

RNA-based immunity in insects

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INTRODUCTION

Drosophila has been an excellent model for the mechanistic studies of innate immunity (Hoffmann, 2003). Recently, a new RNA-based antiviral immunity with features of both innate and adaptive immunities has been described in *Drosophila* and *Anopheles* cells (Li *et al.*, 2002, 2004). This RNA-silencing-mediated immunity is characterized by the production of pathogen-derived, 22-nt small RNAs that serve as specificity determinants inside a multi-subunit complex. Similar to innate immunity, however, the new invertebrate antiviral response is capable of a rapid virus clearance in the absence of a virus-encoded suppressor of RNA silencing. The discovery of a new antiviral pathway in insects opens up the possibility of using this pathway to prevent transmission of vector-borne virus pathogens such as dengue and West Nile viruses.

THE RNA-SILENCING PATHWAY

Homology-dependent gene silencing was discovered in transgenic plants in a form of co-suppression between introduced transgenes or between a transgene and its homologous endogenous gene (Matzke *et al.*, 1989; Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). Similar gene-silencing phenomena have subsequently been described in a wide range of eukaryotic organisms such as fungi, worms, flies and mammals (Denli & Hannon, 2003; Fire *et al.*, 1998). A generic term, RNA silencing (Ding, 2000), has been used to describe these related RNA-guided gene regulatory mechanisms variously termed post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNA interference (RNAi) in animals.

A core feature of RNA silencing detected in all organisms is the production of 21–26-nt small RNAs from structured or double-stranded RNA (dsRNA) by the endoribonuclease Dicer (Bernstein *et al.*, 2001; Hamilton *et al.*, 2002; Hamilton & Baulcombe, 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000). These small interfering RNAs (siRNAs) control the specificity of RNA silencing in a homology-dependent manner in an RNA-induced silencing complex (RISC), of which the Argonaute-2 (AGO2) protein is an essential component (Denli & Hannon, 2003; Elbashir *et al.*, 2001; Hammond *et al.*, 2001). RNA silencing in fungi, plants and worms involves a cellular RNA-dependent RNA polymerase (RdRP); however, the multiple-turnover RISC may mediate RNA silencing in the absence of a cellular RdRP in *Drosophila* and mammalian cells (Cogoni & Macino, 1999; Denli & Hannon, 2003; Martinez *et al.*, 2002; Schwarz *et al.*, 2002).

The first indication for a possible antiviral role of RNA silencing came from the observation that virus infection is able to trigger RNA silencing of a homologous virus-derived transgene in transgenic plants (Lindbo *et al.*, 1993). Subsequent work has established that RNA silencing is a natural antiviral response of plants, which is induced upon virus infection and can specifically target the viral and homologous RNAs for degradation (Baulcombe, 1999). This conclusion is further supported by the demonstration in 1998 that HC-Pro and 2b, two essential pathogenic factors encoded by potyviruses and cucumoviruses, are suppressors of RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998; Li *et al.*, 1999; Li & Ding, 2001).

In contrast to the broad recognition of a natural antiviral role for RNA silencing in higher plants, no experimental evidence was available until recently on whether or not RNA silencing plays a similar antiviral role in the animal kingdom. Here we review recent progress in establishing RNA silencing as a novel nucleic-acid-based antiviral immunity in insects and discuss features of this novel antiviral response as compared to the known innate and adaptive immunities.

VIRUSES ARE TARGETS AND INDUCERS OF RNA SILENCING IN INSECTS

Following the first report in *Drosophila* (Kennerdell & Carthew, 1998, 2000; Pal-Bhadra *et al.*, 1997), effective RNAi induced by dsRNA has been demonstrated in many arthropod species (Table 1). These findings indicate that arthropods encode a functional RNA-silencing pathway. The early evidence implicating viruses as targets of RNA silencing in insects came from the use of alphavirus-based expression vectors (Gaines *et al.*, 1996; Olson *et al.*, 1996). Similar to transgenic plants that contain a virus-derived silencing transgene (Baulcombe, 1999), mosquitoes infected with a

Table 1. RNAi in arthropods

Species	Inducer	Means of delivery	Target RNA	Reference*
Fruit fly <i>Drosophila melanogaster</i>	Transgene dsRNA RNA replication	Transfection Embryo injection Transformation	Cellular and viral	1–5
Medfly <i>Ceratitidis capitata</i>	dsRNA	Embryo injection	Cellular	6
Milkweed bug <i>Oncopeltus fasciatus</i>	dsRNA	Embryo injection	Cellular	7
Mosquito <i>Anopheles gambiae</i>	dsRNA Transgene RNA replication	Transfection Adult injection Transformation	Cellular and viral	8–13, 18
Giant silkworm <i>Hyalophora cecropia</i>	dsRNA	Pupa injection	Cellular	14
Red flour beetle <i>Tribolium castaneum</i>	dsRNA	Pupa injection Embryo injection	Cellular	15–16
Noctuid moth <i>Trichoplusia ni</i>	dsRNA	Transfection	Viral	17
Silkworm <i>Bombyx mori</i>	RNA replication	Larvae injection	Cellular	18
Tick <i>Amblyomma americanum</i>	dsRNA	Soaking Adult injection	Cellular	19–20
Honeybee <i>Apis mellifera</i>	dsRNA	Embryo injection Pupa injection	Cellular	21–22
Spider <i>Cupiennius salei</i>	dsRNA	Embryo injection	Cellular	23

* 1, Pal-Bhadra *et al.* (1997); 2, Kennerdell & Carthew (1998); 3, Kennerdell & Carthew (2000); 4, Hammond *et al.* (2000); 5, Li *et al.* (2002); 6, Pane *et al.* (2002); 7, Hughes & Kaufman (2000); 8, Billecocq *et al.* (2000); 9, Brown *et al.* (2003); 10, Gaines *et al.* (1996); 11, Hoa *et al.* (2003); 12, Levashina *et al.* (2001); 13, Li *et al.* (2004); 14, Bettencourt *et al.* (2002); 15, Bucher *et al.* (2002); 16, Wheeler *et al.* (2003); 17, Beck & Strand (2003); 18, Uhlirova *et al.* (2003); 19, Aljamali *et al.* (2003); 20, Aljamali *et al.* (2002); 21, Amdam *et al.* (2003); 22, Beye *et al.* (2002); 23, Stollewerk *et al.* (2003).

recombinant alphavirus carrying a heterologous viral sequence also develop a RNA-mediated homology-dependent virus resistance (Adelman *et al.*, 2001; Billecocq *et al.*, 2000; Gaines *et al.*, 1996; Olson *et al.*, 1996). Specific targeting of insect viruses by RNA silencing was confirmed by several recent studies. For example, fruit fly cells transfected with dsRNA corresponding to part of the plus-strand RNA genome of flock house virus (FHV) became resistant to FHV (Li *et al.*, 2002). Similarly, mosquito cells transcribing an inverted-repeat RNA targeting the dengue virus type 2 (DEN-2) RNA genome were unable to support replication of DEN-2 (Adelman *et al.*, 2002). Efficient RNAi of two genes encoded by a double-stranded DNA virus from the *Polydnaviridae* family was also demonstrated in noctuid moth (*Trichoplusia ni*) cells (Beck & Strand, 2003).

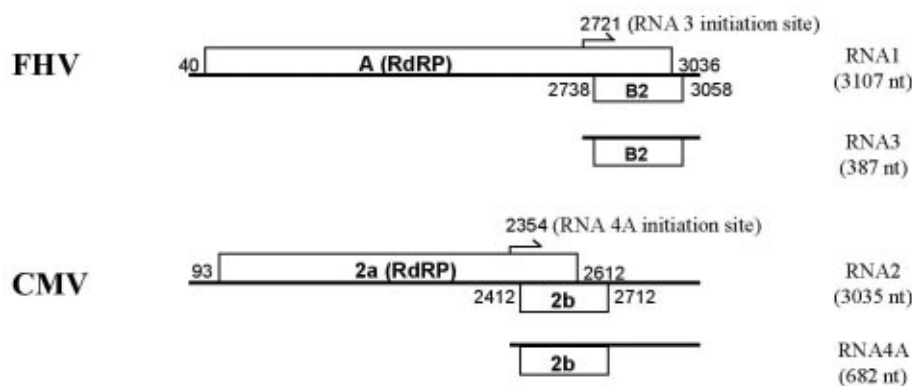


Fig. 1. Genome organization and expression of FHV RNA1 and CMV RNA2. The locations of both start and stop codons in the genomic RNAs for each of the viral genes are indicated. The ORF encoding the viral RdRP, called 2a and A, respectively, is translated from the genomic RNA whereas the smaller overlapping gene (B2 and 2b) is translated from the subgenomic RNA. The conserved GDD motif found in viral RdRPs is encoded by nucleotides 2110–2118 of FHV and nucleotides 1908–1916 of CMV.

Two lines of evidence indicate that specific silencing of the infecting viral RNAs is induced naturally in insect cells upon virus infection. First, infection of either cultured fruit fly cells by FHV or the silkworm (*Bombyx mori*) larvae by Sindbis virus led to the *in vivo* accumulation of the virus-specific siRNAs (Li *et al.*, 2002; Uhlirova *et al.*, 2003). This indicates that the invertebrate Dicer ribonuclease(s) is able to detect the infecting viruses, most likely by recognizing the viral RNA replicative intermediates as the substrate. Second, increased accumulation of FHV RNAs was observed in the fly cells after the RNA-silencing pathway was made partially defective by RISC depletion through RNAi of AGO2 (Li *et al.*, 2002). Thus a functional RNA-silencing pathway naturally restricts FHV accumulation in infected fruit fly cells. A recent study has further provided evidence for the induction of the RNA-silencing-based antiviral response (RSAR) in both fruit fly and mosquito cells following nodamura virus (NoV) RNA replication (Li *et al.*, 2004) (see later).

INVERTEBRATE VIRAL SUPPRESSORS OF RNA SILENCING

The first invertebrate viral suppressor of RNA silencing, the FHV B2 protein (Li *et al.*, 2002), was discovered following a prediction made in an earlier report (Ding *et al.*, 1995). FHV belongs to the *Alphanodavirus* genus, of which NoV is the type member. FHV was originally isolated from the grass grub *Costelytra zealandica* (Coleoptera) in New Zealand. In the laboratory, FHV also infects *Galleria mellonella* (wax moth) and cultured *Drosophila melanogaster* and mosquito cells and replicates efficiently in plant, yeast and mammalian cells (Ball, 1995; Dasgupta *et al.*, 2003). The nodaviral B2 gene

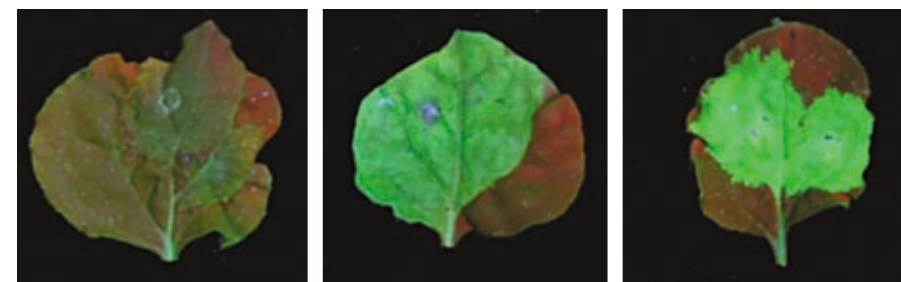


Fig. 2. Cross-kingdom suppression of RNA silencing in plants by an animal viral suppressor of RSAR. The GFP-expressing *Nicotiana benthamiana* leaves were co-infiltrated with a mixture of two *Agrobacterium tumefaciens* strains, as described by Guo & Ding (2002). One directs the expression of GFP and thereby induces GFP RNA silencing, and the other simultaneously expresses the NoV-encoded B2 (right), a plant cucumoviral 2b (middle) or an untranslatable mutant of B2 (left). The leaves were detached and photographed under UV illumination 6 days after infiltration. GFP silencing is visualized in the left leaf as a lack of green fluorescence in the infiltrated patch surrounded by a red colour zone caused by chlorophyll fluorescence.

shares a number of features with the 2b gene of the plant cucumoviruses (Ding *et al.*, 1995), which was demonstrated in 1998 to encode a suppressor of RNA silencing (Brigneti *et al.*, 1998; Li *et al.*, 1999). Both 2b and B2 genes overlap the 3'-end, and occupy the +1 reading frame, of the viral RdRP gene and are translated *in vivo* from a subgenomic RNA, although their encoded proteins do not exhibit any detectable sequence similarities (Fig. 1). Knockout of either B2 in FHV or 2b in cucumber mosaic virus (CMV) resulted in a defect in virus spreading, but neither is required for viral RNA replication (Ball, 1995; Ding *et al.*, 1995).

Using an assay based on transgene RNA silencing in plants, it was shown that the FHV B2 protein indeed is a suppressor of RNA silencing (Li *et al.*, 2002). In this system, B2 suppression was as potent as the 2b protein encoded by tomato aspermy cucumovirus, which is among the strongest plant viral suppressors known (Li *et al.*, 2002). By comparison, the 2b of CMV is a much weaker suppressor that delayed, but did not prevent, the initiation of RNA silencing (Guo & Ding, 2002). A recent study (Li *et al.*, 2004) found that the NoV B2 protein is also a suppressor of RNA silencing in the same *in planta* assay (Fig. 2). Notably, the FHV B2 is able to functionally substitute for 2b of CMV in whole-plant infections, suggesting that the nodaviral B2 is able to inhibit RNA silencing triggered by viral RNA replication (Li *et al.*, 2002).

The nodaviral B2 also suppresses RNA silencing in *Drosophila* cells that is induced by exogenous long dsRNA (Li *et al.*, 2004). Interestingly, RNA-silencing suppression requires B2 expression before dsRNA is introduced into cells to initiate RNAi and suppression was not observed when B2 and dsRNA were simultaneously introduced.

This suggests that B2 suppression may occur before either the production or RISC loading of siRNAs (Li *et al.*, 2004). The fact that the nodaviral B2 protein suppresses RNA silencing in both fruit flies and plants provides the first direct evidence for a conserved RNA-silencing pathway in the plant and animal kingdoms.

SUPPRESSION OF RNA SILENCING IS ESSENTIAL FOR VIRUS INFECTION IN FRUIT FLY CELLS

The important contribution of RNA silencing to invertebrate defence against virus infection was not obvious until the nodavirus-encoded suppressor of RNA silencing was made inactive (Li *et al.*, 2002, 2004). Infection with wild-type FHV rapidly triggers RSAR in infected fruit fly cells, as indicated by the detection of FHV-specific siRNAs 24 h after infection. Nevertheless, FHV accumulation continues to increase in infected cells, suggesting that the induced RSAR fails to provide protection against the nodaviral infection. The FHV mutant containing point mutations that rendered B2 untranslatable, however, does not accumulate to a detectable level in *Drosophila* cells unless it is co-transfected with a B2-expressing plasmid. An efficient rescue of the B2-knockout mutant was also observed when the host machinery RISC was depleted by co-transfection with either a dsRNA or siRNA targeting AGO2 (Li *et al.*, 2002, 2004). These findings show that the B2 deletion did not prevent FHV RNA replication and that the observed essential role of B2 is to suppress the RNA silencing induced by FHV RNA replication that specifically targets the FHV RNAs for destruction. It is important to note that, as occurred in the absence of viral suppression of RNA silencing by B2, the induced RSAR is sufficient to ensure a rapid and complete virus clearance in the challenged *Drosophila* cells.

A mutational analysis on the B2 gene encoded by NoV has been carried out recently (Li *et al.*, 2004). NoV is the only member of the *Alphanodavirus* genus that can lethally infect both insects and mammals in the laboratory and it shares either low or minimal sequence identities with FHV in their encoded proteins (44 % for the viral RdRP and <19 % for B2) (Johnson *et al.*, 2001). The result shows that NoV RNA replication in *Drosophila* cells induces a potent RSAR that is RISC-dependent and capable of eliminating NoV RNAs when its B2 protein is not expressed (Li *et al.*, 2004). In *Drosophila* cells, the RSAR triggered by nodaviral RNA replication can be reciprocally inhibited by heterologous B2 proteins, leading to efficient rescue of either B2-deficient nodavirus mutant (Li *et al.*, 2004). This shows that although targeted destruction of the infecting viral RNAs by RSAR as mediated by siRNAs is specific, viral suppression of invertebrate RSAR is broad-spectrum, implying that the same core pathway is involved in defence against different viruses.

RNA SILENCING AS AN RNA-BASED ANTIVIRAL IMMUNITY IN MOSQUITO CELLS

Mosquito cells support RNAi and homologues of the *Drosophila* RNAi components are encoded by the recently sequenced genome of *Anopheles gambiae*, which transmits both malaria and o'nyong-nyong alphavirus (Christophides *et al.*, 2002; Hoa *et al.*, 2003; Holt *et al.*, 2002; Levashina *et al.*, 2001; Powers *et al.*, 2000). It has been demonstrated recently that replicating virus RNAs are naturally targeted for destruction by RNAi in mosquito cells (Li *et al.*, 2004). This potent RSAR does not require prior activation, e.g. through transformation with an inverted-repeat RNA transgene (Adelman *et al.*, 2002), but it is masked in *A. gambiae* cells by the action of the B2 protein expressed from wild-type NoV RNA1. After its B2 was made untranslatable, the accumulation of NoV RNAs was almost completely abolished in mosquito cells (Li *et al.*, 2004). The NoV RNA1 mutant was rescued by co-transfection with a plasmid expressing B2 of either nodavirus, a dsRNA or siRNA targeting the mRNA of the *A. gambiae* AGO2, but not by an unrelated dsRNA or siRNA. Thus, as has been found in *Drosophila*, NoV RNA replication also triggers RSAR in *A. gambiae* cells which is both AGO2-dependent and sensitive to B2 suppression. These findings establish RSAR as a new RNA-based antiviral immunity in mosquitoes.

FEATURES OF THE INVERTEBRATE RSAR

The RSAR of invertebrates is rapid, effective and adaptive. In contrast to the broad-spectrum innate immunity in *Drosophila* and mosquitoes (Christophides *et al.*, 2002; Hoffmann, 2003), RSAR has features similar to the peptide-based adaptive immunity in vertebrates (Whitton & Oldstone, 2001). The specificity determinants of RSAR are siRNAs, which are derived and processed from the invading virus. After being uploaded into RISC, these virus-specific siRNAs selectively recruit target viral genomic and/or messenger RNAs by base-pairing for RISC-mediated destruction. At the whole-organism level, RNA silencing may also provide a long-term memory, analogous to the lifetime maintenance of specific virus resistance in plants recovered from a virulent primary infection (Covey *et al.*, 1997; Ratcliff *et al.*, 1997, 1999; Xin & Ding, 2003). However, while the peptide-based immunity in vertebrates usually takes more than a week to respond (Whitton & Oldstone, 2001), the RNA-silencing response in fruit flies and mosquitoes is capable of a rapid and complete virus clearance in single cells (Li *et al.*, 2002, 2004), which is similar to the innate immunity. In this regard, it is possible that RNA silencing may contribute to, or be part of, the robust innate immunity occurring at early stages of viral infection in vertebrates (Parham, 2003). This is supported in part by the recent demonstration that a number of mammalian viruses encode suppressors of RNA silencing that were previously shown to inhibit the interferon-regulated innate immunity in vertebrates (Li *et al.*, 2004).

It is known that common components, such as siRNA, Dicer and Argonaute proteins, are involved in PTGS in plants and RNAi in animals (Denli & Hannon, 2003; Tang *et al.*, 2003; Vance & Vaucheret, 2001). Suppression of RSAR in both plants and invertebrates by the same B2 protein provides direct experimental evidence that at least some aspects are conserved in the RNA-silencing pathway between the plant and animal kingdoms (Li *et al.*, 2002; Lindenbach & Rice, 2002). However, it appears that at the single-cell level, RSAR targeting of infecting viruses is not as potent in plants as in insects. For example, most of the plant viral suppressors characterized to date interfere with the cell-to-cell and long-distance spread of RNA silencing, which may explain why plant virus mutants that lack a functional suppressor do not exhibit obvious defect in single cells (Li & Ding, 2001; Silhavy *et al.*, 2002). This is in contrast to an essential role for B2 suppression in nodaviral infection of insect cells (Li *et al.*, 2002, 2004). It is possible that organisms that do not carry a cellular RdRP such as fruit flies and mosquitoes have evolved a more efficient multiple-turnover RISC capable of a potent intracellular silencing, which becomes the target of animal viral suppressors.

CONCLUDING REMARKS

There is now compelling evidence supporting RNA silencing as a novel RNA-based antiviral immunity in insects since RNAi was first demonstrated more than 5 years ago. First, virus infection triggers RNA silencing in insect cells that specifically targets the invading viral RNA. Second, invertebrate viruses such as FHV and NoV encode suppressors of RNA silencing essential for infection of insect cells in which the RNA-silencing pathway is not compromised. It is also clear that the insect RSAR is mediated by the RNAi pathway, which is based mostly on the experimental induction of RNA silencing by exogenous dsRNA. This is because the nodaviral B2 protein suppresses RNA silencing in insect cells induced by either dsRNA or viral RNA replication and the insect RSAR involves Dicer recognition of replicating virus RNAs and is RISC-dependent. The demonstration that RNAi naturally protects insects from virus infection opens up the possibility of using this pathway to control insects that are either crop pests or vectors for crop and human pathogens.

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Specificity of *Borrelia*–tick vector relationships

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BORRELIA

The genus *Borrelia* comprises diverse species of spirochaetes that are associated with haematophagous arthropods (Paster *et al.*, 1991; Paster & Dewhirst, 2000). Some *Borrelia* species are pathogenic for humans or for livestock. Other spirochaete groups with human pathogens are the treponemes, which include the human-restricted agent of syphilis, and the leptospire, which are mostly free-living spirochaetes that infect a wide variety of animals. The spirochaete phylum also contains a number of species that are symbionts of invertebrates, such as molluscs and termites. *Borrelia* spirochaetes characteristically circulate in the blood of their vertebrate hosts and are transmitted between vertebrates by ticks, with the single, epidemiologically important exception of a louse-borne species. A common strategy of *Borrelia* spp. for prolonging spirochaetaemia – thus increasing the probability of vector transmission – is avoidance of the immune response through antigenic variation (Barbour & Restrepo, 2000; Barbour, 2002). Most types of *Borrelia* infections are zoonoses, but humans are the critical reservoirs for at least one species (Barbour & Hayes, 1986; Barbour, 2004).

The number of recognized *Borrelia* species has more than doubled over the last two decades, in part because cultivation methods improved (Barbour, 1988; Cutler *et al.*, 1994) and technologies like PCR allowed identification and taxonomic classification without being able to culture the organism (Anda *et al.*, 1996; Barbour *et al.*, 1996; Kisinza *et al.*, 2003; Scoles *et al.*, 2001). Table 1 is a list of accepted and tentative species designations, as of early 2004. *Borrelia* species have been documented in the Palaearctic, Afro-Tropical, Nearctic, Neotropical and Antarctic ecological regions, and some