible because *dcl2/dcl3/dcl4* and *dcl1/dcl2/dcl3/dcl4* mutants showed unaltered susceptibility to *Cucumber mosaic virus* or *Turnip crinkle virus*, and DCL1-dependent viRNAs were hardly detectable even in the *dcl2/dcl3/dcl4* mutant. Therefore, hierarchy and redundancy link *Arabidopsis* DCLs to dsRNA produced by cytoplasmically replicating (+)ssRNA viruses. In contrast, the four DCLs cooperate in defense against two DNA viruses replicating in the nucleus: *Cauliflower mosaic virus* and a geminivirus (ssDNA virus). DCL4 and DCL3 were the prevalent Dicers, whereas DCL2 activity was mostly evident following DCL4 inactivation (Blevins et al., 2006; Moissiard and Voinnet, 2006). Increased susceptibility to *Cauliflower mosaic virus* and reduced viRNA accumulation were only observed if *DCL4*, *DCL2*, and *DCL3* were all disabled (Moissiard and Voinnet, 2006). Thus, unlike in (+)ssRNA virus infections, DCL3 has clear antiviral roles in natural DNA virus infections. Moreover, DCL1 was required for optimal accumulation

of DCL4- and DCL3-dependent viRNAs from the *Cauliflower mosaic virus* 35S leader (Figure 1). DCL1 likely excises such hairpins from primary transcripts and thereby facilitates their subsequent access by the other DCLs, a process resembling the nuclear and DCL1-dependent pri-miRNA to pre-miRNA conversion step. This process is not likely to affect hairpin structures within RNA viruses, which are cytoplasmic.

The hierarchy of DCL action may be influenced by the subcellular localization of viral dsRNA and DCLs and by intrinsic DCL affinities for various dsRNA forms. All four *Arabidopsis* DCLs, perhaps with the exception of DCL2, appeared to localize to the nucleus based on reporter-gene fusion experiments (Vaucheret, 2006). Yet, under appropriate genetic circumstances, each DCL can process viral dsRNA of either cytosolic or nuclear origin. Thus, either the DCL localization data are inaccurate or some cytoplasmic viral dsRNAs are imported into the nucleus for processing. Alternatively, DCLs might relocalize during infection. How, when, and where are Dicers expressed during infection? How does their cellular distribution compare with the sites of viral RNA replication, translation, or encapsidation? These questions deserve careful attention both in plants and animals.

The different DCL affinities for various dsRNA substrates could depend on dsRNA-binding proteins (DRB) known to physically and specifically interact with Dicers in several organisms. In *Arabidopsis*, DRB1 (also known as HYL1) facilitates DCL1-dependent miRNA production from their fold-back precursors (Bartel, 2004; Vaucheret, 2006), and DRB4 enhances DCL4 processing of long dsRNAs involved in endogenous gene silencing (Vaucheret, 2006; Figure 1). *Drosophila* Dcr1 interacts with Loquacious (Loqs or R3D1) to process miRNAs, and Dcr2 associates with R2D2 during RNA interference (RNAi; Hammond, 2005). The closest *C. elegans* R2D2 homolog, RDE-4, interacts with Dcr-1 (Hammond, 2005). Mutations in *rde-4* enhance vesicular stomatitis virus replication in worm cells (Schott et al., 2005), and *r2d2* mutant flies accumulate greater titers of flock-house virus, cricket paralysis virus, and *Drosophila* X virus (Wang et al., 2006; Zamboni et al., 2006), indicating a key role for those dsRNA-binding proteins in antiviral immunity (Figures 2A and 2B). Assigning such a role to the five *Arabidopsis* dsRNA-binding proteins has been comparatively difficult because of possible functional redundancy.

Specific proteins influence small RNA stability upon dicing. *Arabidopsis* HEN1 methylates the 2' hydroxy groups at the 3'-end termini of cellular small RNAs, protecting them from degradation (Chen, 2007). Resistance to beta-elimination indicates that plant viRNAs are also methylated (Blevins et al., 2006; Figure 1). Moreover, *hen1* mutants accumulate less viRNAs from RNA and DNA viruses and exhibit reduced virus-induced gene silencing (Blevins et al., 2006). 3'-end methylation of animal piRNAs has just been reported (Chen, 2007), and, thus, animal viRNAs might carry similar protective modifications. In *C. elegans*, several mutations enhance

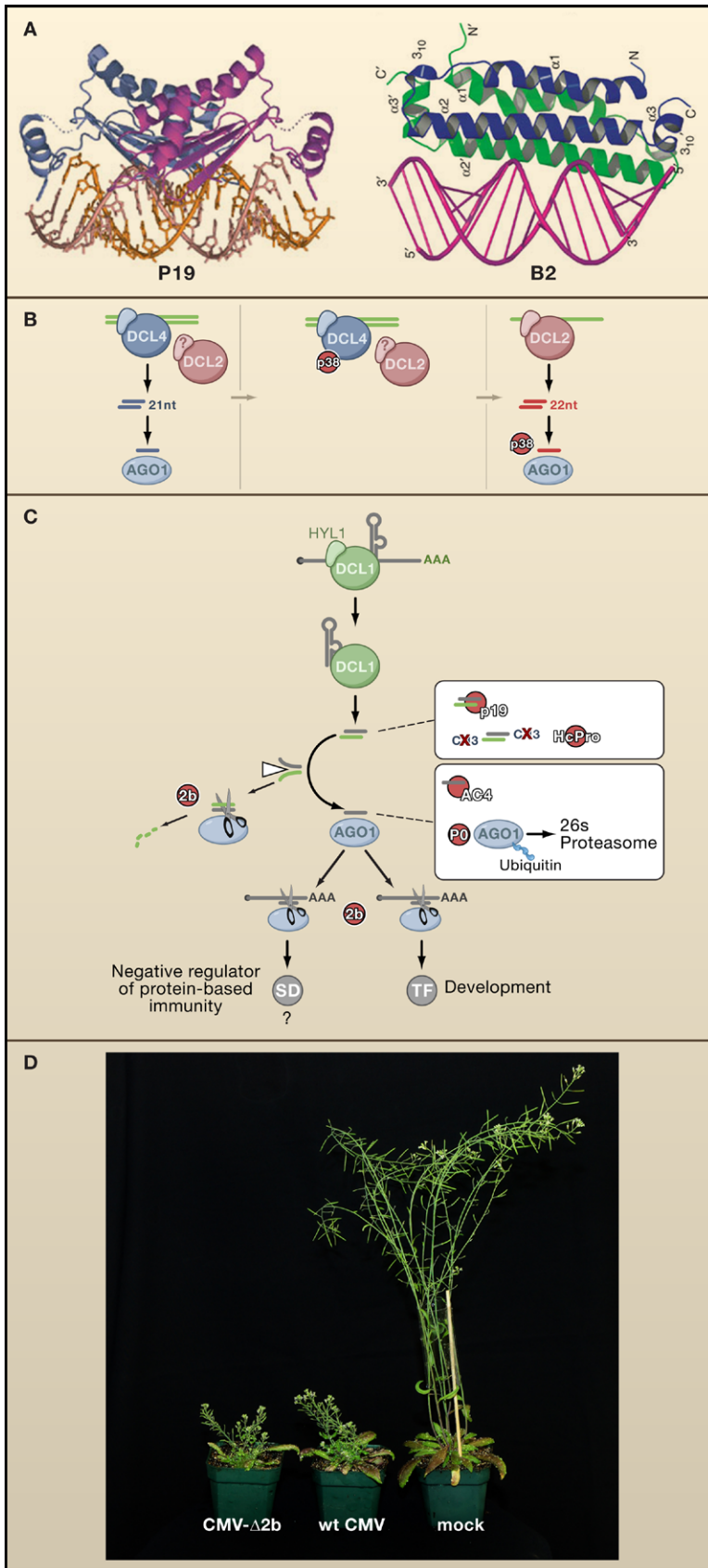


Figure 3. VSRs of Plant and Fly Viruses

(A) The VSRs P19, encoded by *Cymbidium ringspot virus*, and B2, encoded by flock-house virus, both bind dsRNA but with very different structural requirements. P19 acts as a head-to-tail homodimer that binds to and specifically measures 21 bp duplexes that are the products of DCL4. In contrast, B2 forms a four-helix bundle that binds to one face of an A-form RNA duplex, independent of its length. P19 in complex with siRNA, reprinted by permission from MacMillan Publishers Ltd: Nature (98), copyright 2003, and B2 in complex with dsRNA, adapted by permission from MacMillan Publisher Ltd: Nat. Struct. Mol. Biol. (109), copyright 2005.

(B) Inhibition of DCL4 by the P38 VSR of *Turnip crinkle virus* reveals the redundant antiviral function of DCL2 which generates 22 nucleotide instead of 21 nucleotide viRNAs in *Turnip crinkle virus*-infected *Arabidopsis*. The antiviral activity of DCL2-dependent viRNAs is in turn further compromised by P38, possibly through inhibition of AGO1.

(C) Transgenically expressed VSRs interfere with the *Arabidopsis* DCL1-dependent miRNA pathway. In contrast to the genetic interactions in Figure 1, these interactions are likely to be physical. VSRs may interfere both with developmental programs mediated by transcription factors (TF) and innate immune pathways negatively regulated by suppressors of defense (SD).

(D) Both wild-type *Cucumber mosaic virus* (middle) and a version lacking the VSR 2b (CMV-Δ2b, left) induce similarly severe stunting symptoms in *dcl2/dcl4* double mutant plants, demonstrating that the VSR is dispensable for infection and disease induction in a host defective in small RNA-directed immunity (Diaz-Pendon et al., 2007). Image courtesy of R. Lu.

RNAi, one of which is Eri-1, encoding an exonuclease thought to degrade worm small RNAs (Grishok, 2005; Figure 2B). Embryonic cells and adult worms lacking Eri-1 function are only slightly more resistant to vesicular stomatitis virus, an effect strongly enhanced by additional mutations in *lin-15B*, a suppressor of somatic RNAi (Schott et al., 2005).

Antiviral RNA-Induced Silencing Complexes

The mere existence of plant and invertebrate antiviral RISCs has been debated because Dicer-mediated processing of viral dsRNA is, in principle, sufficient to impede virus replication. Nonetheless, viRNAs inhibit expression of homologous cellular transcripts in *trans*. This has been shown with plant and insect recombinant viruses whose genomes incorporate fragments of host or reporter transcripts. Upon infection, the viral symptoms mimic knockdown mutations in the corresponding mRNA, a process called virus-induced gene

silencing (Ruiz et al., 1998; Uhlirova et al., 2003). Virus-induced gene silencing elicited by *Tobacco rattle virus* was used to explore the requirements of a putative RISC in *Arabidopsis* antiviral defense (Deleris et al., 2006). Virus-induced gene silencing was unaltered in *dcl2/dcl3* and *dcl3/dcl4* double mutants, but was abolished in *dcl2/dcl4* plants, which exhibited strong symptoms and high viral titers. However, there were equivalent levels of 21, 22, and 24 nucleotide viRNAs in *dcl2/dcl3*, *dcl3/dcl4*, and *dcl2/dcl4* plants, respectively, suggesting that dicing per se is not sufficient for antiviral immunity (Deleris et al., 2006). Direct evidence for a viRNA-loaded RISC came from studies of *Cymbidium ringspot virus* (a (+)ssRNA tombusvirus; Pantaleo et al., 2007). Because *Cymbidium ringspot virus* viRNAs derive mostly from discrete hot spots within the (+)RNA strand, a viRNA-guided RISC was expected to target symmetrical positions in the (–)RNA strand. These cleavage fragments were indeed detected and carried nontemplated U residues at predicted cut sites, a signature of RISC-directed cleavage. A likely antiviral slicer in plants is AGO1, a major component of the miRNA-guided RISC (Figures 1 and 3C). First, AGO1 has the catalytic residues diagnostic of cleavage-competent AGOs (Baumberger and Baulcombe, 2005). Second, *ago1* hypomorphic mutants are more susceptible to viruses (Morel et al., 2002). Third, AGO1 immunoprecipitates with viRNAs in infected tissues (Zhang et al., 2006), and fourth, *Cymbidium ringspot virus*-derived viRNAs and cellular miRNAs cofractionate in two protein complexes that likely correspond to free AGO1 (~150 kDa) and partially or fully assembled (holo) RISC (~650 kDa), respectively (Pantaleo et al., 2007). A similarly sized complex was isolated in separate experiments involving a virus related to *Cymbidium ringspot virus*. This complex contained viRNAs and exhibited in vitro nuclease activity that was virus sequence preferential and ssRNA specific (Omarov et al., 2007).

Drosophila Ago2 was the first component identified in the antiviral RNA silencing pathway of the animal kingdom (Li et al., 2002). Dcr2 expression fails to restrict flock-house virus replication in fly embryos deprived of the siRNA-guided slicer Ago2 (Wang et al., 2006), whereas *ago2* mutants are hypersusceptible to *Drosophila* C virus and *Drosophila* X virus (van Rij et al., 2006; Zambon et al., 2006). Likewise, knockdown of *Anopheles gambiae* Ago2 increases viral titers in cell culture and adults (Li et al., 2004; Keene et al., 2004). Therefore, as in plants, dicing alone is insufficient for insect antiviral immunity, and the additional function of Ago2 is required (Figure 2A). Other mutations downstream of dicing in the dsRNA-siRNA pathway enhance *Drosophila* and *C. elegans* susceptibility to viruses. Flock-house virus and vesicular stomatitis virus replicate more in worms deprived of the Ago2 paralog, RDE1, and of RDE4, which links Dcr-1 to RDE1 (Lu et al., 2005; Schott et al., 2005; Wilkins et al., 2005). Similarly, components of the Ago2-associated RISC restrict *Drosophila* X virus accumulation in fly, including the Vasa intronic gene dsRNA

helicase (VIG), armitage, and r2d2, required for RISC assembly and siRNA loading (Zambon et al., 2006; Figure 2). Nonetheless, although these factors likely form an antiviral holoRISC, sliced viral RNA fragments await characterization in *Drosophila* and *C. elegans*.

viRNA-loaded RISCs could also inhibit viral mRNA translation, as do plant and animal miRNA-loaded RISCs (Bartel, 2004). Furthermore, RISCs directing DNA/histone methylation could recruit viRNAs to impact DNA viruses. For example, de novo asymmetric cytosine methylation—a hallmark of DCL3/Ago4-directed heterochromatic silencing—occurs on both DNA strands of *Tomato leaf curl virus* and restricts its replication (Bian et al., 2006; Figure 1). A general issue of antiviral RISCs is functional diversification: there are 26, 10, and 5 AGO-like proteins in *C. elegans*, *Arabidopsis*, and *Drosophila*, respectively, and many have the catalytic residues of authentic Slicers (Tolia and Joshua-Tor, 2007). Perhaps there is no single antiviral RISC in those organisms but, rather, a multilayered network of defensive complexes (Lu et al., 2005) with redundancy and specialization. Hence, the antiviral function of worm and fly miRNA-directed Agos has not been examined yet. Moreover, Piwi and Aubergine, two *Drosophila* Slicers involved in the germline piRNA pathway that silences retrotransposons, inhibit *Drosophila* X virus replication (Zambon et al., 2006). Another unresolved issue concerns target accessibility: the quasi-rod-shaped RNA genome of viroids is an effective DCL substrate but is largely inaccessible to slicing, presumably because RISC cannot resolve extensive intramolecular RNA folds (Itaya et al., 2007). By extension, dicing hot spots found within virion RNA strands may not be efficiently sliced, agreeing with the mapping of several RISC-directed cleavage sites on the *Cymbidium ringspot virus* genomic (+)ssRNA (Pantaleo et al., 2007). Interestingly, the same study showed no cleavage of the replicating (–)ssRNA even though cleavage was detected if a homologous RNA was expressed transgenically, suggesting that replication within membrane-bound vesicles normally protects the (–)ssRNA from (+)viRNA-loaded RISC.

Amplification and Spread of Antiviral Silencing

RNA silencing can be amplified by cellular RNA-dependent RNA polymerases identified in plants, *C. elegans*, and fungi, but not in insects or vertebrates which do not possess these enzymes (Wassenegger and Krczal, 2006). In plants, amplification entails at least two mechanisms. In the first mechanism, primary siRNAs derived from viral- or transgene-dsRNA recruit RNA-dependent RNA polymerases to homologous ssRNA, from which complementary RNA synthesis and subsequent secondary siRNA production ensues. These secondary RNAs are not related to the primary targeted site. This process—called transitivity—occurs in virus-induced gene silencing of transgenes, during which transgene-specific siRNAs from regions absent in the recombinant viral genome accumulate (Voinnet, 2005). Targeting transgenes through transitivity in *Arabidop-*

sis requires RDR6, the RNA-helicase SDE3, and SDE5, which resembles human mRNA export factor TAP (Voinnet, 2005; Hernandez-Pinzon et al., 2007). Together with AGO1 and the coiled-coil protein SGS3, RDR6, SDE3, and possibly SDE5 mediate a second RNA-dependent RNA polymerase mechanism, in which primary siRNA production is dispensable (Voinnet, 2005; Vaucheret, 2006). This mechanism likely perceives aberrant RNAs spuriously produced by transgenes, transposons, or viruses and converts them de novo into dsRNA. Aberrations include the lack of 5' cap or polyA tail, frequent in aborted viral transcription products (Figure 1; Brodersen and Voinnet, 2006).

The two RNA-dependent RNA polymerase mechanisms are not mutually exclusive, and both are likely required to keep pace with viral replication. *Arabidopsis* mutants lacking components of the AGO1-RDR6-SGS3-SDE5 pathway support higher *Cucumber mosaic virus* titers comparable to those in *dcl2/dcl4* mutant plants (Bouché et al., 2006; Deleris et al., 2006; Vaucheret, 2006). These results—together with those from recent studies of transgene silencing (Moissiard et al., 2007)—suggest that the same DCL2-DCL4 consortium is required for synthesis of primary and secondary small RNAs, although secondary viRNA accumulation has been experimentally verified only recently (Diaz-Pendon et al., 2007). Tobacco plants with reduced RDR6 activity also exhibit temperature-dependent hypersusceptibility to several unrelated (+)ssRNA viruses (Qu et al., 2005; Schwach et al., 2005). No antiviral role has been ascribed so far to RDR2, involved in the DCL3-AGO4 heterochromatic silencing pathway (Matzke and Birchler, 2005). However, the existence of six *Arabidopsis* RNA-dependent RNA polymerase paralogs (of which only RDR1, RDR2, and RDR6 have established functions) suggests both redundancy and specialization in antiviral defense, as seen with DCLs and, possibly, with DRBs and AGOs. Consistent with this idea, reducing the activity of RDR1 in tobacco increases susceptibility to tobamo-, tobra- and potex-viruses, but not to *Cucumber mosaic virus* (Yang et al., 2004; Yu et al., 2003).

C. elegans RNA-dependent RNA polymerase mechanisms differ from those of plants. In worms, siRNAs accumulating in response to exogenous dsRNA are predominantly secondary siRNAs produced through unprimed RNA synthesis (Grishok, 2005). Unlike Dcr-1-dependent primary siRNAs, secondary siRNAs are exclusively antisense, have 5' di- or triphosphates, and are thought to occur as individual RNA-dependent RNA polymerase products, or “copy” (c)RNA, directly synthesized from target mRNA upon primary siRNA-guided cleavage (Pak and Fire, 2007; Sijen et al., 2007; Figure 2B). Three of the four *C. elegans* RNA-dependent RNA polymerases have been studied: RRF-1 is mandatory for somatic RNAi, and EGO-1 is its germline-specific counterpart; RRF-3 negatively regulates RRF-1 and EGO-1, possibly by competing for RNAi effectors (Grishok, 2005). RRF-1 function might require the RNAi component RDE-3, a putative

beta-nucleotidyltransferase that could stabilize RRF-1 templates. Because vesicular stomatitis virus replication is enhanced in *rrf-1* and *rde-3* mutant cells and decreased in *rrf-3*, RNAi amplification likely contributes to antiviral immunity (Schott et al., 2005; Wilkins et al., 2005; Figure 2B). It will be important to determine which proportion of viRNAs have the distinctive features of (c)RNAs because the (c)RNAs involved in experimental RNAi are only in their minority associated with the AGO homolog RDE1 (Yigit et al., 2006), which is, nonetheless, crucial for antiviral defense.

In plants, RNA-dependent RNA polymerases influence the extent to which silencing moves from cell-to-cell through plasmodesmata and over long distances through the phloem (Voinnet, 2005). Cell-to-cell RNAi spread involves two interrelated processes differing in their requirement for transitivity. In short-range signaling (10–15 cells) the specific activity of DCL4, but not of RDR6 or SDE3, is mandatory for synthesis or detection of a signal, whose movement necessitates at least three *SILENCING MOVEMENT DEFICIENT* genes (*SMD1* to *-3*; Voinnet, 2005). Long-range cell-to-cell signaling requires that RDR6, SDE3, and possibly SDE5 convert homologous transcripts into new dsRNA in cells receiving the short-range signal (Voinnet, 2005). DCL4 processes the new dsRNA into secondary 21 nucleotide siRNAs, and movement proceeds over another 10–15 cells. Short- and long-range signaling events are related to primary and secondary viRNA synthesis, respectively, and likely impact virus movement (Figure 1). Hence, VSR-deficient *Cymbidium ringspot virus* and *Turnip crinkle virus* accumulate in vascular bundles, yet fail to unload into neighboring cells, which, although virus-free, exhibit sequence-specific resistance to secondary infection (Deleris et al., 2006; Havelda et al., 2003). As this phenomenon is alleviated in *Arabidopsis* loss-of-function *dcl4* mutant, cell-to-cell spread and amplification of DCL4-dependent signals likely immunize tissues ahead of the infection (Deleris et al., 2006; Figure 1). Long-distance silencing movement through the vasculature, demonstrated with *Potato virus X* (Voinnet, 2005), also likely has antiviral roles because it is precluded by the *Cucumber mosaic virus*-encoded VSR protein 2b (Guo and Ding, 2002). Upon vascular transport, silencing amplification helps immunizing recipient tissues, as RDR6 activities enabling detection/amplification of long-distance transgene silencing exclude viruses from meristems—the stem cell niches at apical growing points (Qu et al., 2005; Schwach et al., 2005).

Systemic RNAi in *C. elegans* involves many tissue-specific components (Voinnet, 2005). However, a near-ubiquitous player is SID-1, a transmembrane channel enabling long dsRNA uptake in target organs (Figure 2B). Although no biological function has been ascribed to SID-1, systemic antiviral defense is now a testable possibility due to the establishment of flock-house virus replication in adult worms (Lu et al., 2005). The impact of RNA-dependent RNA polymerases in systemic antiviral

silencing might be harder to evaluate because their function is, above all, mandatory for cell-autonomous silencing. Nonetheless, systemic transitive RNAi observed in *C. elegans* (Voinnet, 2005) could entail antiviral effects of RRF-1 and RDE-3 in whole organisms. The cell-autonomy of RNAi triggered by an inverted-repeat transgene and the absence of recognizable genome-encoded RNA-dependent RNA polymerases in *Drosophila* have prompted the idea that arthropods lack systemic silencing. However, injected dsRNA allows robust systemic gene knockdown in adult *Drosophila*, *Tribolium castaneum* and *A. gambiae* (Voinnet, 2005). Furthermore, injected dsRNA, but not siRNAs, confers systemic, sequence-specific antiviral immunity to adult shrimps (Robalino et al., 2005). Therefore, dsRNA dissemination mechanisms, together with recently discovered dsRNA uptake mechanisms based on receptor-mediated endocytosis (Saleh et al., 2006), may well contribute to arthropod antiviral defense.

Viral Suppressors of RNA Silencing **dsRNA Binding by VSRs: Separating the Wheat from the Tares**

More than 35 individual VSR families have been identified from virtually all plant virus types, unraveling a necessary and ubiquitous counterstrategy (Li and Ding, 2006). VSRs were also isolated from insect and fungus viruses including flock-house virus (B2), cricket paralysis virus/*Drosophila C virus* (1A), and *Cryphonectria parasitica* hypovirus (P29; Li et al., 2002; Segers et al., 2006; Wang et al., 2006; van Rij et al., 2006). VSRs are strikingly diverse within and across kingdoms because they are often encoded by novel, out-of-frame overlapping genes contained within more ancient genes. Their acquisition is through fast evolutionary convergence, confining VSRs within tight lineages in virus phylogenies. Consequently, similar silencing suppression strategies may evolve independently several times such that unrelated VSRs might share analogous biochemical properties. For example, both *Cymbidium ringspot virus*-encoded P19 and flock-house virus-encoded B2 proteins display dsRNA-binding activities required for suppression, but X-ray crystallography studies reveal very distinct protein folds in these proteins (Figure 3A). B2 dimerizes into a four-helix bundle that binds A-form RNA duplexes independently of length (Chao et al., 2005). In contrast, P19 head-to-tail homo-dimers form a “siRNA caliper” that specifically sequesters DCL4-dependent 21 bp RNA duplexes (Vargason et al., 2003; Ye et al., 2003). B2 inhibits long dsRNA processing by Dcr2 in vitro, as does the *Drosophila C virus*-encoded 1A protein (Lu et al., 2005; Fenner et al., 2007; van Rij et al., 2006). Moreover, point mutations disrupting dsRNA binding by B2 and 1A abolish their VSR activity. viRNA sequestration by P19 prevents their incorporation into RISC, and plants infected with P19-deficient *Cymbidium ringspot virus* (CymRSV- Δ P19) contain vastly reduced virus titers (Lakatos et al., 2004). Therefore, dsRNA binding by B2, 1A, and P19 is nec-

essary to silencing suppression. These examples have promoted dsRNA binding as a popular feature in characterizing VSRs, but recent results obtained with many additional recombinant viral proteins with suspected or established VSR functions should be cautiously interpreted (Lakatos et al., 2006). First, RNA binding is often nonspecific: the octameric ring formed by monomers of P21, a plant closterovirus VSR, shows equal affinity for long, short, single-, or double-stranded RNAs, providing no obvious insight into its mode of action (Ye and Patel, 2005). In fact, dsRNA binding might often reflect additional VSR functions, unrelated to suppression, that require close association to viral nucleic acids. Moreover, in vitro binding assays for many VSRs usually lack crucial negative controls involving stable loss-of-function VSR alleles. Consequently, whether dsRNA binding is a bona fide feature of silencing suppression remains, in most cases, unresolved.

Emerging Themes in VSR Functions

VSRs are often studied as isolated proteins that are transgenically or transiently expressed with a second reporter transgene (Li and Ding, 2006), an approach showing increasing limitations. Any given virus might produce multiple VSRs—as do *Citrus tristesa virus* and geminiviruses (Lu et al., 2004; Vanitharani et al., 2004)—with probable temporally or spatially restricted functions that could be overlooked in transgenic/transient expression. The experimental silencing systems used in VSR studies can also have misleading outputs: VSRs acting upstream of viRNA synthesis will be inactive against siRNA-induced RNAi; those inhibiting secondary viRNA synthesis will not be recognized in the context of silencing triggered by inverted-repeat-transgenes that produce fold-back RNAs, which does not require RDR activities. Additionally, the level and timing of VSR and silencing trigger expression are usually set arbitrarily such that the results from independent systems might not be comparable and may provide exaggerated or inaccurate views of VSR function during authentic infections. These and other caveats emphasize the value of recent genetic rescue experiments involving viruses with disabled or modified VSRs. For example, out of many *dcl* combination mutants, only in *dcl2/dcl4* double mutants were the VSR-deficient mutants of *Turnip crinkle virus* (TCV- Δ P38) and *Cucumber mosaic virus* (CMV- Δ 2b) as virulent as their wild-type counterparts in wild-type plants (Deleris et al., 2006; Diaz-Pendon et al., 2007). This clearly implicates pathways initiated by DCL2 and DCL4 as genetic targets of both P38 and 2b (Figures 3B and 3D). Similarly, rescue of B2-deficient flock-house virus in Ago2-depleted cells and in *dcr2* and *ago2* *Drosophila* mutant embryos unequivocally implicated the Dcr2-Ago2 pathway in anti-flock-house virus silencing, establishing a molecular framework to characterize B2 function (Li et al., 2002; Wang et al., 2006).

Studying TCV- Δ P38 provided insights into the P38 mode of action and simultaneously unraveled the functional redundancy between DCL2 and DCL4 in viRNA

synthesis. DCL2-dependent, 22 nucleotide viRNAs normally accumulate in *Turnip crinkle virus*-infected wild-type *Arabidopsis*. However, 21 nucleotide, instead of 22 nucleotide, viRNAs accumulate in response to TCV- Δ P38, indicating that, although DCL4 is the primary Dicer to access *Turnip crinkle virus* dsRNA, its action is suppressed by P38. Consequently, DCL2 substitutes DCL4 to produce viRNAs 22 nucleotide in length whose activity is, in turn, also compromised by P38 (Deleris et al., 2006; Figure 3B). This dual P38 action readily explains why inactivating DCL2 or DCL4 separately has little impact on wild-type *Turnip crinkle virus* infection. Therefore, a full appreciation of antiviral silencing requires the use of VSR-deficient viruses because only under these conditions are the redundant effects of VSRs and those of genetic mutations in host silencing factors uncoupled. Further emphasizing this notion, only with CymRSV- Δ P19 was the demonstration of an active viRNA-loaded RISC possible, because most viRNAs are normally sequestered by P19 in wild-type *Cymbidium ringspot virus* infection (Lakatos et al., 2004; Pantaleo et al., 2007).

Viral Antisilencing Strategies

In *Drosophila*, the flock-house virus B2 and *Drosophila* C virus/cricket paralysis virus 1A proteins directly inhibit viral dsRNA processing (Lu et al., 2005; Fenner et al., 2007; van Rij et al., 2006). Nonetheless, VSRs may suppress many additional steps, sometimes simultaneously (Figures 1 and 3C). Tobamovirus infection and the potyviral VSR HcPro mimic mutations in *hen1*: viRNAs become oligo-uridylylated and partially degraded because they lack 2'-O methylation (Ebhardt et al., 2005; Yu et al., 2006), as do P19-bound viRNA duplexes (Yu et al., 2006). The geminivirus VSR AC4 seems to prevent holoRISC assembly by capturing single-stranded small RNAs normally bound by AGOs (Chellappan et al., 2005), whereas the *Cucumber mosaic virus* 2b protein physically interacts with siRNA-loaded AGO1 and inhibits slicing (Zhang et al., 2006). Because AGO1 immunoprecipitates with *Cucumber mosaic virus*-derived viRNAs, this finding supports the idea that AGO1 is an antiviral slicer, as does the observation that the P0 VSR of poleroviruses contains an F-box-like domain that likely promotes ubiquitin-dependent proteolysis of AGO1 (Pazhouhandeh et al., 2006). Suppression of sense transgene silencing (RDR6-dependent) but not of silencing induced by inverted repeats (RDR6-independent) by the *tymoviral* P69 protein suggests that it inhibits viRNA amplification (Chen et al., 2004), although direct suppression of an RNA-dependent RNA polymerase activity is yet to be documented. Several VSRs that sequester, degrade, or inactivate primary viRNAs also inhibit transitivity and de novo dsRNA production by RNA-dependent RNA polymerases (Moissiard et al., 2007), providing possible insights into the suppression of silencing movement ahead of the infection (Figure 1).

AC2 from begomoviruses is a viral transcriptional activator whose nuclear localization, DNA-, and zinc-binding domains are all necessary to its VSR function.

Transgenic AC2 indeed acts on host DNA by inducing \sim 30 genes, of which one possibly encodes a cellular silencing suppressor (Trinks et al., 2005). Similarly, HcPro interacts with a calmodulin-related protein (rgs-CaM) that suppresses silencing when overexpressed (Anandalakshmi et al., 2000). Silencing suppression by AL2, the AC2 paralog of curtoviruses (another genus of the *geminiviridae*), relies, in contrast, on direct protein-protein interaction with adenosine kinase (Wang et al., 2005). Because adenosine kinase sustains the cellular methyl levels, its inhibition could prevent methylation of viral episomes by DCL3-dependent viRNAs. RNA- rather than protein-based suppression has been reported with *Red clover mosaic virus*, where RNA elements required for (-)-strand synthesis likely recruit and titrate DCL activities (Takeda et al., 2005). Suppression might not even entail specific VSR functions. Hence, the silencing defense response of *Arabidopsis* against transfer-DNAs (T-DNA) of virulent *Agrobacterium tumefaciens* is overcome by expression of T-DNA-encoded oncogenes promoting host cell proliferation (Dunoyer et al., 2006). The dramatic reduction of DCL activities during cell proliferation may also benefit plant geminiviruses that typically reactivate host DNA replication to amplify their genome (Matthews, 1991). Viroid genomes encode no protein yet resist silencing because they are structurally protected against RISC activity (Itaya et al., 2007). Viral genome association with capsid or movement proteins also likely contributes to reduced access by silencing ribonucleases, as does the compartmentalization of their replication into vesicles or organelles. Finally, the high rates of replication and movement of viruses might often outcompete the silencing machinery, both at cellular and tissue levels.

Suppression of Cellular Silencing Pathways

Many antiviral silencing factors are components of cellular pathways regulating host gene expression, and, thus, VSRs are expected to interfere with those pathways. VSRs that sequester small RNAs (e.g., P19) or inhibit slicing by AGO1 (e.g., 2b) commonly stabilize accumulation of host miRNAs in an inactive duplex form because the normally labile passenger strand, called miRNA*, is no longer unwound and degraded (Figure 3C). Consequently, cellular miRNA targets, including developmentally important transcription factor mRNAs, accumulate in cells where they should be cleared by AGO1-dependent miRNA activities (Figure 3C). Therefore, VSR transgenic plants frequently exhibit recurrent developmental anomalies resembling those of *hyl1*, *ago1*, or *dcl1* mutants (Chapman et al., 2004; Dunoyer et al., 2004; Zhang et al., 2006; Figure 3D). However, the fact that incidental inhibition of miRNA-directed functions brings out some of the symptoms elicited by viruses is probably true only to some extent because these studies involve constitutive or inducible VSR expression in a much broader tissue range than in natural infections. Second, miRNAs and other cellular small RNAs have recently emerged as key regulators

of basal and race-specific disease resistance in *Arabidopsis* (Katiyar-Agarwal et al., 2006; Navarro et al., 2006). These protein-based processes protect plants against many pathogens, including viruses. Therefore, inhibition of endogenous small RNA pathways by VSRs might reflect a deliberate viral strategy to inhibit such immune systems (Figure 3C). Studies of the virulence-attenuating hypovirus of *Cryphonectria parasitica* (chestnut blight fungus) also illustrate how VSR might affect important host traits. The decreased fungal pathogenicity is partly due to reduced asexual sporulation (conidiation), recently ascribed to the hypovirus-encoded P29 papain-like protease (Segers et al., 2006). Because P29 resembles the potyviral HcPro and has VSR activities in *C. parasitica*, perturbation of fungal RNA silencing pathways required for conidiation could contribute to hypovirus-mediated attenuation of fungal fitness.

RNA Silencing in Vertebrate Viral Infections

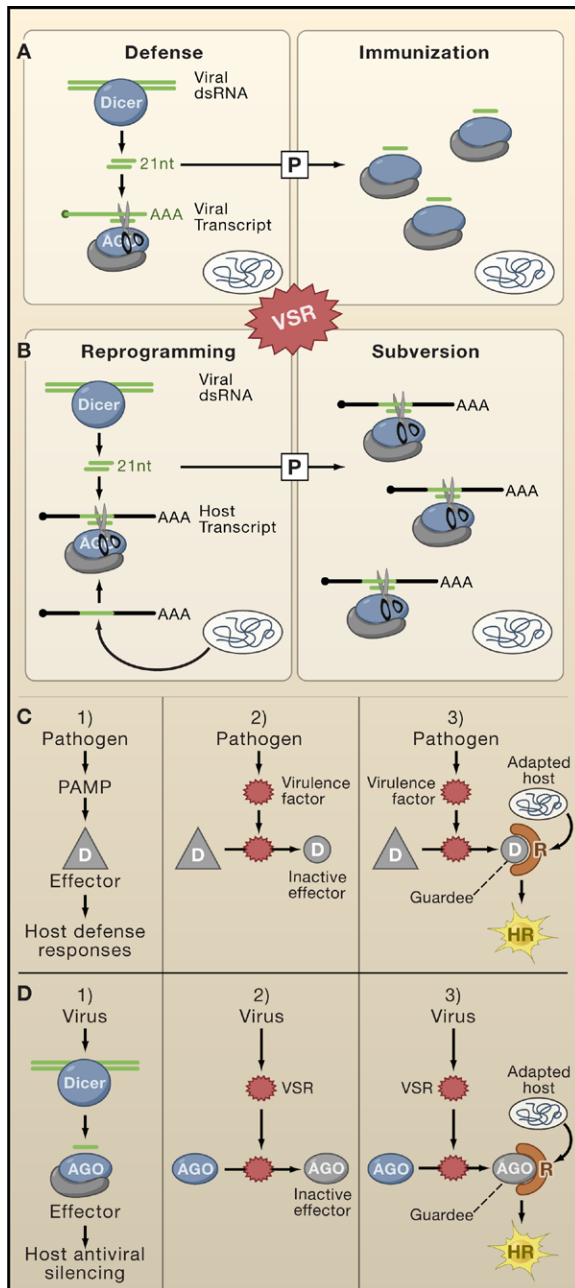
Toll-like receptor (TLR)-mediated innate immunity was first established in *Drosophila*, and subsequent studies showed that TLR-dependent NF- κ B signaling is highly conserved between flies and mammals (Imler et al., 2004), as is the case for RNAi. The demonstration of an antiviral role for RNAi in insects intuitively suggested a similar function for RNAi in mammals, and, indeed, some mammalian viral proteins suppress antiviral silencing in fly cells, and experimental RNAi in mammalian cells (Li and Ding, 2006). However, despite their requirement for viral infection, suppression of antiviral silencing has never been demonstrated in mammalian cells for any of these factors. Furthermore, cloning and sequencing of small RNAs from (+ss)RNA virus-infected mammalian cells failed to identify viRNAs (Pfeffer et al., 2004), which are otherwise readily detected in infected plants and invertebrates. On the one hand, mammalian virus infections commonly produce dsRNA, known to trigger broad-spectrum immune responses via extra- (TLR3) and intracellular (PKR/RIG-I/MDA-5) sensors. These responses are modulated by host proteins, such as PACT and Tar-binding protein (TRBP; Gupta et al., 2003; Garcia-Sastre and Biron, 2006). On the other hand, human Dicer processes dsRNA into siRNAs in vitro and in some human cultured cells (Hammond, 2005), and both PACT and TRBP play important roles in mammalian RNA silencing (Lee et al., 2006). Therefore, the mammalian RNAi machinery may have roles in dsRNA-mediated immunity in a manner awaiting further characterization.

It is clear, however, that mammalian viruses entertain intimate interactions with the host miRNA pathway. Several studies suggest that virus infection in mammalian cells might be indirectly counteracted by cellular miRNAs, in contrast to plants and invertebrates, in which viRNAs derive from viral genomes. The miRNA target is the virus itself in the case of the

primate foamy retrovirus and vesicular stomatitis virus (Lecellier et al., 2005; Otsuka et al., 2007), whereas a host factor critical to viral gene expression is suppressed in HIV-infected cells (Triboulet et al., 2007). Accordingly, knockdown of Dicer or Drosha enhance HIV replication, whereas accumulation of the cellular antiviral miRNAs is suppressed during infection. Other examples linking viruses to mammalian miRNAs include PACT and TRBP—activator and inhibitor of antiviral innate immunity respectively (Gupta et al., 2003)—both of which are required for human miRNA biogenesis/activity (Tolia and Joshua-Tor, 2007; Lee et al., 2006), whereas the adenoviral PKR-antagonist VA1 RNA inhibits miRNA processing and nuclear export (Cullen, 2006). Several groups of DNA viruses replicating in the nucleus intercept the mammalian miRNA pathway, producing viral miRNAs that may act both in *cis*, to regulate viral genome expression, and in *trans*, to alter host gene expression (Cullen, 2006). *Cis* effects are illustrated by SV40-encoded miRNAs that mediate slicing of the perfectly complementary SV40 early transcripts (Sullivan et al., 2005). This decreases viral T antigen expression, attenuating susceptibility to cytotoxic T cells without reducing virus yield. *Trans* effects are exemplified by the HSV1-encoded anti-apoptotic miR-LAT, contributing to virus persistence in sensory neurons (Gupta et al., 2006). Several additional studies support the view that vertebrate viruses might extensively usurp the host miRNA pathway to complete their infection cycle (Jopling et al., 2005; Stern-Ginossar et al., 2007). For instance, hepatitis C virus (HCV) subverts the liver-specific human miR-122 to facilitate its replication (Jopling et al., 2005), although the mechanism involved is unknown.

The Molecular Arms Race RNA Silencing and Evolution of Virus and Host Genomes

The reprogramming of host gene expression by mammalian virus-encoded miRNAs has parallels in plant-virus interactions. Several viRNAs derived from the *Cauliflower mosaic virus* 35S leader exhibit near perfect complementarity to *Arabidopsis* transcripts that are effectively targeted for sequence-specific downregulation during infection (Moissiard and Voinnet, 2006). In fact, dozens, if not hundreds of host transcripts might be targeted by viRNAs from *Cauliflower mosaic virus*, and at least two plant RNA viruses (Moissiard and Voinnet, 2006; O.V. and V.R. Ferrer, unpublished data). Host mRNA silencing by viRNAs might be fortuitous, with little consequence on virus fitness. However, some viRNAs could be selected by viruses, for instance, if they target host defense factors. At present, the sheer density and diversity of plant viRNAs precludes a clear estimation of the role of positive selection versus chance in this process, but this certainly prompts a re-evaluation of the current models for antiviral silencing. As much as some viRNAs might promote defense, others might



benefit viruses (Figure 4A). Cell-to-cell silencing spread, thought to immunize naive tissues, might similarly be hijacked by viruses to create an optimal environment in tissues about to be invaded. These notions deserve careful attention, as they could partly explain the profound modifications in cell metabolism commonly elicited by plant viruses, some of which occur several cells ahead of infection fronts. A further layer of complexity in this double-faced scheme involves the action of VSRs, which should, in principle, inhibit both defensive and subversive effects of viRNAs (Figure 4A), implying that their deployment must be tightly regulated. Harnessing

Figure 4. The Virus-Host Arms Race

Complex and multilayered interactions exist between RNA silencing pathways, VSRs, and other plant immune pathways.

(A and B) This model depicts the possibility that viRNAs produced in virally-infected plants not only contribute to antiviral defense (upper panels) but might sometimes benefit viruses if viRNAs share sequence homologies with host transcripts (lower panels). The model also depicts the possibility that immunization and subversion by viRNAs might operate in cells ahead of the infection front. The extent of defense and subversion is influenced by the timing and level of VSR expression.

(C) This model illustrates the guard hypothesis, which proposes that suppression of PAMP-elicited basal defense responses in plants by pathogens' effector proteins is detected by dedicated host-encoded R proteins, sometimes resulting in a hypersensitive response (HR).

(D) An adaptation of the guard hypothesis model explains how VSRs might elicit a hypersensitive response in specific plant ecotypes. The model entails that some effectors of antiviral silencing (e.g., AGO1) are modified by VSRs (such as P0 of poleroviruses; Figure 3C).

the dynamics of VSR expression and activity in space and time is surely a major challenge in plants and, perhaps, in animals, as related models could well apply to invertebrate infections.

Mechanisms directed by viRNAs to turn off host genes in plants, mammals, and possibly invertebrates have implications for viral and host genome evolution. Single-nucleotide polymorphisms that prevent viRNA-directed repression of certain cellular transcripts might be under strong positive selection in the host genome. However, this might, in turn, favor the emergence of virus quasi-species carrying compensatory mutations that restore viRNA activities. Conversely, persistent virus infection might contribute to select random mutations that promote resistance of some cellular transcripts to some viRNAs. A similar rationale applies to host-encoded small RNAs interacting directly with viral RNA, either positively or negatively. The liver-specific miR-122 enhances HCV replication by binding to the 5' end of the virion RNA, given that mutations of the viral miRNA-binding site reduce HCV titers (Jopling et al., 2005). Therefore, sequence polymorphisms in either cellular miR-122 or viral miR-122 target site could influence the susceptibility of specific individuals to specific HCV strains. Another aspect deserving attention is that repertoires and polymorphisms of cell-specific miRNAs might influence tissue permissiveness to certain viruses: the prevalence of miR-122 in the liver might contribute to the tropism of HCV for hepatocytes (Jopling et al., 2005).

Defense, Counterdefense, Countercounterdefense...

Components of plant and invertebrate antiviral silencing pathways must be under constant challenge by highly diverse VSRs and so should be continuously and rapidly evolving. Accordingly, in insects, the evolution rate of the antiviral defense factors Dcr2, R2D2, and Ago2 is considerably faster than that of their miRNA-pathway counterparts Dcr1, R3D1, and Ago1 (Obbard et al., 2006). Dcr2, R2D2, and Ago2 are evolving among the fastest 3% of all *Drosophila* proteins and display markedly reduced genetic diversity indicating recent selective sweep. These signatures of host-pathogens arms race strongly suggest that viruses shape host RNAi/antiviral functions in *Drosophila*

and, presumably, in other invertebrates and plants. This probably explains the emergence of DCL2 as a surrogate for DCL4 in *Arabidopsis* (Figure 1) and the fluidity of VSR genes, presumably required to face the rapid changes in host antiviral proteins. This host-pathogens arms race could, therefore, result in paralogous VSRs sometimes having different modes of action, as for the AL2/AC2 proteins of begomovirus and curtovirus in the plant *geminiviridae* (Trinks et al., 2005; Wang et al., 2005).

Plant protein-based innate immune responses typically involve NBS-LRR genes, known as resistance (R) genes (Jones and Dangl, 2006). Their products, the R proteins, monitor or “guard” the integrity of specific host defense components called “guardees,” which are primary targets of pathogen’s virulence factors. Changes in guardees’ status usually result in R proteins triggering host defense reactions sometime culminating in a form of programmed cell death (called the hypersensitive response, HR), which is thought to restrict pathogen’s growth (Jones and Dangl, 2006; Figure 4C). VSRs are, by their very own nature, viral virulence factors, of which several are known to trigger R gene-dependent hypersensitive response in specific hosts; in at least one case, VSR mutations compromising silencing suppression also compromised induction of the hypersensitive response (Li et al., 1999; Ren et al., 2000). Therefore, some R genes may have evolved to specifically sense the damages incurred by VSRs to host antiviral silencing components (Figure 4D). Avoiding R gene recognition could constitute another strong selective pressure driving VSR gene evolution. Presumably, hosts could also directly neutralize VSRs through activities that degrade or relocate them into inappropriate subcellular compartments. The former probably explains the failure of specific alleles of the *Cucumber mosaic virus*-encoded VSR 2b to accumulate in *Arabidopsis*, possibly due to proteolysis (Zhang et al., 2006), whereas the latter is suggested by the nuclear relocation of the tombusvirus P19 caused by ALY proteins, which interact with this VSR (Canto et al., 2006). These observations suggest that the varying efficacy of host-directed VSR suppression mechanisms and the polymorphism among VSR alleles may well contribute to the differences in viral susceptibility between ecotypes or between species.

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