RNA silencing is a new immunity mechanism that protects fruit flies, mosquitoes, and nematodes as well as plants against viral infections.

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The innate immune system provides diverse organisms, including the fruit fly *Drosophila melanogaster*, with an early-acting defense against a broad range of microorganisms. Because *D. melanogaster* is relatively simple to work with and its genetics have been extensively analyzed, it provides a useful model for researchers to study innate immunity. For instance, the mammalian innate immune system against pathogens initiated by Toll-like receptor (TLR)-mediated nuclear factor κB (NF-κB) signaling was discovered in *Drosophila*. Bacterial and fungal infections induce antimicrobial peptide effectors by the Toll and immune deficiency (Imd) pathways in *D. melanogaster*. However, whether either pathway helps to protect *Drosophila* against viruses remains controversial.

Research in my laboratory showed that a distinct immunity mechanism is induced in *D. melanogaster* after virus infection. This viral immunity, discovered in plants during the 1990s, is controlled by RNA silencing, also known as RNA interference (RNAi). RNA silencing, an enzyme called Dicer, which is a dsRNA-specific endoribonuclease (RNase), hydrolyzes double-stranded RNA (dsRNA) into small interfering RNA molecules (siRNAs) of 21 to 26 nucleotides. These siRNAs then guide an RNaseH-like protein called Argonaute in an RNA-induced silencing complex (RISC) to specifically degrade single-stranded RNA molecules such as mRNA, thereby shutting down gene expression. RNA silencing is a conserved, gene-silencing mechanism that is widely distributed among plants, invertebrates, and vertebrates.

We refer to this viral immunity as small RNA-directed immunity (RDI), which also is active in both mosquitoes and nematodes. In RDI, virus-derived small interfering RNAs (viRNAs) are processed by Dicer directly from the infecting virus and used to guide specific degradation of virus genomic RNA and mRNA in infected cells. In counterdefense, viruses encode diverse proteins that can block viral immunity and facilitate virus infection.

**An Animal Viral Protein Suppresses RNA Silencing in Plants**

In 1995 we proposed that the B2 protein of Flockhouse virus (FHV), which infects insects including *D. melanogaster*, and the 2b protein of cucumber mosaic virus (CMV) function in similar ways. In 1998, my group along with David Baulcombe and his collaborators at the John Innes Centre in Norwich, England, identified the CMV 2b protein as one of the first two plant viral suppressors of RNA silencing (VSR). FHV is a member of the virus family *Noda-*

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**Summary**

- *Drosophila* cell culture studies provided early evidence that RNAi-mediated viral immunity occurs in animals.
- The immune system produces virus small interfering RNAs (viRNAs) from the infecting virus for guiding specific degradation of virus genomic RNA and mRNA.
- Key components in the immunity pathway have been identified by genetic studies in *D. melanogaster* and *C. elegans*.
- An effective counterdefense strategy is illuminated by the B2 protein of Flockhouse virus that inhibits production of viRNAs.
viridae, which includes pathogens of insects and fish. An exception is the Nodamura virus (NoV) that can lethally infect not only insects but also mammals. The animal Nodaviruses and the plant cucumoviruses contain segmented positive-strand RNA genomes that replicate via dsRNA intermediates. Both 2b of CMV and the FHV B2 open reading frames (ORFs) overlap the carboxyl terminus of the viral RNA-dependent RNA polymerase ORF and are translated from a subgenomic RNA (RNA 4A and RNA 3, respectively) produced after the genome replicates (Fig. 1). Although the B2 and 2b proteins are each conserved in amino acid sequence within their virus genera, we find no striking similarities between their primary sequences.

B2 of FHV suppresses RNA silencing in plants and, in a standard assay, is as potent as the plant viral 2b protein (Fig. 2). That assay is based on the transient, mixed expression of two transgenes that are co-infiltrated with two Agrobacterium tumefaciens strains. One strain induces RNA silencing of a green fluorescent protein (GFP) reporter gene in infiltrated leaves, visualized as a bright red color zone surrounding the infiltrated patch caused by chlorophyll autofluorescence and loss of GFP expression (Fig. 2, middle leaf). The other strain directs high-level expression of the candidate viral protein in co-infiltrated patches to test suppression of the induced GFP silencing.

Coexpression of either B2 of FHV (Fig. 2, left leaf) or a cucumoviral 2b (Fig. 2, right leaf) suppresses FHV silencing, leading to high-intensity green fluorescence in the infiltrated patch and loss of the red zone, according to my former postdoctoral fellow Hongwei Li, who is now at the University of Hawaii in Manoa. B2 also can substitute for 2b of CMV in whole-plant infections. The activity of this insect viral protein in plants struck us as so unusual that we suspected it plays a similar role in insects during viral infections.

**Induction and Suppression of RDI in Fruit Fly Cells**

We obtained direct evidence for RNAi-mediated viral immunity in invertebrates by studying fruit fly cells. Thus, we searched for virus-specific small RNAs by infecting Drosophila Schneider 2 (S2) cells with virions of FHV and then probing RNA from those cells for FHV-specific
small RNAs. Those samples contain abundant amounts of FHV viRNA molecules that are 22 nucleotides long, according to Li. Moreover, those viRNAs exhibit both virion and anti-virion RNA polarities. They can be detected in S2 cells one day after being infected with FHV, reaching a peak two days later. Thus, FHV infections trigger production of FHV-specific viRNAs.

To determine whether B2 activity is required for FHV to infect these insect cells, we introduced point mutations that block translation of this gene. Indeed, blocking B2 expression inhibits FHV replication, according to Hongwei Li and Wan-Xiang Li in my lab. Thus, the B2-deficient FHV mutant proves difficult to detect in S2 cells. However, when such cells are transfected with a plasmid containing the wild type FHV genome, the virus is readily detected. Thus, B2 plays an essential role in FHV infections of insect cells.

We suspected that B2 acts as a viral suppressor of RNAi in fly cells because FHV infection in fly cells triggers FHV-specific viRNAs. Moreover, in the absence of RNAi suppression, even if the B2-deficient mutant FHV replicated, RNAi would destroy invading viral RNAs and block production of viral progeny.

If true, it should be possible to rescue the B2-deficient mutant FHV in fly cells that cannot launch the RNAi response. Indeed, treatment of S2 cells with dsRNA that specifically targets the mRNA of Argonaute-2 rescues the B2-deficient mutant FHV, indicating that this mutant virus can replicate. The rescue is specific and is not a result of nonspecific dsRNAs saturating the RNAi pathway. Shortly before we started these experiments, Greg Hannon and colleagues of Cold Spring Harbor Laboratory in Cold Spring Harbor, N.Y., reported that Argonaute-2 is essential for RNAi as depletion of Argonaute-2 severely weakens RNAi potency in S2 cells.

These findings led us to several important conclusions. First, FHV infection triggers RNAi-mediated immunity that clears viral RNAs from infected Drosophila cells. Second, Argonaute-2 is a key host component—and the first to be identified—with this invertebrate immunity pathway. Third, the B2 protein of FHV, which is functionally similar with the plant viral 2b protein, actively suppresses RNAi in animal cells, making it the first such suppressor identified in animals. Fourth, suppressing RNAi immunity is essential for viruses to infect fruit fly cells.

Replication of Nodamura virus (NoV), which is similar to FHV, can also induce Argonaute2-dependent RNAi immunity in fruit cells, according to Wan-Xiang Li. B2 of NoV, whose sequence differs substantially from that of B2 (less than 19% identity) from FHV, also is a potent suppressor of RNAi in fruit fly cells and in plants. Notably, a plasmid that expresses B2 of either NoV or FHV can rescue in trans B2-deficient FHV and NoV. Thus, the same small RNA-directed immunity is induced by replication of either FHV or NoV in fruit fly cells, and each virus is equipped with a protein that can block RDI.

**Restriction of Viral Replication by RNAi in Mosquito Cells**

After the genome sequence of the malaria mosquito Anopheles gambiae was published in October 2002, Monica Dus in my lab scanned it for genes resembling those required for RNAi in fruit flies. This search led us to identify mosquito versions of the Argonaute-2 and Dicer-2 genes, which Liangbiao Zheng and colleagues of Yale University School of Medicine in New Haven, Conn., also identified.

Although NoV replicates robustly in wild-type malaria mosquito cells, a B2-deficient NoV mutant replicates poorly, and is detectable only in cells that were depleted of Argonaute-2 or cotransfected with a plasmid that directs expression of a nodaviral B2 protein, according to Rui Lu in my lab. These 2004 results provide direct
evidence for RNAi-mediated viral immunity in mosquitoes. Consistent with those findings, Ken Olson and colleagues at Colorado State University in Fort Collins later that year detected enhanced accumulation of an arbovirus that was coinjected with the Argonaute-2 dsRNA from mosquitoes.

RNAi Immunity Prevents Diverse Viruses from Infecting Fruit Flies

We also are studying true genetic, loss-of-function mutant fruit flies, including those defective in Dicer-2 (*dcr-2*), R2D2 (*r2d2*) and Argonaute-2 (*ago-2*) that were isolated by Richard Carthew of Northwestern University in Evanston, Ill., and Mikiko Siomi of the University of Tokushima in Todushima, Japan, respectively, and their collaborators.

With assistance from Richard Carthew and Peter Atkinson in the Department of Entomology at the University of California, Riverside, Xiao-Hong Wang and Wan-Xiang Li in my lab compared how the wild-type and the B2-deficient mutant FHV replicate when they are injected into wild-type and *dcr-2* and *ago-2* mutant embryos. They then extracted total RNAs from the embryos 30 hours later and probed for FHV RNAs. Unlike wild-type FHV, which replicates to high levels in the wild-type and mutant embryos, we could detect the B2-deficient mutant only in the *dcr-2* and *ago-2* mutant embryos. These findings recapitulate our data in cell culture by RNAi knockdown of Argonaute-2. Moreover, they also point to a specific role for Dicer-2—namely, initiating RNAi-mediated clearance of FHV RNAs in fruit fly embryos.

FHV is of low virulence, and at least 50% of adult flies remain alive 15 days after being infected with this virus. However, infecting *dcr-2* and *r2d2* mutant flies with the same dose of FHV results in nearly 100% mortality within 10 days (Fig. 3), according to Xiao-Hong Wang. R2D2 contains tandem dsRNA-binding domains and forms a heterodimer with Dicer-2 that is required for siRNA loading into RISC. Thus, fruit flies that are defective in RNAi are more susceptible to a low-virulence virus.

These findings provided the first evidence that RNAi immunity indeed protects adult flies from viral infections. Notably, although RNAi is defective in both *dcr-2* and *r2d2* mutants and FHV accumulates to much higher levels in both of the mutants than in wild type flies, abundant viral siRNAs are detected only in *r2d2* flies but not in *dcr-2* flies. Thus, we conclude that Dicer-2 is required for producing viRNAs, whereas R2D2 is required for those viRNAs to be active.

RNAi Immunity Works against Other Fly-Infecting Viruses

To determine whether RNAi immunity works against other viruses, Xiao-Hong Wang injected Cricket paralysis virus (CrPV), which contains a
nonsegmented positive-strand RNA genome and belongs to a group of polio-like viruses, into both mutant and wild type flies. CrPV is substantially more virulent than FHV in fruit flies. For example, introducing CrPV at much lower titers kills 70% of wild type flies by 15 days. CrPV is more virulent and accumulates more rapidly and to greater levels in both

\[ \text{dcr-2} \]

and

\[ \text{r2d2} \]

mutant flies (Fig. 3). Thus, both Dicer-2 and R2D2 are required to protect fruit flies against CrPV. However, inducing antimicrobial peptide genes via Toll and Imd signaling pathways is not compromised in

\[ \text{dcr-2} \]

, and

\[ \text{r2d2} \]

mutant flies, according to Roghiyh Aliyari in my lab. This finding suggests that NF-κB-like signaling does not play a role in RNAi-mediated viral immunity.

\[ \text{dcr-2} \]

and

\[ \text{r2d2} \]

mutant flies after they are infected with several different positive-strand RNA viruses, including FHV, Sindbis virus, and

\[ \text{Drosophila} \] C virus.

Moreover, Dicer-2, R2D2, and Argonaute-2 are key components, each playing a specific role in the dsRNA-siRNA pathway. However, none of the three genes plays a detectable role in fly development or the biogenesis and activity of microRNAs (miRNAs), which in

\[ \text{Drosophila} \]

Dicer-1 processes from stem-loop structures of nuclear transcripts, thereby silencing the endogenous genes.

From these results, we draw several conclusions. First, fruit flies are equipped with a conserved RNAi pathway to defend against genetically diverse groups of positive-strand RNA viruses. Second, this protective system includes several genetically required molecular components, including Dicer-2, R2D2, and Argonaute-2. Dicer-2 recognizes dsRNA as its substrate, but does not produce miRNAs from intramolecular stem-loop structures in miRNA precursors. The viral dsRNA replicative intermediates serve as the pathogen trigger—and Dicer-2 as the host sensor—of this innate immunity system (Fig. 4). Meanwhile, the viral siRNAs apparently guide Argonaute-2 in destroying viral RNAs. Interestingly, Louisa Wu and colleagues in the University of Maryland Biotechnology Institute demonstrate that both

\[ \text{r2d2} \]

and

\[ \text{ago-2} \]

mutant flies, but not

\[ \text{dcr-2} \]

flies, exhibit enhanced susceptibility to

\[ \text{Drosophila} \]

X virus, suggesting induction of a related but distinct immunity by the dsRNA virus.

**Viral Counterdefenses Depend on RNAi Suppressors**

Plant viruses employ diverse means for suppressing RNAi-mediated immunity. Similarly, animal viruses encode proteins that can suppress RNAi-mediated degradation of nonviral RNA targets in animal cells. However, the only data demonstrating a specific suppressor role for these animal viral proteins involves the nodaviral B2 protein. The B2 protein of
FHV binds dsRNA, according to my former graduate student Feng Li. Recognizing this activity proved a major milestone in understanding how viruses suppress RNAi immunity. Working with Hongwei Li, Feng Li showed that B2 inhibits Dicer-2 production of siRNAs from dsRNA in vitro. Replacing Arg with Gln at position 54 of B2 abolishes both the dsRNA-binding activity and the inhibitory effect on siRNA production. Viruses with this mutant B2 are defective in suppressing RNAi immunity in Drosophila cells.

B2 of FHV is indeed a dsRNA-binding protein distinct from the canonical dsRNA-binding proteins, according to James Williamson and colleagues at Scripps Research Institute in San Diego, Calif., and Michael Sattler and his colleagues at the European Molecular Biology Laboratory in Heidelberg, Germany. Working separately, they and their respective colleagues found that Arg-54 is located in the center of the dsRNA binding surface. Thus, insect nodaviruses keep Dicer-2 from producing viral siRNAs, thereby suppressing RNAi-mediated innate immunity.

*C. elegans—a New Model for Genetic Studies on Virus-Host Interactions*

The nematode *Caenorhabditis elegans* is a widely studied organism. For instance, Nobel Prizes were awarded to Sydney Brenner, Robert Horvitz, and John Sulston in 2002 for their discoveries concerning genetic regulation of organ development and programmed cell death, and in 2006 Andrew Fire and Craig Mello for discovering dsRNA-triggered RNAi in *C. elegans*. Unlike plants and insects that encode multiple Dicers, *C. elegans* encodes a single Dicer, as do vertebrates and humans.

In 2003 five research groups detected viral replication and induction of RNAi-mediated immunity in *C. elegans*. For example, with assistance from Morris Maduro at the University of California, Riverside, Rui Lu in my lab observed replication of the FHV genome in *C. elegans* strains carrying chromosome-integrated, heat-inducible transgenes. The RNAi immunity induced by FHV in *C. elegans* is effectively suppressed by B2 expressed from the viral genome. Notably, the nematode immunity requires RDE-1, an Argonaute protein similar to Argonaute-2 of *Drosophila*. RDE-1 is essential for siRNA activity but not that of miRNAs. Based on vesicular stomatitis virus studies in both cases, Marie Chow and colleagues at the University of Arkansas for Medical Sciences in Little Rock and Craig Hunter and colleagues at Harvard University in Cambridge, Mass., similarly found evidence of RNAi immunity in *C. elegans*.

These studies, together with those by Yi-Chun Wu, Ching-Len Liao, and their colleagues from the National Taiwan University in Taipei, demonstrate that viruses can infect, replicate, and assemble within *C. elegans* cells. Thus, *C. elegans* provides another relatively simple eukaryotic organism for further understanding the intricacies of virus-host interplay, including RNAi-mediated host immunity.

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**SUGGESTED READING**


