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REVIEW

Infection Defects of RNA and DNA Viruses Induced by Antiviral RNA Interference

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SUMMARY Immune recognition of viral genome-derived double-stranded RNA (dsRNA) molecules and their subsequent processing into small interfering RNAs (siRNAs) in plants, invertebrates, and mammals trigger specific antiviral immunity known as antiviral RNA interference (RNAi). Immune sensing of viral dsRNA is sequence-independent, and most regions of viral RNAs are targeted by virus-derived siRNAs which extensively overlap in sequence. Thus, the high mutation rates of viruses do not drive immune escape from antiviral RNAi, in contrast to other mechanisms involving specific virus recognition by host immune proteins such as antibodies and resistance (R) proteins in mammals and plants, respectively. Instead, viruses actively suppress antiviral RNAi at various key steps with a group of proteins known as viral suppressors of RNAi (VSRs). Some VSRs are so effective in virus counter-defense that potent inhibition of virus infection by antiviral RNAi is undetectable unless the cognate VSR is rendered nonexpressing or nonfunctional. Since viral proteins are often multifunctional, resistance phenotypes of antiviral RNAi are accurately defined by those infection defects of VSR-deletion mutant viruses that are efficiently rescued by host deficiency in antiviral RNAi. Here, we review and discuss *in vivo* infection defects of VSR-deficient RNA and DNA viruses resulting from the actions of host antiviral RNAi in model systems.

KEYWORDS RNA interference, antiviral immunity, antiviral RNAi, viral suppressors of RNAi

INTRODUCTION

Viruses are obligate intracellular pathogens and cause devastating diseases in plants, animals, and humans. In response to viral infection, hosts activate distinct innate and adaptive immune responses to restrict virus entry, replication, cell-to-cell and systemic spread, and disease development. Over the past two decades, virus

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infection in plants, invertebrates, and mammals has been shown to induce production of small interfering RNAs (siRNAs) to direct specific virus clearance by RNA interference (RNAi) (1–3). First discovered in the nematode *Caenorhabditis elegans* (4), RNAi refers to the sequence-specific RNA degradation pathway which is conserved broadly in eukaryotes and includes two sequential RNase cleavage reactions (1, 2). In the first reaction, long double-stranded RNA (dsRNA) is cleaved by the RNase III enzyme Dicer into siRNAs 21 to 24 nucleotides in length. The subsequent target RNA cleavages are carried out by the RNA-induced silencing complex (RISC), which contains one strand of siRNA and at least one Argonaute protein (AGO) that harbors an enzyme domain homologous to RNase H.

In antiviral RNAi, viral genome-derived dsRNA molecules synthesized during infection are recognized intracellularly and processed into siRNAs by a Dicer enzyme (1, 2, 5–9). These virus-derived siRNAs (vsiRNAs) act as specificity determinants inside the antiviral RISC to direct virus clearance by base-pairing with the viral RNAs. Because the vsiRNAs produced by the host immune system overlap extensively in nucleotide sequences, it is possible to identify viruses by deep sequencing and bioinformatic assembly of the pools of vsiRNAs (10–14).

It is known that virus-host coevolution drives the rapid emergence of new virus variants which escape immunity mechanisms dependent on specific virus recognition by host immune proteins such as antibodies in mammals and resistance (R) proteins in plants (1–3). However, high mutation rates of viruses do not drive immune escape from antiviral RNAi because Dicer recognition of the viral dsRNA molecules is not sequence-dependent and the repertoire of vsiRNAs generated by the host immune system is enormous. Therefore, as their major counter-defensive strategy, plant and animal viruses have evolved proteins known as viral suppressors of RNAi (VSRs) to actively suppress antiviral RNAi (15–19). Notably, VSRs encoded by distinct virus families often exhibit no detectable sequence similarity, suggesting independent origins (16, 18).

VSRs target almost all key steps in the antiviral RNAi pathway to promote virus infection (15–19). Some viruses express VSRs to suppress vsiRNA biogenesis so that virulent infection with these viruses induces undetectable or low abundant accumulation of vsiRNAs. In contrast, many viruses develop virulent systemic infection in host organisms despite production of abundant vsiRNAs because they encode VSRs to suppress the antiviral activity of vsiRNAs without interfering with their biogenesis. Therefore, the function, regulation, and physiological significance of the antiviral RNAi response in an infected host are best defined by characterizing infection with mutant viruses incapable of RNAi suppression.

In this article, we review the infection-defective phenotypes and mechanisms of RNA and DNA viruses in model animal and plant hosts after each is rendered defective in RNAi suppression. In particular, we highlight *in vivo* infection defects of VSR-inactivated mutant viruses whose robust infection can be restored by genetic deficiency in the host antiviral RNAi pathway. Examining the known range of *in vivo* resistance phenotypes of antiviral RNAi should facilitate future studies on the mechanisms and physiological significance of antiviral RNAi in plants, insects, and mammals against additional viral pathogens.

INFECTION-DEFECTIVE PHENOTYPES AND MECHANISM OF VSR-DEFICIENT INSECT VIRUSES

The fruit fly *Drosophila melanogaster* has been an excellent model for understanding innate immunity against bacterial and fungal pathogens, which is mediated by Toll and immune deficiency (Imd) pathways that lead to the transcriptional induction of antimicrobial peptide effectors (20, 21). *D. melanogaster* encodes two distinct genetic pathways for the biogenesis and function of microRNAs (miRNAs) and siRNAs, respectively (1). Synthetic long dsRNA transfected into fly cells or injected into flies is cleaved by Dicer-2 into 21-nt siRNAs, which associate with Argonaute 2 (AGO2) to direct specific slicing of complementary target mRNAs (22–26). In contrast, Dicer-1 produces predominantly 22-nt miRNAs from single-stranded RNA precursors with local hairpin

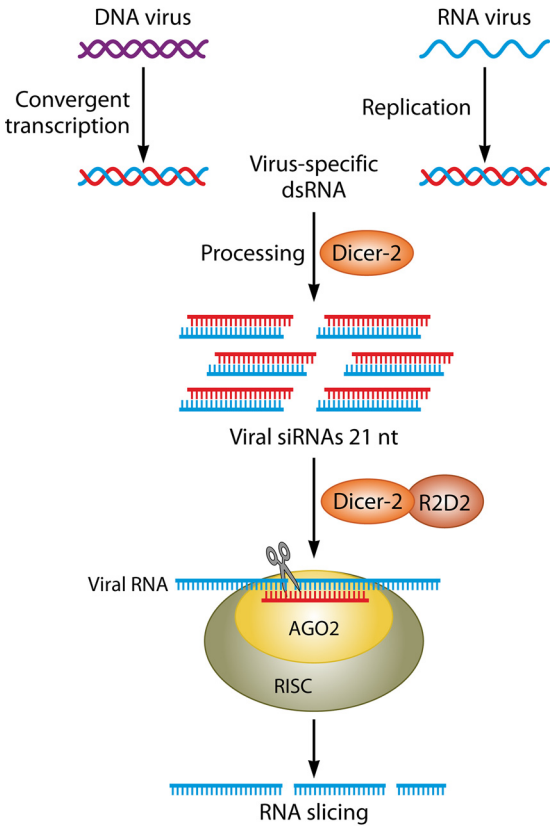

FIG 1 The antiviral RNAi pathway in *Drosophila melanogaster*. The virus-specific double-stranded RNA (dsRNA) produced during infection with both positive-stranded RNA viruses and a large dsDNA virus from the *Iridoviridae* (with circularly permuted genomes) are processed into 21-nucleotide (nt) virus-derived small interfering RNAs (siRNAs) by Dicer-2 to direct Argonaute 2 (AGO2)-dependent antiviral RNAi. Both R2D2, a protein with tandem dsRNA-binding domains participating in the assembly of siRNAs into RNA-induced silencing complex (RISC), and AGO2 are required for the antiviral activity, but not the production, of the viral siRNAs.

structures to inhibit translation of partially complementary mRNA targets in AGO1 effector complex. None of the long dsRNA-siRNA pathway genes are essential in the miRNA pathway, so flies do not show obvious developmental defects after genetic inactivation of genes encoding Dicer-2, AGO2, or the dsRNA-binding protein R2D2 required for siRNA loading (1).

Studies of the fruit fly model have made important contributions to understanding the function and mechanisms of antiviral RNAi (Fig. 1). For example, it was first shown in fruit flies that the long dsRNA-siRNA pathway, not the miRNA pathway or Toll/Imd signaling, mediates an essential defense mechanism against distinct viruses (27–30). Efficient rescue of the VSR deletion-associated defect in virus RNA accumulation by host deficiency in RNAi was also first demonstrated in fly cells (27). Further *in vivo* studies in fruit flies, discussed in detail below, have revealed specific infection defects of VSR-deficient mutant RNA and DNA viruses that are rescued by genetic loss-of-function in the RNAi pathway (Fig. 1).

Flock House Virus

Flock house virus (FHV) is an insect virus of the *Nodaviridae*, located in branch 3 of positive-stranded RNA viruses, which also includes alphaviruses (31). FHV belongs to the same genus as American nodavirus, which persistently infects *Drosophila* Schneider 2 (S2) cell cultures in many labs (12), and production of animal vsiRNAs was first detected in S2 cells in response to FHV infection (27). FHV contains a bipartite RNA genome that encodes three functional proteins in total (Fig. 2A). The viral RNA-dependent RNA polymerase (RdRP) and capsid protein (CP) are translated directly from

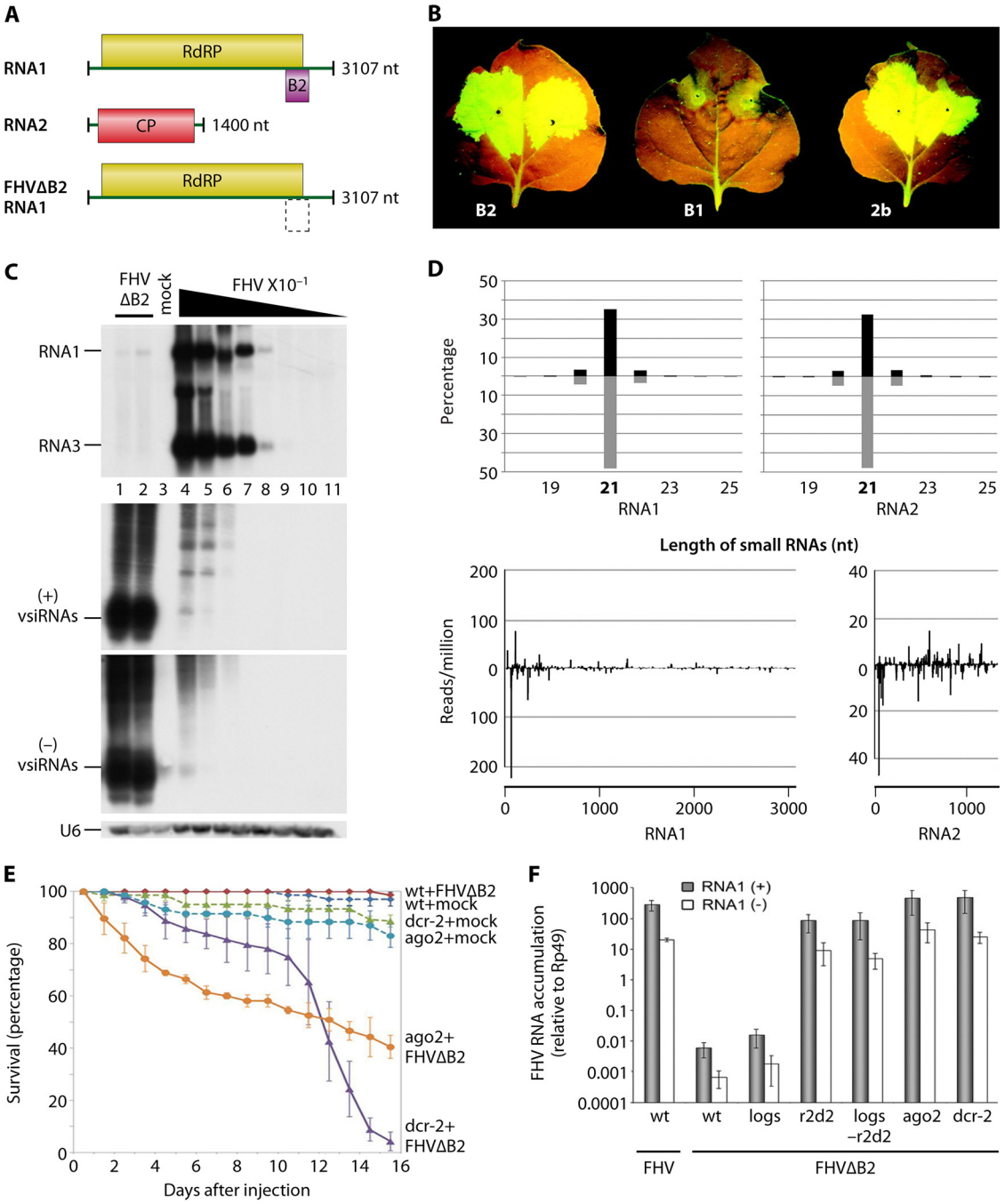

FIG 2 Infection defects of viral suppressor of RNAi (VSR)-deficient flock house virus (FHV). (A) Genome organization of FHV and B2-deficient mutant virus FHVΔB2. Open reading frames (ORFs) in yellow, red and purple represent viral RNA-dependent RNA polymerase (RdRP), capsid protein (CP), and VSR, respectively. (B) Cross-kingdom suppression of transgene-induced post-transcriptional gene silencing in plants by FHV B2. Green fluorescent protein (GFP)-expressing *Nicotiana benthamiana* plants were co-infiltrated with transgenes directing expression of GFP together with B2 (left), B1 (a C-terminal region of FHV RdRP, middle), or the plant VSR-2b (right). Leaves were photographed under UV illumination 6 days post-infiltration (reprinted with permission from Li et al. [27]). (C) Accumulation of FHV RNAs 1 and 3 (upper panel) and virus-derived siRNAs (vsiRNAs; lower panel) 72 h postinoculation (p.i.) with virions of FHVΔB2 or FHV in a series of 10-fold dilutions (lanes 4 to 11) in *Drosophila* S2 cells. U6 RNA was probed as a loading control (reprinted with permission from Aliyari et al. [10]). (D) Relative abundance of different-sized vsiRNAs (upper panel) and distribution patterns of the 21-nt vsiRNAs mapped to viral genomic RNAs 1 and 2 (lower panel) in wild-type (WT) fruit flies inoculated with FHVΔB2. Reads of 21-nt vsiRNAs per million of total sequenced small RNAs were shown. (E) Survival of WT and RNAi-defective *dcr-2* and *ago2* fruit flies until 16 days p.i. with buffer (mock) or FHVΔB2. Each data point represents the mean value of triplicates and error bars indicate corresponding standard deviation. (F) Relative abundance of FHV positive- and negative-stranded RNA1 in FHVΔB2-inoculated fruit flies. FHV-infected WT flies were used as a control. FHVΔB2 RNA1 accumulated to statistically significant lower levels in WT flies than in all of the RNAi-defective fly mutants ($P < 0.05$). Panels D to F reprinted with permission from Han et al. (38).

genomic RNAs 1 and 2, respectively. RNA3 is the subgenomic RNA of RNA1 synthesized by the viral RdRP to express the B2 protein, initially shown to suppress transgene RNA silencing in plants (Fig. 2B) (27). It is known that nodaviral RNA1 self-replicates efficiently in the absence of RNA2 (32). However, the self-replication of FR1ΔB2, a mutant FHV RNA1 made incapable of expressing B2 due to point mutations (Fig. 2A), does not lead to readily detectable accumulation of viral RNAs 1 and 3 in S2 cells unless the host RNAi pathway is disrupted by AGO2 depletion (27). FR1ΔB2 accumulation is also rescued by ectopic expression of the B2 protein from a co-transfected plasmid (27). These findings revealed an antiviral function of the AGO2-dependent RNAi pathway and a counter-defense function of the B2 protein, which acts as a VSR to enhance viral RNA accumulation by suppressing antiviral RNAi (27).

Biochemical and crystal structural studies have shown that nodaviral B2 protein functions as a dsRNA-binding protein to sequester both long dsRNA and duplex siRNA (33–35). Notably, B2 inhibits *in vitro* Dicer processing of long dsRNA into siRNAs and substitution of the conserved Arg at position 54 with Gln abolishes both activities of FHV B2 in dsRNA binding and dicing suppression (33). The counter-defense function of B2 is best illustrated by its activity to dramatically enhance FHV RNA accumulation in wild-type *Drosophila* embryos, but not in mutant embryos carrying a homozygous null mutation in either *dicer-2* or *ago2* (10, 28). In the absence of B2 expression, FR1ΔB2 self-replication activates vsiRNA biogenesis and viral RNA clearance in wild-type embryos so that elimination of vsiRNA biogenesis in *dicer-2* mutant embryos allows abundant accumulation of FR1ΔB2. FR1ΔB2 also accumulates at high levels in *ago2* mutant embryos that produce more abundant vsiRNAs than wild-type embryos, indicating that AGO2 is essential for the antiviral activity, but not the biogenesis, of vsiRNAs. In the presence of B2 expression, robust self-replication of wild-type FHV RNA1 does not trigger detectable vsiRNA production in wild-type or *ago2* embryos, demonstrating strong suppression of vsiRNA biogenesis by the B2 protein.

The VSR activity of B2 is essential for authentic FHV infection of both S2 cells and adult flies. Infection of S2 cells with wild-type FHV leads to high viral titers but low levels of vsiRNA accumulation. In contrast, infection with B2-deficient FHVΔB2 (FR1ΔB2 + wild-type RNA2) triggers production of highly abundant vsiRNAs that potently inhibit the accumulation of mutant virus genomic RNAs (10) (Fig. 2C). Co-immunoprecipitation experiments have demonstrated that the vsiRNAs associate with AGO2, but not with AGO1, and similar to the endogenous siRNAs, AGO2-bound vsiRNAs are methylated at their 3′ ends by the *Drosophila* ortholog of *Arabidopsis thaliana* HEN1 (10, 36, 37). Sequencing of total small RNAs from FHVΔB2-infected S2 cells with two deep sequencing platforms consistently reveals a population of predominantly 21-nt vsiRNAs divided approximately equally into positive and negative strands (10, 38) (Fig. 2D), providing further support for these vsiRNAs as Dicer-2 products processed from long dsRNA viral replicative intermediates (1, 39, 40) These vsiRNAs overlap extensively in nucleotide sequence to allow the discovery of new viruses by deep sequencing and bioinformatic assembly of total vsiRNAs (10, 12, 14, 41).

Adult flies are susceptible to FHV and up to 50% of infected flies are dead by 15 days postinoculation (28, 30). Wild-type flies inoculated with FHVΔB2 exhibit no difference in survival from mock-inoculated controls (Fig. 2E) and do not accumulate viral genomic RNAs and CP at levels detectable by Northern and Western blotting (38). Notably, FHVΔB2 becomes highly virulent in *dicer-2* and *ago2* mutant flies and replicates to titers at least 10,000-fold higher in these mutant flies than in wild-type flies (Fig. 2F). Deep sequencing of small RNAs reveals production of a typical population of 21-nt vsiRNAs in FHVΔB2-inoculated wild-type flies which is not detectable in *dicer-2* mutant flies (38). Unlike in *dicer-2* mutant flies, however, 21-nt vsiRNAs are highly abundant in FHVΔB2-infected *ago2* mutant flies. Moreover, *r2d2* mutant flies are as susceptible as *ago2* mutant flies to FHVΔB2 and produce abundant vsiRNAs (38) (Fig. 2F). Therefore, active Dicer-2 processing of the viral dsRNA replicative intermediates into vsiRNAs is not sufficient to inhibit FHVΔB2 infection in *ago2* and *r2d2* mutant flies, illustrating that the

activity of vsiRNAs to direct virus clearance in the antiviral RISC is essential for antiviral RNAi. These findings together demonstrate an *in vivo* function of the RNAi pathway mediated by Dicer-2, R2D2, and AGO2 in insect defense against virus infection (Fig. 1).

As a consequence of RNAi suppression by B2, the viral small RNAs sequenced from FHV-infected wild-type flies do not exhibit the properties of vsiRNAs made by Dicer-2 (38). Instead, these viral small RNAs share striking similarities with those sequenced from FHVΔB2-infected *dicer-2* mutant flies, as both populations of viral small RNAs are overwhelmingly positive-stranded without an obvious size preference. Thus, the vast majority of viral small RNAs sequenced from wild-type and *dicer-2* mutant flies infected with FHV and FHVΔB2, respectively, most likely correspond to the nonspecific degradation products of the abundant viral genomic RNAs. Nevertheless, negative-stranded 21-nt vsiRNAs are readily detectable by Northern blotting in FHV-infected wild-type flies, and FHV accumulates to higher titers and is more virulent in *dicer-2* and *r2d2* mutant flies than in wild-type flies (28, 30). Thus, B2 suppression of RNAi is incomplete and antiviral RNAi remains partially active against FHV infection in wild-type flies. These FHV studies together show that sequencing of small RNAs yields inconclusive results from wild-type virus-infected fruit flies and defining the physiological significance and mechanisms of antiviral RNAi requires VSR identification and characterization of VSR-deficient mutant viruses.

Cricket Paralysis Virus

Cricket paralysis virus (CrPV) belongs to the *Dicistroviridae* in branch 2 of the positive-stranded RNA viruses, which also includes picornaviruses (31). CrPV contains a non-segmented RNA genome that encodes two open reading frames (ORFs) translated from the dicistronic genomic RNA using independent internal ribosome entry sites (42, 43). ORF1 encodes a polyprotein for the mature nonstructural proteins involved in replication, whereas the viral capsid proteins are processed from the ORF2 polyprotein (Fig. 3A). CrPV infects diverse species of insects and is substantially more virulent than FHV in fruit flies (28). Like FHV, however, CrPV triggers vsiRNA production in S2 cells, accumulates to higher titers, and is more virulent in *dicer-2*, *r2d2*, and *ago2* mutant flies than in wild-type flies (28, 44). Thus, CrPV infection of wild-type flies also induces antiviral RNAi which confers partial protection against wild-type CrPV.

The use of a cell-based assay (45) has led to the identification of a VSR encoded by CrPV (28). Active suppression of antiviral RNAi induced by FHV RNA1 self-replication was detected in S2 cells expressing a protein corresponding to the first 140 codons of CrPV ORF1. Under the same conditions, expression of a truncated protein corresponding to the first 107 codons of CrPV ORF1 failed to suppress antiviral RNAi (28). Similarly, assaying for suppression of synthetic dsRNA-induced RNAi revealed VSR activity for the CrPV ORF1 N-terminal segments of either 168 or 148 residues, but not those of 128 or 108 residues (46). The VSR protein of CrPV was designated 1A because a mature protein around 160 residues in size accumulates in CrPV-infected cells, as shown by Western blotting analysis using an antiserum raised against the first 148 N-terminal residues of CrPV ORF1 (46). Interestingly, the N-terminal segment of the ORF1 polyprotein encoded by *Drosophila* C virus (DCV) from the same genus as CrPV also exhibits VSR activity but shares no significant sequence similarity with CrPV-1A (29). DCV-1A contains a canonical dsRNA-binding domain (dsRBD) and suppresses Dicer processing of long dsRNA into siRNA, thus exhibiting functionary similarity with the nodaviral B2 protein. In contrast, CrPV-1A binds to AGO2 and inhibits the RNA-slicing activity of AGO2-RISC, most likely by blocking vsiRNA-mediated recognition of the target mRNA (46, 47).

Structural and functional studies of CrPV-1A have mapped the residues essential for the VSR activity to a flexible loop region at the C-terminal region of CrPV-1A (48). These residues include proline 106 (P106) and phenylalanine 114 (F114), and alanine substitution of either amino acid abolishes both the AGO2-binding and RNA-slicing inhibition activities of CrPV-1A. The VSR activity of CrPV-1A is essential for CrPV infection of S2 cells because CrPV mutants designed from an infectious CrPV cDNA clone (49) to carry either a P106A or F114A mutation replicate to high viral titers only after AGO2

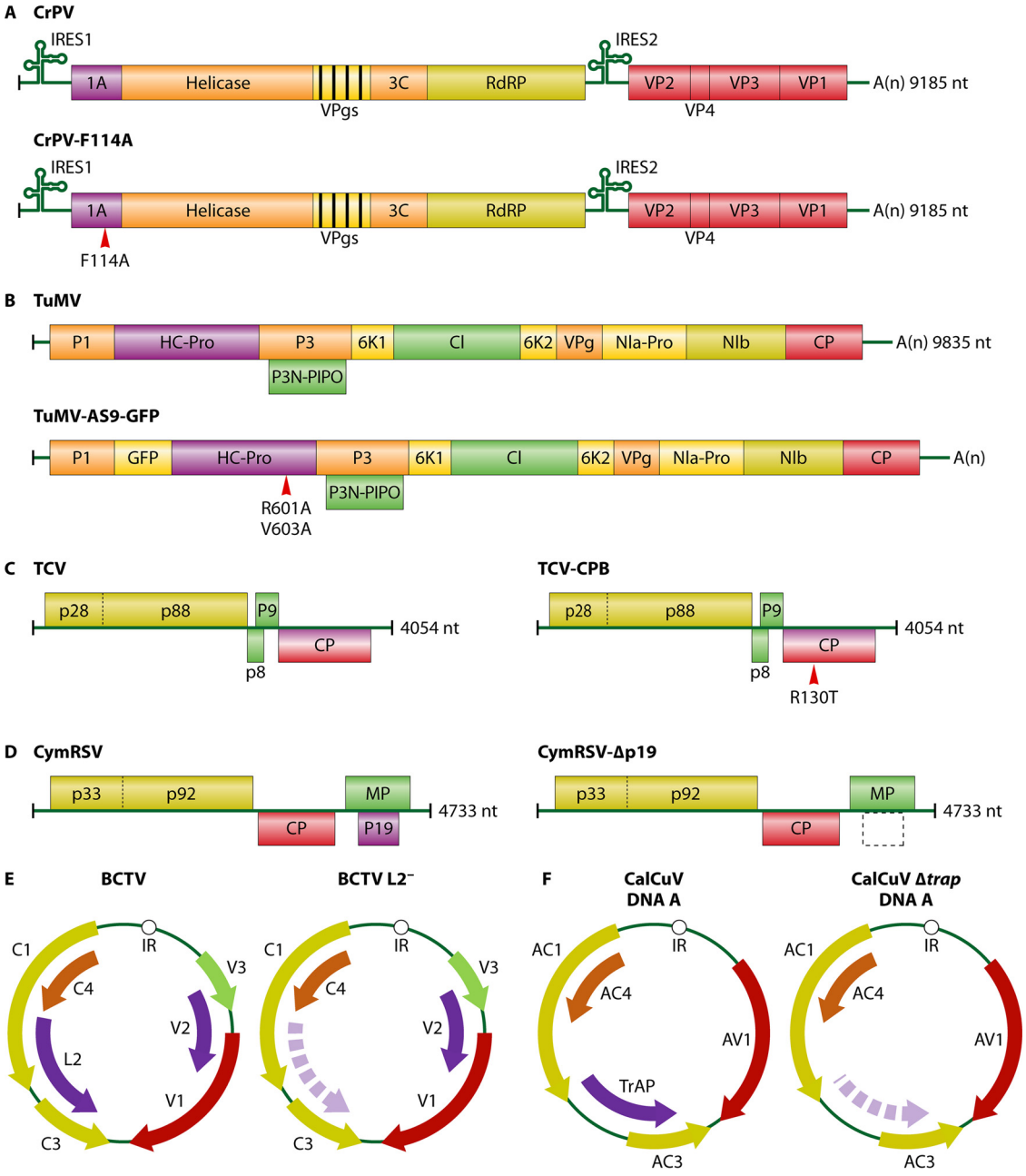

FIG 3 Genome organization of wild-type and VSR-deficient viruses discussed in this article. (A) Cricket paralysis virus (CrPV). (B) Turnip mosaic virus (TuMV). (C) Turnip crinkle virus (TCV). (D) *Cymbidium* ringspot virus (CymRSV). (E) Beet curly top virus (BCTV). (F) Cabbage leaf curl virus (CaLCuV). ORFs encode proteins required for genome replication such as RdRP (yellow), CP (red), movement protein (MP; green), VSR (purple), and other proteins (brown). Specific mutations introduced into the genomes of VSR-deficient mutant viruses to inactivate VSR function (A to C) or prevent VSR translation (D to F) are indicated by red triangle and purple dash line, respectively.

has been depleted by dsRNA soaking (48). Moreover, the mutant virus CrPV-F114A accumulates at lower viral titers and is less virulent than wild-type CrPV in adult flies. In contrast, wild-type and mutant CrPV exhibit no significant differences in either virulence or accumulation levels in *ago2* mutant flies. These findings show that CrPV infection *in vivo* triggers an AGO2-dependent antiviral RNAi (1, 39, 40).

It is interesting that the mutant virus CrPV-F114A remains virulent to wild-type adult flies, suggesting the presence of an independent CrPV-encoded activity to antagonize antiviral RNAi. Indeed, the N-terminal region of CrPV-1A contains a motif that

participates in the assembly of an E3 ligase in the infected cells to target AGO2 for degradation in a proteasome-dependent manner (48). Introduction of mutation L17A or A21D into this motif in the viral infectious cDNA to disrupt E3 ligase assembly led to drastically decreased viral titers in S2 cells. However, the infection-defective phenotype of CrPV-L17A and CrPV-A21D mutant viruses was significantly rescued by AGO2 depletion, demonstrating dual RNAi suppression mechanism by CrPV (48).

Invertebrate Iridescence Virus-6

Invertebrate iridescence virus-6 (IIV6) belongs to the *Iridoviridae* and contains a large dsDNA genome (212,482 bp). IIV6 has a broad host range and establishes a productive infection in fruit flies under laboratory conditions. Several lines of evidence show restriction of IIV6 infection by the same long dsRNA-siRNA pathway that confers protection against RNA viruses in fruit flies (Fig. 1). IIV6 infection induces production of a typical population of 21-nt vsiRNAs processed from long dsRNA formed between viral convergent transcripts in S2 cells as well as in wild-type and *ago2* mutant flies, but not in *dicer-2* mutant flies (50, 51). IIV6 was shown to replicate to higher titers and caused more severe disease in *dicer-2* and *ago2* mutant flies than in wild-type flies (50, 51). Notably, IIV6 encodes protein 340R, which contains a canonical dsRBD observed in other VSRs (29, 45), binds long dsRNA and inhibits its Dicer processing *in vitro*, and suppresses long dsRNA-triggered RNAi in S2 cells (52). Protein 340R also binds siRNA duplexes and blocks siRNA loading into RISC and siRNA-induced RNAi without interfering with the RNA-slicing activity of pre-assembled mature RISC, indicating that 340R may suppress antiviral RNAi by preventing vsiRNA biogenesis, vsiRNA loading, or both (52).

Bronkhorst et al. (53) recently reported the construction and characterization of a 340R-deletion mutant of IIV6, IIV6 Δ340R. IIV6 Δ340R consistently replicates to significantly lower viral titers than wild-type IIV6 in adult flies. However, the wild-type and VSR-deficient mutant IIV6 accumulate to similar levels in *dicer-2* and *ago2* mutant flies defective in the long dsRNA-siRNA pathway of RNAi. Instead of the slightly higher wild-type IIV6 levels previously observed in RNAi-defective flies (50, 51), careful reexamination found that neither *dicer-2* nor *ago2* mutant flies accumulated higher viral levels than WT flies after infection with wild-type IIV6 in contrast to IIV6 Δ340R infection (53). Therefore, infection with the large DNA virus triggers a potent antiviral RNAi response in adult flies that is completely suppressed by the cognate VSR 340R (53). Moreover, IIV6 Δ340R does not trigger enhanced vsiRNA production and has no obvious replication defects in S2 cells compared to wild-type IIV6 (53). In this regard, IIV6 Δ340R is similar to several VSR-deficient plant viruses discussed below and differs from the VSR-deficient mutants of FHV and CrPV that exhibit strong defects in viral RNA accumulation in single-cell replication assays (10, 27, 48). These findings suggest that the VSR of IIV6 most likely acts by sequestering siRNA duplexes and that antiviral RNAi inhibits IIV6 Δ340R infection at a step after viral replication in the initially infected cells.

INFECTION-DEFECTIVE PHENOTYPES AND MECHANISMS OF VSR-DEFICIENT PLANT VIRUSES

Plant viruses serve as a model for understanding the structure and biology of viruses since the discovery of the first virus in plants (54, 55). The vast majority of plant viruses contain single-stranded (ss) positive-sense RNA genomes or circular ssDNA genomes (54, 55). Unlike those that infect insects and other animals, plant viruses enter plant cells via wounds created by mechanical means or vector feeding and spread from the initially infected cell to neighboring cells through plasmodesmata, requiring the unique function of viral cell-to-cell movement proteins (54–57). Subsequently, viruses are transported over long distances to establish systemic infection via the phloem, the vascular tissue responsible for the translocation of soluble organic compounds (54–57).

Pioneering studies on plant viruses have provided early evidence for an RNA-mediated virus resistance mechanism in plants (58). For example, plant viruses are known to serve as both the inducers and targets of the homology-dependent post-transcriptional gene silencing (PTGS) of transgenes when there is sufficient nucleotide sequence

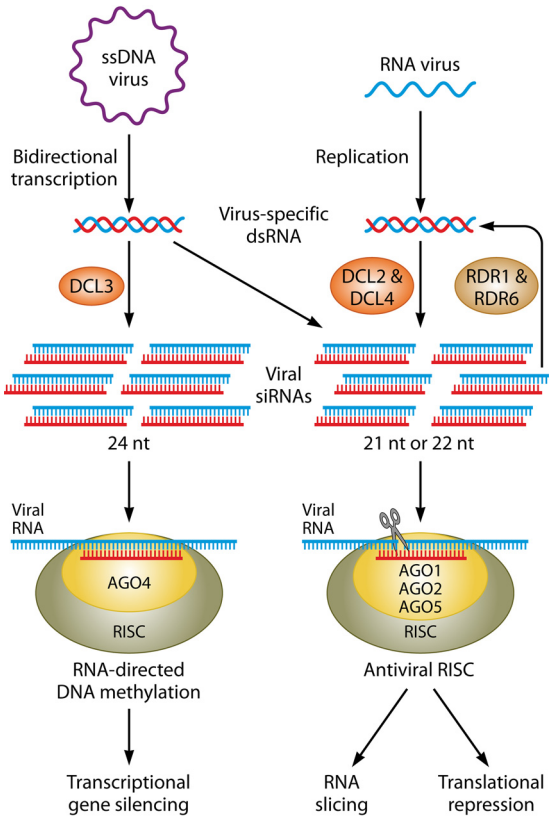

FIG 4 Antiviral RNAi pathways in *Arabidopsis thaliana*. The virus-specific double-stranded RNA (dsRNA) precursors produced as RNA virus replicative intermediates or from bidirectional transcription of viral circular DNA genomes are processed into 21-, 22-, or 24-nt viral siRNAs by host Dicer-like 4 (DCL4), DCL2, and DCL3, respectively. Antiviral RNAi against many viruses further depends on the production of secondary viral siRNAs processed from virus-specific long dsRNA precursors synthesized by the host RNA-dependent RNA polymerases RDR1 and RDR6. Multiple host Argonaute proteins (AGOs) direct specific siRNA-guided antiviral RNAi in RISC or another effector complex by directing RNA slicing and translational repression of viral RNAs or RNA-directed DNA methylation and transcriptional gene silencing of viral DNA genomes.

homology between the transgene and the viral genome (59–61). The first VSRs were identified from plant virus virulence proteins that exhibited novel activity to suppress PTGS of transgenes (62–65). Notably, viral infection of wild-type plants activates PTGS-like RNA degradation and induces accumulation of ~25-nt virus-derived small RNAs similar to those that target transgenes undergoing PTGS (66–68). Moreover, distinct *A. thaliana* mutants defective in transgene PTGS show enhanced susceptibility to cucumber mosaic virus (CMV) (69–71). However, key questions regarding the function and mechanisms of RNA-mediated virus resistance in plants remained unaddressed before the dsRNA-siRNA pathway was shown to confer antiviral defense and be targeted for suppression by VSRs to enhance virus accumulation in both *D. melanogaster* and *C. elegans* (27–30, 33, 72). For example, it was unclear whether the 25-nt virus-derived small RNAs were essential for RNA-mediated virus resistance and corresponded to the siRNA processed from viral long dsRNA molecules or the miRNA from viral single-stranded RNA precursors with local hairpin structures (58). It was also unknown whether viral suppressors of transgene PTGS promoted virus infection by suppressing the function of either the virus-derived small RNAs or host miRNAs (58).

The first set of answers to these questions came from genetic studies of the model plant *A. thaliana* (58), especially those involving the use of virus mutants rendered defective in RNAi suppression, reviewed in detail below. The biogenesis and mechanisms of siRNAs differ between insects and plants (73–75) (Fig. 1 and 4). *Arabidopsis* plants produce siRNAs in 21-, 22-, and 24-nt size classes by Dicer-like 4 (DCL4), DCL2, and

DCL3, respectively. Unlike insects, *A. thaliana* encodes six cellular RdRP genes (RDR1 to -6), of which RDR1, -2, and -6 are known to synthesize long dsRNA as the substrates of DCLs for siRNA amplification. Moreover, of the 10 *Arabidopsis* AGOs, AGO1 is the main RISC effector for both miRNAs and siRNAs. Like DCL1, which is responsible for the biogenesis of most miRNAs, AGO1 is essential for plant development, so only partial loss-of-function mutants of AGO1 are fertile and available for infection studies. Therefore, it is critical to determine whether the AGO1-dependent antiviral phenotype observed in plants, unlike that in fruit flies, is specifically mediated by an antiviral RNAi pathway independent of host miRNAs. In addition, plant 24-nt siRNAs are produced by DCL3 to direct transcriptional gene silencing by inducing DNA methylation and histone H3 lysine 9 dimethylation (H3K9me2) in a self-reinforcing loop (76, 77).

Cucumber Mosaic Virus

Cucumber mosaic virus infects more than 1,200 plant species and belongs to the genus *Cucumovirus* in the family *Bromoviridae* in branch 3 of the positive-stranded RNA viruses, which also includes animal alphaviruses (31, 78, 79). CMV was the first virus shown to replicate to higher titers in *A. thaliana* plants carrying mutations in genes that control PTGS of transgenes, including RDR6 (also known as SGS2 and SDE1), SGS3, SDE3, and AGO1 (69–71). CMV was also one of the viruses used to reveal the antiviral activity of the 21- and 22-nt vsiRNAs produced in a hierarchically redundant manner by DCL4 and DCL2, respectively (80–83).

CMV contains three genomic RNAs coding for five proteins in total (Fig. 5A). The 1a and 2a proteins are required for virus replication, whereas the 3a protein and CP are essential for both cell-to-cell and long-distance virus movement (78). The 2b protein of cucumoviruses, translated from subgenomic RNA 4A (84), was one of the first viral proteins discovered to exhibit suppression of PTGS of transgenes (6, 65, 85, 86). Based on the known role of 2b as a virulence protein to promote long-distance virus transport and enhance virus accumulation levels in the upper uninoculated leaves in diverse host species (87, 88), it was hypothesized that CMV infection induces an antiviral PTGS response antagonized specifically by 2b to develop systemic infection (65, 86, 89). Subsequent genetic studies in *A. thaliana* have shown that plants produce 21- and 22-nt vsiRNAs by DCL4 and DCL2, respectively, to act redundantly in antiviral RNAi against CMV (81–83). The most abundant class of vsiRNAs detected in CMV-infected plants are the 21-nt class vsiRNAs made by DCL4. However, CMV replicates to similar levels in wild-type, *dcl2*, and *dcl4* single-mutant plants, and exhibits significantly enhanced virulence with higher virus titers only in the *dcl2 dcl4* double-mutant plants (81–83).

Compared to wild-type CMV, the 2b-deletion mutant of CMV (CMV-Δ2b) (Fig. 5A) accumulates to dramatically reduced levels and induces no visible symptoms in *A. thaliana* ecotype Columbia-0 (Col-0) plants, as shown previously in diverse host species (83, 87, 88). However, the accumulation of 21-, 22-, and 24-nt vsiRNAs is significantly higher in CMV-Δ2b–infected Col-0 plants than in CMV-infected plants despite low virus replication levels (83). Strikingly, both the *in vivo* accumulation and symptom development (Fig. 5B) of CMV-Δ2b are indistinguishable from that of wild-type CMV in both the *dcl2 dcl4* and *dcl2 dcl3 dcl4* mutant plants which are defective in antiviral RNAi (83, 90–92). Thus, CMV-Δ2b exhibits no functional defects in virus replication, cell-to-cell movement, or long-distance transport, demonstrating that the infection-defective phenotypes of the mutant virus in wild-type plants result solely from antiviral RNAi initiated by DCL2 or DCL4 in response to infection. Together, these findings have confirmed the 2b protein of CMV as a VSR that functions to promote *in vivo* infection specifically by suppressing antiviral RNAi and established the VSR-deficient CMV-Δ2b as a sensitive reporter virus for the antiviral activities of vsiRNAs produced by the host immune system. As reviewed elsewhere (15–19), VSR-2b inhibits both the biogenesis and antiviral activity of vsiRNAs by interacting with long dsRNA and siRNA duplex as well as with the Argonaute and RDR proteins of the RNAi pathway (93–97).

Although VSR-2b dramatically enhances CMV accumulation at the whole-plant level, it has no effect on CMV replication in protoplast single-cell assays (88, 98).

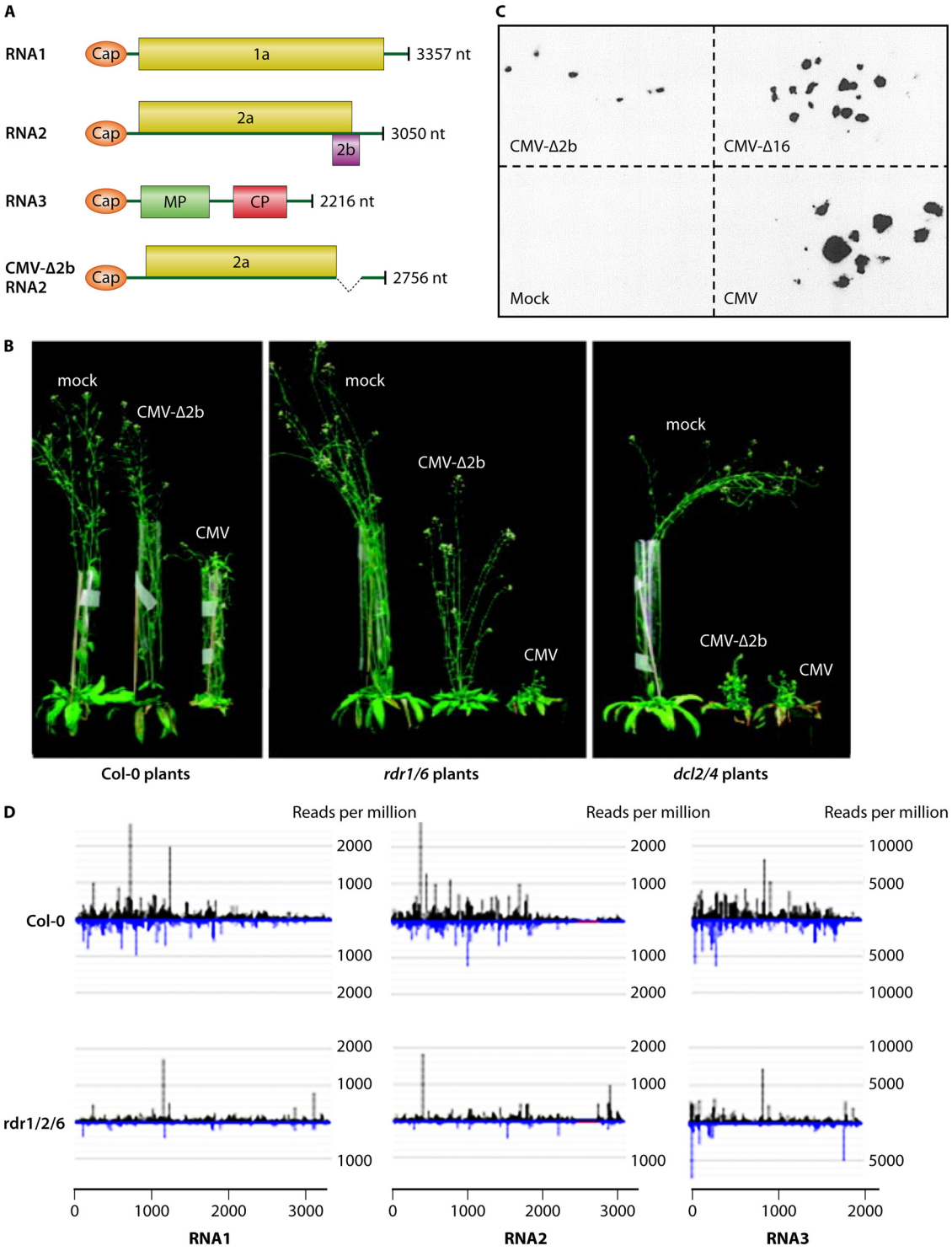

FIG 5 Infection defects of VSR-deficient cucumber mosaic virus (CMV). (A) Genome organization of CMV. ORFs encode proteins required for replication (yellow), CP (red), MP (green), and VSR (purple). (B) Distinct virulence phenotypes of CMV and its VSR-deficient mutant (CMV-Δ2b) 6 weeks postinfection in wild-type (Columbia-0 [Col-0]) and mutant plants as indicated. In the absence of viral RNAi suppression, CMV-Δ2b is symptom-free in wild-type plants but becomes as virulent as CMV in *dcl2* and *dcl4* double-knockout plants (*dcl2/4*), which produce neither primary nor secondary viral siRNAs. The primary viral siRNAs in *rdr1* and *rdr6* double-knockout plants (*rdr1/6*) confer a basal defense so that CMV-Δ2b virulence is only partially restored (reprinted with permission from Ding and Lu [92]). (C) Distribution of CMV RNAs in cucumber cotyledons as revealed by press-blot hybridization at 4 days postinoculation with water (mock), CMV, CMV-Δ2b, or CMV-Δ16 (reprinted with permission from Ding et al. [84]). CMV-Δ16 expressed a mutant 2b protein with the 16 C-terminal amino acids deleted, which was shown later to retain its VSR activity (85). (D) Mapping of CMV-Δ2b produced viral siRNAs to viral genomic RNAs in Col-0 and *rdr1*, *rdr2*, and *rdr6* triple-knockout plants (*rdr1/2/6*). Perfect matched reads of 21-nt vsiRNAs per million total sequenced small RNAs were shown (reprinted with permission from Wang et al. [104]).

Moreover, CMV-Δ2b constructed from either Q-CMV in the subgroup II strains (Fig. 5C, top left) or Fny-CMV in the subgroup I strains replicates efficiently in the initially infected cells and spreads to neighboring cells to form local infection foci in the directly inoculated leaves (87, 98). These findings indicate that the antiviral RNAi response triggered by CMV does not inhibit virus accumulation at the single-cell level or the initial cell-to-cell virus movement. Instead, the induced antiviral RNAi suppresses long-distance virus transport and virus accumulation in the upper uninoculated leaves of wild-type plants.

The use of CMV-Δ2b as a reporter for antiviral RNAi has facilitated the identification and mechanistic characterization of several additional components in the defense pathway. For example, the mechanisms of plant RDR genes known to confer virus resistance has remained unclear since the year 2000 (69, 71, 99–103). Examination of a full panel of *rdr1*, *rdr2*, and *rdr6* single-, double-, and triple-mutant plants has revealed that the infection defects of CMV-Δ2b are rescued when neither RDR1 nor RDR6 is functional (104) (Fig. 5B). Northern blotting and deep sequencing of small RNAs revealed that knockout of both RDR1 and RDR6 leads to at least 20-fold reduction in the vsiRNA biogenesis induced by CMV-Δ2b (Fig. 5D). These findings have provided the first evidence for a specific role of the host RDR1 and RDR6 genes in antiviral RNAi by synthesizing virus-specific long dsRNA precursors of a dominant pool of vsiRNAs (104). Similarly, CMV-Δ2b establishes virulent systemic infection with high viral load in several *A. thaliana* mutants defective in vsiRNA amplification, including those carrying loss-of-function mutations in Suppressor of Gene Silencing 3 (SGS3), phospholipid flippase genes ALA1/ALA2, Antiviral RNAi-defective 2 (AVI2), or Reduced Dormancy 5 (RDO5) (91, 105–108). Notably, RDR6-mediated vsiRNA amplification to target CMV is inhibited by autophagy mediated by a virus-inducible small peptide in *A. thaliana* (109) and upregulated by transcription factor CAMTA3 in *Nicotiana benthamiana* (110). As in nematodes (111, 112), the RDR-independent and RDR-dependent vsiRNAs have been designated primary and secondary vsiRNAs, respectively (104, 113, 114). The primary vsiRNAs processed from viral RdRP products without RDR amplification also direct active antiviral RNAi in plants as has been found in insects and mammals, which do not encode an RDR homolog. For example, CMV-Δ2b replicates to lower levels and induces less severe disease symptoms in *rdr1 rdr6* or *ala1 ala2* mutant plants, which produce only primary vsiRNAs, than in *dcl2 dcl4* mutant plants (Fig. 5B), in which neither the primary nor the secondary 21- and 22-nt vsiRNAs are produced.

CMV-Δ2b also establishes virulent systemic infection in *ago1 ago2* double-mutant plants despite the production of highly abundant primary and secondary vsiRNAs (105). The antiviral activities of AGO1 and AGO2 are additive and non-redundant. Consistently, AGO1 and AGO2 are associated *in vivo* with two distinct populations of vsiRNAs (105) which differ in both the ratio of 21-nt to 22-nt vsiRNAs and the 5′-terminal nucleotide (U for AGO1 and A for AGO2) found previously for endogenous small RNAs (115, 116). These results demonstrate that without the subsequent assembly of vsiRNA-AGO effector complex, Dicer processing of the long dsRNA precursors of the vsiRNA is insufficient to confer virus resistance (105), which is similar to antiviral RNAi in insects (10, 38).

CMV-Δ2b was used in a recent genome-wide association studies (GWAS) screen to determine whether natural variations among re-sequenced wild *A. thaliana* populations were enriched in specific pathways known to confer virus resistance in plants (108). Genetic and infection studies have demonstrated that the highest-ranked gene significantly associated with quantitative virus resistance functions in antiviral RNAi by promoting RDR6-dependent amplification of vsiRNAs. Remarkably, a negative regulator of antiviral RNAi was identified as the highest-ranked gene from a similar GWAS screen using wild-type Q-CMV (108). Since CMV is a natural pathogen of *A. thaliana* in wild ecosystems (117), these findings suggest that antiviral RNAi drives host adaptation to viral infection in plants (108, 118).

Turnip Mosaic Virus

Potyvirus is the largest genus of the *Potyviridae* in branch 2 of positive-stranded RNA viruses, which also includes animal picornaviruses (31, 79, 119). Potyviruses,

including tobacco etch virus (TEV) and turnip mosaic virus (TuMV), produce their mature proteins by proteolytic cleavages of a large polyprotein translated from a non-segmented RNA genome (Fig. 3B). Potyviral helper component-proteinase (HC-Pro) was one of the first viral suppressors of transgene PTGS reported in 1998 (63, 64). The silencing suppressor activity of TEV HC-Pro is completely abolished in two HC-Pro mutants (AS9 and AS10) with alanine replacement of 2 or 3 charged amino acid residues in the central region that are dispensable for proteinase activity and invariable among potyviruses (120, 121). Consistently, TuMV HC-Pro containing the conserved AS9 substitutions is also defective in silencing suppression assays, and the AS9-containing mutant TuMV triggers vsiRNA production and is unable to initiate infection in *A. thaliana* plants (122). However, TuMV-AS9 establishes efficient systemic infection in both *dcl2 dcl4* and *dcl2 dcl3 dcl4* mutant plants, with severe disease symptoms indistinguishable from those of wild-type TuMV. These findings demonstrate that the VSR-deficient TuMV-AS9 exhibits no functional defects in virus replication, cell-to-cell movement, or long-distance transport when antiviral RNAi is defective (122), providing functional validation of HC-Pro as the potyviral VSR.

The use of VSR-defective potyviruses marked by a beta-glucuronidase gene (GUS) or green fluorescent protein (GFP) tag has revealed important insights into the antiviral activities of vsiRNAs. TuMV-AS9-GFP (Fig. 3B) is especially powerful because it allows visual inspection of live infection, developing local green fluorescent foci following virus replication and cell-to-cell movement in inoculated leaves before long-distance transport into the uninoculated bolt tissue, cauline leaves, and inflorescence clusters (122). The VSR-deficient TEV-AS9-GUS replicates to at least 4-fold lower levels than TEV-GUS in protoplast assays and exhibits defects in long-distance movement (120). Similarly, TuMV-AS9-GFP causes no detectable infection in either inoculated leaves or uninoculated tissues of wild-type plants (122), indicating the induction of an antiviral RNAi response which inhibits potyviral accumulation in the initially infected cells. The induction of local antiviral RNAi requires production of DCL4-produced 21-nt secondary vsiRNAs by both RDR1 and RDR6 since the resistance is lost in *dcl4*, *rdr1*, and *rdr6* single mutants (122), allowing not only local infection in inoculated leaves, but also systemic infection of bolt tissue and cauline leaves. Interestingly, further systemic infection of the inflorescence tissues with TuMV-AS9-GFP is successful only in mutant plants in which both DCL2 and DCL4 or both RDR1 and RDR6 are inactivated (122). Therefore, a qualitatively distinct population of 22-nt vsiRNAs produced by DCL2 and amplified by either RDR1 or RDR6 can direct another module of antiviral RNAi to prevent inflorescence invasion in the absence of the 21-nt vsiRNAs made by DCL4. By comparison, TuMV-AS9-GFP invasion of the inflorescence clusters is more efficient and virus titers in the inoculated leaves and uninoculated cauline leaves and inflorescence are higher in *dcl2 dcl4* plants than in *rdr1 rdr6* or *rdr1 rdr2 rdr6* plants, revealing a basal level of resistance against the VSR-deficient potyvirus conferred by the RDR-independent primary vsiRNAs (122).

Two overlapping sets of AGOs play essential roles in the two modules of antiviral RNAi directed by the 21- and 22-nt vsiRNAs, respectively (113, 123, 124). Among the three clades of *A. thaliana* AGOs (125, 126), all three members of clades AGO1/5/10 and AGO2 and AGO7 (ZIP-1) from clade AGO2/3/7 are required for the induction of local antiviral RNAi. For example, TuMV-AS9-GFP infection is visible in inoculated leaves and uninoculated cauline leaves of *ago2*, *ago5*, *ago7* and *ago10* single-mutant plants (113), and TuMV-AS9-GFP spread into the uninoculated systemic leaves is observed in *ago1-57* plants (127). By comparison, AGO2 plays a more prominent role, and its RNA-slicing activity is necessary for antiviral RNAi (113, 123, 124). In contrast, either AGO1 or AGO10 can function independently to mediate systemic antiviral RNAi by DCL2-produced 22-nt vsiRNAs. Interestingly, AGO2 further contributes to restricting viral spread to inflorescence clusters because inflorescence infection occurs in a larger proportion of *ago1 ago2 ago10* triple-mutant plants compared to *ago1 ago10* double-mutant plants (113). Consistently, both 21- and 22-nt vsiRNAs induced to target TuMV-AS9-GFP associate with AGO1, AGO2, and AGO10, and HC-Pro sequesters the vsiRNAs away from all three antiviral AGOs in TuMV-infected plants (113). Thus, HC-Pro may suppress

antiviral RNAi *in vivo* by preventing the loading of vsiRNAs into the AGOs in addition to its multiple previously reported VSR activities (113, 123, 124, 128–131).

Turnip Crinkle Virus and Cymbidium Ringspot Virus

Viruses in the family *Tombusviridae* represent a distinct lineage in branch 3 of positive-stranded RNA viruses (31, 79) and include several viruses that serve as models for antiviral RNAi. Turnip crinkle virus (TCV) belongs to the genus *Betacarmovirus* (Fig. 3C) and was among the first plant viruses shown to trigger antiviral RNAi mediated by DCL2 and DCL4 (80, 132). Unlike cucumoviral and potyviral VSRs, which are viral nonstructural proteins, TCV VSR is the capsid protein (CP) (also known as P38) responsible for the encapsidation of the viral genomic RNA. TCV CP is dispensable for viral replication in protoplast assays and cell-to-cell movement, but virus exit from the vasculature in the systemically infected leaves after long-distance transport requires active virion assembly by CP in a process independent of its VSR activity (57, 133–137). In the absence of CP expression, a VSR-deficient TCV mutant tagged with GFP (TCV GFPΔCP) is potently silenced in *A. thaliana* plants by DCL2/DCL4-dependent vsiRNAs so that the mutant virus causes virulent systemic infection only when both DCL2 and DCL4 are inactivated (80, 133, 134, 138). In the inoculated leaves of wild-type plants, TCV GFPΔCP replication is accompanied by cell-to-cell movement, resulting in fluorescent foci (139). VSR-deficient TCV mutants accumulate at higher levels in the inoculated leaves after genetic inactivation of DCL4, DRB4, RDR6, AGO1, AGO2, AGO3, or AGO7 (80, 133, 134). Moreover, a TCV mutant (CPB, Fig. 3C) with a single amino acid substitution to inactivate the VSR activity of CP without disrupting virion assembly can spread long-distance to infect (i) uninoculated cauline leaves in *ago2*, *ago3*, and *ago7* single-mutant plants and (ii) both the uninoculated cauline leaves and inflorescence tissues in *dcl2 dcl3 dcl4* mutant plants (133, 134). These findings indicate that TCV infection also triggers two similar modules of antiviral RNAi to reduce the accumulation of virus available for long-distance transport after the initial virus replication and cell-to-cell movement in the inoculated leaves (134). Interestingly, only single fluorescent cells are visible in *N. benthamiana* leaves inoculated by TCV GFPΔCP (139). Thus, TCV infection may induce antiviral RNAi to inhibit cell-to-cell virus movement in *N. benthamiana* but not in *A. thaliana*, indicating a host-specific difference in blocking virus spread by antiviral RNAi.

Cymbidium ringspot virus (CymRSV) encodes an extensively characterized VSR p19 conserved in the genus *Tombusvirus*, which includes tomato bushy stunt virus (TBSV) (140, 141) (Fig. 3D). Because *A. thaliana* is a non-host for these viruses, the Laboratory (LAB) strain of *N. benthamiana* naturally defective in RDR1 (101) has been used to characterize the infection-defective phenotypes of VSR-deficient tombusviral mutants (140–142). Deep sequencing of total small RNAs from CymRSV-infected *N. benthamiana* plants has identified the precursors of vsiRNAs as the long dsRNA, not ssRNA with local foldback structures (143). Expression of p19 is dispensable not only for viral replication in protoplast assays, but also for the initial cell-to-cell and long-distance virus movement in infected plants, so that wild-type and VSR-deficient tombusviruses accumulate to similar levels in both the inoculated leaves and the first systemically infected leaves (140–142). VSR-p19 expression does not alter the accumulation levels of vsiRNAs, probably because it acts by sequestering duplex siRNAs (140, 144). Unlike wild-type viruses, however, VSR-defective viruses are gradually cleared from the subsequently emerged tissues that are free of disease symptoms. For example, *in situ* analyses of viral RNAs and proteins reveal that the presence of VSR-deficient mutant CymRSV (Fig. 3D) is confined to and around the vascular bundles in the systemically infected leaves, indicating defective virus exit from the vasculature following phloem-dependent transport (145).

The phloem exit block of the VSR-deficient CymRSV and TCV is phenotypically similar but mechanistically distinct. TCV GFPΔCP or TCV mutants expressing assembly-defective CP variants remain confined to the main veins of the systemically infected leaves in *dcl2 dcl3 dcl4 A. thaliana* plants that cannot initiate antiviral RNAi (133). In contrast, the p19-deficient CymRSV spreads as broadly as wild-type CymRSV in the

systemically infected leaves of *N. benthamiana* plants maintained under a low temperature insufficient to trigger vsiRNA production (146). Notably, immunization of *N. benthamiana* with engineered expression of individual vsiRNAs to target TBSV genomic RNA confers strong antiviral protection (147). Moreover, knockdown and/or knockout of the AGO2 or RDR6 gene in *N. benthamiana* enhances the accumulation of VSR-defective mutant tombusviruses and delays the recovery of infected plants (114, 148–150). The observed inhibition of CymRSV-Δp19 infection in mutant *N. benthamiana* plants defective in both RDR1 and RDR6 (114) resembles the incomplete rescue of CMV-Δ2b and TuMV-AS9-GFP in *rdr1 rdr6* plants (104, 122), providing evidence for antiviral activity of RDR-independent primary vsiRNAs in a different host species. It will be interesting to determine whether plants defective in the biogenesis of both primary and secondary vsiRNAs can support more efficient systemic infection with p19-deficient tombusviruses, including the BS3Ng isolate of TBSV recently shown to develop systemic infection in *A. thaliana* (151).

Beet Curly Top Virus and Cabbage Leaf Curl Virus

Geminiviridae is a large family of plant small DNA viruses (79) targeted not only by the 21- and 22-nt vsiRNAs made by DCL4 and DCL2, respectively, but also by DCL3-produced 24-nt vsiRNAs (152–156) (Fig. 4). In plants, 24-nt siRNAs induce DNA methylation and H3K9me2, which inhibit target gene transcription (76, 77). The geminiviral genome comprises one or two circular ssDNA (2.5 to 3.0 kb) and depends on host machinery in the nucleus for rolling-circle DNA replication and on bidirectional transcription from episomal mini-chromosomes to yield virion- and complementary-sense transcripts as mRNAs for the expression of 6 to 7 proteins (8). The multifunctional L2/AL2 (also known as TrAP) protein is an extensively characterized VSR of geminiviruses (8). VSR-L2 of beet curly top virus (BCTV, genus *Curovirus*) is not required for replication or systemic infection, and the initial disease symptoms of *Nicotiana bethamiana* and *A. thaliana* plants caused by wild-type and L2-deficient ($L2^{-}$) BCTV (Fig. 3E) are also indistinguishable (157, 158). However, BCTV $L2^{-}$-infected plants subsequently develop an enhanced recovery phenotype, especially in new growth after removal of the primary infected shoots, which is free of symptoms and contains drastically reduced viral titers (157, 158). Notably, *A. thaliana* recovery from BCTV $L2^{-}$ infection requires DCL3, AGO4, and plant-specific RNA polymerases IV and V to promote cytosine methylation and H3K9me2 of the viral chromatin, demonstrating antiviral activity of the 24-nt vsiRNAs against the DNA virus (157, 159–162).

VSR-TrAP of cabbage leaf curl virus (CaLCuV, a bipartite geminivirus from genus *Begomovirus*) is essential for systemic infection in *A. thaliana*, but mutant plants with deletion of the H3K9me2 histone methyltransferase gene Kryptonite (KYP) support systemic infection of TrAP-deficient CaLCuV (Fig. 3F) (163). Interestingly, tomato RDR genes Ty-1 and Ty-3, which are closely related to *A. thaliana* RDR3/4/5 clade, confer resistance to Tomato yellow leaf curl virus (TYLCV, a monopartite begomovirus) by a mechanism associated with enhanced amplification of 21-, 22-, and 24-nt vsiRNAs (155, 164, 165). Moreover, the *A. thaliana* RDR6 gene plays an indispensable role in non-host resistance to the mixed infection of TYLCV-China with its betasatellite DNA (166). Together, these studies indicate that RDR amplification of vsiRNAs and repressive DNA and histone methylation of the geminiviral chromatin initiated by the 24-nt vsiRNAs are critical to the development of plant resistance to geminiviruses.

CONCLUSIONS AND PERSPECTIVES

We have recently reviewed the studies on the functional validation of VSRs encoded by mammalian RNA viruses from the families *Flaviviridae*, *Nodaviridae*, *Orthomyxoviridae*, and *Picornaviridae* (18). Although they share no similarity in their primary protein sequences, all of these mammalian VSRs suppress Dicer production of vsiRNAs by sequestering the viral long dsRNA replicative intermediates with a similar fold of two antiparallel α-helices (167–170). Moreover, mutant viruses defective in the VSR function trigger production of abundant vsiRNAs to direct potent antiviral RNAi so that they replicate to high titers only in mammalian cells with knockout of either the Dicer gene or the RNA-slicing activity

TABLE 1 Antiviral RNAi-induced inhibition of virus accumulation at distinct stages of infection[a]

VSR-deficient virus	Family (genome type)[b]	Virus accumulation inhibited in:[c]			Reference(s)
		Inoculated cells	Inoculated tissues	Systemic tissues	
FHV	*Nodaviridae* (+RNA[3])	yes	yes	yes	10, 27, 28, 38, 45
CrPV	*Dicistroviridae* (+RNA[2])	yes	nd	yes	28, 46, 48
CMV	*Bromoviridae* (+RNA[3])	no	yes	yes	65, 83, 87, 88, 90, 91, 98, 104–106, 108
TuMV	*Potyviridae* (+RNA[2])	yes	yes	yes	63, 64, 113, 120, 122, 128
TCV	*Tombusviridae* (+RNA[3])	no	no/yes	yes	80, 133–139
CymRSV	*Tombusviridae* (+RNA[3])	no	no	yes	114, 140, 141, 143–146, 148
IIV6	*Iridoviridae* (dsDNA)	no	nd	yes	50–53
BCTV	*Geminiviridae* (ssDNA)	no	no	yes	157–162
CaLCuV	*Geminiviridae* (ssDNA)	nd	nd	yes	163

[a]RNAi, RNA interference; VSR, viral suppressor of RNAi; FHV, flock house virus; CrPV, cricket paralysis virus; CMV, cucumber mosaic virus; TuMV, turnip mosaic virus; TCV, turnip crinkle virus; CymRSV, Cymbidium ringspot virus; IIV6, invertebrate iridescence virus-6; BCTV, beet curly top virus; CalCuV, cabbage leaf curl virus; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.
[b]The positive-stranded RNA viruses are from branches 2 or 3 as shown by Wolf et al. (31).
[c]nd, not determined; yes/no, the observed inhibition of TCV in the inoculated tissues is host-specific (133–139).

of Argonaute-2 (167–172). These VSR-deficient mutant viruses are also defective in *in vivo* infection and mutant virus clearance in infected mice is correlated with the production of abundant vsiRNAs, with the purified vsiRNAs-RISC exhibiting specific RNA-slicing activity (167, 169, 170, 172). When the VSR of enterovirus A71 (EV-A71) is rendered non-functional by synthetic VSR-targeting peptides, *in vivo* infection of wild-type EV-A71 is inhibited and vsiRNA biogenesis is activated (173). Notably, the VSR-deficient Nodamura virus (NoV) acts as a novel live-attenuated vaccine since it induces complete protection against lethal NoV infection in newborn mice only 2 days postvaccination (174). Unfortunately, it has not been possible to demonstrate *in vivo* rescue of any mammalian VSR-deficient mutant virus by deficiency in antiviral RNAi, largely because RNAi-defective mutant vertebrates are nonviable (18).

This review highlights the infection defects of VSR-deletion mutant viruses that can be rescued at the whole-organism level by genetic deficiency in antiviral RNAi. These specific infection defects define the range of virus resistance phenotypes conferred by antiviral RNAi in animals and plants because we excluded infection defects that result from loss of a VSR's function independent of RNAi suppression. Our analyses show that antiviral RNAi can inhibit virus accumulation at all three stages of infection in both plants and insects (Table 1). Antiviral RNAi-mediated clearance of viral RNAs is induced in insect cells inoculated directly with positive-stranded RNA viruses from branches 2 and 3. By contrast, activation of antiviral RNAi in plants restricts the spread of VSR-deficient CMV, TCV, and CymRSV into neighboring cells and/or distinct tissues without detectable effects on their accumulation in the initially infected cells (Table 1). Interestingly, leaves inoculated with the same VSR-deficient TCV marked by GFP develop fluorescent foci in *A. thaliana*, but only single fluorescent cells in *N. benthamiana*, suggesting a host-specific difference in blocking local and systemic virus spread by antiviral RNAi (Table 1). However, although GFP-marked VSR-deficient TuMV establishes local and systemic infection in mutant plants defective in antiviral RNAi, it appears that single fluorescent cells are not visible in the inoculated leaves of wild-type plants (122). This finding supports previous observations that the accumulation of VSR-deficient TEV is inhibited in the inoculated protoplasts, suggesting antiviral RNAi-mediated clearance of potyviral RNAs in the initially infected plant cells (120), similarly to that in insect cells inoculated with positive-strand RNA viruses FHV and CrPV. Moreover, the available evidence indicates that antiviral RNAi inhibits the accumulation of VSR-deleted DNA viruses only in the systemically infected tissues in both plants and insects (Table 1).

It is currently unclear why many mutant RNA and DNA viruses with a VSR deletion remain resistant to antiviral RNAi in the directly inoculated cells (Table 1). It is possible that potent viral RNA degradation by vsiRNA-RISC is activated against these viruses during or after the exponential phase of viral replication. Alternatively, these viruses may encode another VSR that can suppress antiviral RNAi in the directly inoculated

cells, as *Citrus tristeza* virus and geminiviruses (Fig. 3E and F) are known to express multiple mechanistically distinct VSRs (8, 175–177).

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