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R Gopalakrishnan
Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, India

S Subramanian
Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Vinay Kalia
Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, India

Corresponding Author:
R Gopalakrishnan
Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi, India

RNAi machinery in insects and its challenges in pest management: A review

R Gopalakrishnan, S Subramanian and Vinay Kalia

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Abstract
RNA interference (RNAi) is a gene silencing mechanism mediated by double-stranded RNA (dsRNA) through post-transcriptional regulation. RNAi is capitalized for studying gene function in a variety of organisms and it is commonly employed for down regulating gene expression in cell. The principle of RNAi silencing involves custom steps such as processing of dsRNA, formation of RNA-induced silencing complex (RISC), target mRNA binding and cleavage. Nucleolytic degradation of the besieged mRNA by the RNase I enzyme (Argonaute - in case of insects) and RNase H enzyme (slicer - in case of nematode) results in gene silencing. The dsRNA uptake mechanism is mediated either through transmembrane channel or through endocytosis in insects. Among them SID-1 protein with 4 transmembrane domains is the major portal site for dsRNA uptake in insect midgut [15]. RNAi was successfully established in several insect orders like Coleoptera, Orthoptera, Diptera, Hemiptera and Lepidopteran pests such as corn planthopper, whitefly and pea aphid [51]. RNAi was highly efficient in coleopteran insects and have very low efficient in lepidopteran insects and the range of efficiency varies from 40% to 90% in insects. The variation is due to the inherent difference in RNAi machinery in insects influenced by different factors such as RNase, RNA-dependent polymerase (RdRP), SID 1 transmembrane channels and gut pH [32]. The presence of RNA nuclease (*RNase*) in the midgut and absence of RdRP in insects results in degradation of dsRNA in insect and trapping of dsRNA in acidic bodies in the midgut cells yields poor RNAi response in lepidopteran insects [45]. Understanding the inherent difference in RNAi machinery will help to resolve the strategies for efficient pest management through RNAi.

Keywords: RNAi, insects, SID 1, insensitivity

1. Introduction
Plants had originated above 700 million years ago and insects had originated 500 million years ago. Both insects and plants are in co-evolution for millions of years and insects started using plants as a source of food, shelter and reproduction. The natural enemies such as predators and parasitoids play a major role in diminishing the insect pest population in ecosystem. Apart from natural control, human intervention also aims at reducing the population level of insect pests and evolution of technology had resulted in modern means of pest management tools like biopesticides, insecticides with novel mode of action, microbial pesticides and biotechnological approaches like RNA interference etc.

1.1 RNA Interference
RNAi is a conserved process occurring naturally in all organism for gene regulation and defense against pathogens. The major principle of RNAi is post-transcriptional sequence specific gene silencing induced through double stranded RNA (dsRNA) [61]. RNAi can be divided into three pathways based on the small RNA biogenesis and the Argonaute protein: microRNA (miRNA), small interfering RNA (siRNA) and piwi-interacting RNA pathways. Among them, the siRNA pathway is the most prospective tool in insect pest control. The efficacy of RNAi varies among different insect orders and also depends upon various factors, including the target gene selection, method of dsRNase delivery, and expression of dsRNAs and presence of off-target effects. RNAi-mediated silencing of different insect genes involved in various physiological processes was found tobe detrimental to insect growth, development and survival RNA interference (RNAi), usually referring to the small interfering (si) RNA pathway [56].

1.2 History of RNAi

RNAi, the post-transcriptional gene silencing is known as cosuppression in Petunia plant and quelling in Neurospora fungi. In 1990, Napoli and Jorgensen defined RNAi-phenomenon in Petunia plant and called it as "*cosuppression*". There experiment was based on Chalcone synthase (CHS), a key enzyme for flavonoid biosynthesis in plants. It is the rate-limiting enzyme in anthocyanin biosynthesis resulting purple coloration in petals. The experiment was done to increase the expression of CHS enzyme but the result was massive reduction in expression of chalcone synthase gene in flowers producing white colour petals. Later on in 1992 Carlo Cogoni and Guiseppe Macino identified RNAi mechanism in fungus *Neurospora crassa*. They introduced a gene (al-1 gene), a carotenoid synthesis gene in the fungus but instead of over expression, radical inactivation of the mold's own gene was noticed. They called this gene inactivation in fungus as "quelling". In1995, GuoS and Kemphues KJ were the first to put forth that sense RNA and antisense RNA were able to suppress the gene expression in worm in *Caenorhabditis elegans*. Later on in 1998, it was Andrew Fire and Craig Mello reported that it was dsRNA was most effective in RNAi than sense RNA by injecting dsRNA into *C. elegans*. They described this sequence-specific silencing and coined the term "RNA Interference ". In 2006 Andrew Fire and Craig Mello were awarded with Noble Prize in Physiology or Medicine for discovering RNAi mechanism [1, 39].

2. Overview of RNAi in insects

RNAi includes three major pathways lie small interference (siRNA), micro RNA (miRNA) and Piwi-interacting RNA (piRNA) pathway. RNAi pathway is centered in two major steps, initially dsRNA or microRNA is processed into a siRNA by the RNaseIII enzymes Dicer and Drosha. Later on siRNAs are complexed into the RNA-induced silencing complex (RISC). The siRNA is unwound in a strand specific manner during RISC assembly and this single-stranded siRNA locates target mRNA. Gene silencing is a result of the nucleolytic degradation of the targeted mRNA by the RNase H enzyme (slicer - in case of nematode) and RNase I enzyme (Argonaute - in case of insects). While in case of microRNAs the gene silencing is a result of translational inhibition due to mismatch of duplex [21]. The major aspects of RNAi in insects were

i. RNAi (siRNA) is one of the fundamental antiviral pathways in insects. Insects mostly lack an adaptive immunity system, which results in well-defined immunity network in insects [14].
ii. Viral infection is the core reason for induction of RNAi pathway, which results in imbalance in homeostasis of the organism [36, 43].
iii. RNAi depends on the silencing of vital genes for survival in the target insect pest thus resulting in mortality or inhibition of the pest populations [37].
iv. Gene silencing efficiency is decreased by nuclease activity of dsRNA in the insect gut as well as in the hemolymph [16].
v. RNAi activity is also inhibited by another RNAi factor called virus-encoded suppressors

2.1 Major component for siRNA, miRNA and shRNA generation

1. Drosha
2. Dicer
3. RNA-Induced Silencing Complex (RISC)
4. RNA-Dependent RNA Polymerase (RdRP)
5. Argonaute (Ago)

2.1.1 Drosha

Drosha main function is to processes the pri-miRNA into pre-miRNA in miRNA pathway, leaving 3' overhangs on pre-miRNA. The processing of drosha needs co-factor Pasha [24].

2.1.2 Dicer

Dicer is an RNAse III like dsRNA specific ribonuclease (digest dsRNA into uniformly sized small RNAs (si RNA) present in RNAi mechanism in insects and plants. Usually the dsRNA should be with 25 nucleotides in length, complementary to target mRNAs for successful completion of target. Dicer belongs to ATP- dependent nucleases type of proteins. Dicer homologs are found in
C. elegans, Drosophila, yeast and humans.

2.1.3 RNA-Induced Silencing Complex (RISC)

RISC complex is the core processing factor in RNAi mechanism in insects and plants. It is a nuclease complex made up of different proteins and siRNA complex. It specifically targets the target nucleotide and destroys endogenous mRNAs complementary to the siRNA present in its complex [24].

2.1.4 Argonaute (Ago)

Argonaute is a "homology seeking" protein complex which helps in specific binding to target mRNA and it is the part of RISC complex in siRNA, miRNA and shRNA pathways. Argonaute family members reported in insects includes AGO1, AGO2, Piwi, and Sting [38].

2.1.5 Difference between miRNA and siRNA

The siRNA originates with dsRNA and it progress as a response to entry of foreign RNA in to the system (usually viral). It is mostly 100 percent complementary to the target mRNA. While miRNA originates with ssRNA (hairpin secondary structure) and it helps in the regulation of post-transcriptional gene expression. The miRNA is not 100 percent complementary to the target and plays a major role in regulation of gene expression [12].

3. Mechanism of RNA interference

The first phase of RNAi is production of dsRNA either in plant system or in incorporation into the insect body. The ingested dsRNA on reaching the insect midgut it selectively binds to the receptors in the midgut (Transmembrane SID like receptors). This binding leads production of charge in the receptor which results in changing of the configuration of the SID domain. This change in configuration creates a pore in the receptor through which the dsRNA will enter the midgut and reaches the midgut cells. In the midgut cells actual the dsRNA is acted upon by dicer enzyme and cleaved into small siRNAs. The siRNAs then go and binds with aragonite protein complex to form RISC complex, which cleaves them on targeted binding to target mRNA [18].
The RNAi response in insect can be classified in to two lie cell-autonomous and non-cell-autonomous response. In cell-autonomous type the silencing process is only limited to the cell where the dsRNA is introduced and encompasses the RNAi process within individual cells. In non-cell-autonomous

type the silencing process take place in the whole tissues/cells different from the location of application or production of the dsRNA. Further the non-cell-autonomous type can be grouped into environmental RNAi and systemic RNAi. If the dsRNA is up taken from outside the insect body from environment or surrounding and RNAi induced in non-systemic manner it is called environmental RNAi, while if the RNAi response is induced by injection of dsRNA in a systemic manner in insect body is called systemic RNAi [11, 53, 56].

Potential RNAi target genes in major insect pest

Insect Species	Target Gene	Delivery method	Reference
Coleoptera			
Diabrotica undecimpunctata	*V-ATPase A*; *V-ATPase E*	Feeding	(Baum *et al*., 2007)
Leptinotarsa decemlineata	*b-actin*; protein transport protein *sec23*	Feeding	(Zhu *et al*., 2011)
Tribolium castaneum	Chitin synthase genes *TcCHT-A*	Injection	(Arakane *et al*., 2008)
Diptera			
Bactrocera dorsalis	Fatty acid elongase *Noa*; GTPase*Rab11*	Feeding or injection	(Li *et al*., 2011)
Lepidoptera			
Helicoverpa armigera	Acetylcholinesterase gene *AChE*	Feeding	(Kumar *et al*., 2009)
Plutella xylostella	Cytochrome P450 *CYP6BG1*	Feeding	(Bautista *et al*., 2009)
Spodoptera litura	Vitellogenin receptor *SNgR*	Injection	(Shu *et al*., 2011)
Hemiptera			
Acyrthosiphon pisum	Gut digestive enzyme *cathepsin-L*	Injection	(Mutti *et al*., 2006)
Nilaparvata lugens	Glutathione-*S*-transferase gene *nlgst1-1*	Feeding	(Sun *et al*., 2013)
Orthoptera			
Schistocerca gregaria	Cytochrome P450 gene *CYP6H1*	Injection	(Marchal *et al*., 2012)
Blattaria			
Blattella germanica	Hyper-trehalosemic hormone gene *HTH*	Injection	(Huang and Lee, 2011)
Acari			
Tetranychus urticae	b subunit of co-atomer protein complex *T-COPB2*;	Feeding	(Kwon *et al*., 2013)

4. Challenges in Applying RNAi for Pest Control

The approach of RNAi in insect pest management was first commercialization in the western corn rootworm (*Diabrotica virgifera virgifera*) and while in case of other insects there are still in the lab stages and need further work for functional gene analysis [41, 61].

Major challenges in RNAi are

The major factors deterring the use of RNAi for successful insect pest management are [37]

1. dsRNA delivery in practice
2. Efficiency of RNAi in Pest Control
3. Resistance Development to RNAi

4.1 dsRNA delivery in practice

The dsRNA delivery system is the major factor which determines the success rate of RNAi across the different major orders of insects. Gene silencing is mostly limited to cells that uptake the target gene dsRNAs hence more the uptake of dsRNA into the cell the more is the success rate. The different types of delivery system used to transfer the dsRNA into insect
system are [61]

The main dsRNA delivery methods:

i. Soaking
ii. Feeding
iii. Injection and transgenic plants expressing dsRNA.

4.1.1 Levels of dsRNA uptake in insects:

1. dsRNA uptake from the environment into the insect body.
2. Cellular uptake and the stability of dsRNA in insects after uptake of the dsRNA from the environment into the insect body.

4.1.2 dsRNA ingestion Methods

1. Diet ingestion or direct uptake through insect exocuticle.
2. Transgenic plants express specific dsRNAs (eg. *D. virgiferavirgifera*)
3. Sprayable formations (*Leptinotarsa decemlineata*) [13]

4.2 Efficiency of RNAi in Pest Control

The major factors influencing the efficiency of RNAi are delivery method, the presence of key enzymes in midgut and gut pH of the targeted insect. Expression level of the target gene is higher in injection method of dsRNA delivery in to haemolymph than oral method [10].

4.3 Resistance Development to RNAi

The first case offield resistance of RNAi was reported in resistant strains of *D. virgifera* against dsRNA (dsDvSnf7) which showed more than 130-fold resistance under field condition. In *D. virgifera*, DvSnf7 gene was targeted which encodes for protein ESCRT (Endosomal Sorting Complex Required for Transport) -III complex an important transmembrane protein sorting present in midgut cells [20]. The lower level of dsRNA content in resistant strain of the insect haemolymph makes them less vulnerable with susceptible type strain. This proves that resistant strain was able to reduce the uptake of dsRNA into midgut cells and the introduced dsRNAs were also degraded or sequestrated by special cells in the gut region of insect [20].

5. Possible causes for RNAi insensitivity in insects

5.1 Intrinsic of the species

Several factors play a critical role in influencing the sensitivity of RNAi to insects were the efficiency of degradation of ingested alien dsRNAs, deficient amplification, spreading of the RNA signal with in the nearby neighboring tissues and low level of response of core RNAi genes after dsRNA treatment. In corn rootworm, *Diabrotica virgifera*, if the ingested dsRNAs is larger than 60 bp, the efficiency of RNAi was reported to attain maximum response. It was due to the fact that the extracellular domain of the SID-

1 transmembrane protein selectively binds only to long dsRNAs than smaller ones [27, 57].

5.2 RNAi mechanism in Nematode and Insect a comparison

The efficiency of RNAi varies from order to order in insect and also with other organisms like nematode. It was found the variation in mechanism of RNAi was the major reason behind this scenario and it urges to study and explore the capricious mechanism of RNAi in different organism for effective application of RNAi over those organisms. In many insect species, the gene knockdown was found to be less than 60% and often the mechanism was temporary which in turn is influenced by many factors [25].

There are two types of SID transmembrane protein in nematodes lie SID 1 and SID 2. The SID 1 is present in the outer membrane of midgut transferring the dsRNA from midgut lumen into the midgut cells, while the inner protein SID 2 helps in systematization of introduced dsRNA in the neighboring tissues enabling systemic effect in the nematode. While in case of insect only SID 1 protein is reported which may influence the efficiency of uptake of dsRNA in to the midgut
cells [31].

5.3 dsRNA translocation in insect midgut

The translocation of dsRNA into insect midgut is mediated by several transmembrane proteins present in the midgut cells. The major one include SID 1 and SID 2 proteins of which Sid-1 proteins are reported to be present in most of the insect species and there is no report of presence of *Sid-2* proteins in insect species through genomes sequencing. In *Drosophila* it is reported that the uptake of dsRNA is mediated through endocytic pathway and Pattern recognition receptors (PRRs) receptors present in insect midgut. Apart from that several scavenger receptors were are also present in midgut cells and silencing them with RNAi diminished the uptake of dsRNA up to 90% in *D. melanogaster* [8, 50].

In case of Coleopteran insects both the Sid-1-like channel proteins and the receptor-mediated endocytosis plays a major role in dsRNA uptake, resulting in higher dsRNA uptake hence improved efficiency of RNAi in Coleoptera [7, 40]. In *C. elegans* (as well as in plants and fungi) there is a peculiar protein enzyme complex called RdRP (ego-1 transcript) is present which is stated to amplifies the siRNAs after the cleavage of mRNA by RISC complex resulting in systemic pathway mechanism. Insects mostly lack these RdRP mutants in there RNAI mechanism making them insensitive to dsRNA (only SID 1 protein is present). In *C. elegans* both RRF-1 and EGO-1 were reported to be present and plays an essential role in secondary siRNA production from RNAi-targeted transcripts [49].

5.3.1 SID 1 receptor in midgut with SER protein complex in Insects

The SID 1 receptor complex is the only means of transmembrane protein helpful to transfer dsRNA into the midgut cells. The SID 1 protein consists of 11 domains which are sandwiched in above the other forming a complex. At domain one there is a long loop consisting of several glycosylated sites and one H_2N element at tip giving stability to the SID 1 protein complex. There is also an inner loop present between domain 1 and 2 and between preceding domains which carry several S and T phosphorylated sites giving net positive charge to the SID 1 complex so that negatively charged dsRNA gets specifically attached to the protein. Once the dsRNA is attached to the domain an electric charge is produced which results in change in configuration of SID 1 complex leading to pore formation. Once the dsRNA has entered the complex the pore is than closed. The pore is of larger size so as to accommodate the larger sized dsRNA and the pore is created only after the binding of dsRNA in to the receptors present in SID 1 protein [48].

5.3.2. Intrinsic of the tissue

The uptake of dsRNA into epithelial cells of the insect midgut brush border membrane is an important step in RNAi mechanism in insects. The inner layering of peri-microvillar membrane in Hemiptera and peritrophic membrane in Coleoptera and Lepidoptera species mid-gut acts as a mechanical barrier for delivery of dsRNA. The cellular uptake of dsRNA through endocytosis pathway is effective only if the entrapped dsRNA in endosomes are able to discharge or escape from the endosome and get transferred into the cytoplasm or else in results in reduced efficacy. Though the dsRNA is more stable than ssRNA they are quickly degradation the salivary nucleases in the insect midgut [55]. Several dsRNases are being reported in the saliva enzymes of *Ligus lineolaris*, which quickly digest the ingested dsRNA [2].

5.3.2.1 Length and Concentration of dsRNA

Length of dsRNA also affects the efficiency of RNAi mechanism. In insect species an optimum range of 140 to 500 nucleotides in length are required for effective binding silencing. Length of 60 and 30-bp of dsRNAs can induce 70 and 30% of gene knockdown in *Tribolium*,
respectively [33]. Multiple injection of different dsRNAs at a time leads to competition within them cellular uptake leading to oversaturation and results in poor RNAi response [19].

5.4 RNA interference in the termite *Reticulitermes flavipes*

Termite is the major destructive pest in the world often attacking the whole plant system from root to top of the plant. The RNAi mechanism is almost success in case of termites due to their peculiar behavior of trophallaxis, which leads to rapid transfer of dsRNA throughout entire colonies in short periods of time [59]. Two genes were tested for RNAi in termites such as Hex 2 gene and larval cuticle protein gene, which showed increased levels of dsRNA among the individuals of termite colony due to the trophallaxis behavior.

5.5 dsRNA degrading enzymes in gut of desert locust, *Schistocerca gregaria*

The dsRNase is a *Sg-endoG* transcript (four *Sg-dsRNase* transcripts), an EndoG family which have potent dsRNA degrading capacity in midgut of desert locust. Bm-alkaline nuclease is also expressed in the middle and posterior midgut, which are potent dsRNA degrading
enzymes [4].

6. Barriers for successful RNAi in Lepidopteran

The reduced efficiency of RNAi in Lepidopteran insects due to presence of alkaline pH in gut and presence of specific gene encoding for a nuclease that contributes to the RNAi insensitivity in this insect. The transcriptome analysis in Asian corn borer *Ostrinia furnacalis* revealed that "*up56"* gene is up-regulated in response to dsRNA, which codes for

dsRNase enzyme. This nuclease has three-dimensional structure similarity to human exonuclease I. This protein is well-known as "RNAi Efficiency–related nuclease (*REase*). Presence of double-stranded ribonucleases (dsRNase) in the lumen and hemolymph is the main reason for variability in the uptake and transport of dsRNA into and within the cells [16, 46, 52]. The list of dsRNase present in several insect groups have been given below

The list of dsRNase present in several insect groups have been given below

Insect Species	Reference
Silkmoth, *Bombyx mori*	(Arimatsu *et al*., 2007)
Migratory locust, *Locusta migratoria*	(Luo *et al*., 2013)
Desert locust, *Schistocerca gregaria*	(Wynant *et al*., 2014)
Pea aphid, *Acyrthosiphon pisum*	(Christiaens *et al*., 2014)
Tarnished plant bug, *Lygus lineolarus*	(Allen and Walker, 2012)

6.1 dsRNase (Bm-dsRNase) in silkworm, *Bombyx mori*

The presence of dsRNase is also reported in the midgut of silkworm, *Bombyx mori*, resulting in reduced uptake of dsRNA into system due to lower level of dsRNA in in midgut after enzymatic lysis by Bm-dsRNase. The Bm-dsRNase is concerned only in the midgut cells of silkworm and its concentration varies with in the mid gut region.

6.2 Reduced stability and intracellular transport of dsRNA in insects

Coleopterans insects mostly have systemic RNAi response due to presence of several transmembrane proteins to uptake the dsRNA while in case of lepidopteran insects it is non-systemic and less efficient due to presence of fewer proteins, entrapment of dsRNA in endosomes in midgut and gut pH which destabilizes the dsRNA in midgut. Though the dsRNA escapes the dsRNase digestion and enters lepidopteran cells it is not processed into small interference RNA (siRNA) in further progress [44]. Entrapment of dsRNAs in endosomes is an evolutionary selective advantage of sequestering exogenous dsRNA viruses in response of antiviral immune response system in insects [42]. Degradation of dsRNA and trapping of dsRNA in acidic bodies cell is the major reason behind poor RNAi response in lepidopteran insects [44].

7. Pyramiding RNAi with Bt to Counters Insect Resistance

Several report of resistance of Bt cotton have been arising, to solve this problem there comes the emergence of new concept called Gene Pyramiding. Cotton plant producing both Bt toxin Cry1Ac and dsRNA (JH acid methyl transferase) for *Helicoverpa armigera* was produced which reduce resistant development against Bt cotton due to combined effect [29, 58]. Refuges (non-transgenic) crops in Bt cotton filed will delay the resistance development by 14 to 75 years in pyramided cotton than Bt cotton alone [29, 35].

8. Conclusion

RNAi is a potent pest management tool and there is a need for understanding the RNAi machinery in insects for successful insect pest management. RNAi was found to be efficient against Coleopteran while it was insensitive to other orders like Lepidoptera and Dipterans. The presence of Rnase, RdRp, SID 1 specificity, pH of insect midgut and storage dsRNA in endosomes affect the efficiency of RNAi in insects. The availability of transcriptomic sequences or genomic data from different species makes RNAi more achievable. The major disadvantage of RNAi in the agricultural system is the off-target gene silencing effect on non-target organisms. The combined strategy of gene pyramiding, RNAi and Bt plays a vital role in managing insect pest resistance development in future and pyramiding genes with different MOA against insects helps in efficient management of targets pest.

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