Chapter 17

RNAi — Implications in Entomological Research and Pest Control

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Additional information is available at the end of the chapter

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Abstract

RNA interference (RNAi) has progressed swiftly in the past decade to become a convenient and dominant genetic tool that has immense utility in diverse fields. The entomological research, ranging from functional genomics to agriculture, has gained enormous momentum due to this technology. RNAi tool helped to discover the functions of new genes and study the complicated genetic networks, thus providing an evolutionary insight into various processes. RNAi is also becoming a method of choice for controlling insect pest populations. It is envisaged as tailor-made insecticide, which is highly species specific. However, the efficiency of this mechanism is limited by various factors such as the stability of the trigger molecule, the candidate gene selection, delivery system adopted and, most importantly, the choice of the target species. Apart from the successful implication in diverse areas, there are certain drawbacks of this technology such as ‘off-target’ effects, lack of sensitivity of various species, etc. Further research would relieve these limitations and support the manifestation of this genetic tool with much more reliability.

Keywords: RNAi, insects, pest management, efficiency of RNAi

1. Introduction

RNA interference (RNAi) is a highly conserved, sequence-specific mechanism of gene silencing which is triggered by the presence of double-stranded RNA (dsRNA). Since its discovery in 1998, RNAi has attained the status of a powerful genetic tool [1]. This reverse genetics technique is now immensely used in biomedical research, functional genetics and many other areas of biological research. Broadly, all the reactions that take place for RNA silencing are initiated when a long dsRNA is processed into small dsRNAs of about 21 to 24 bp by the RNaseIII enzyme, called Dicer. These small dsRNAs are called small interfering RNA (siRNA),

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which when unwound using ATP-dependent activity are incorporated into the multi-subunit RNA-induced silencing complex (RISC). Here, the siRNA guides the RISC complex to degrade cellular RNA molecules that are complementary to its sequence [2]. Earlier this process was described in the experimental RNAi studies, and now it is the most accepted tool for gene knockdown studies.

The advent of RNAi also revolutionised the entomological research, as novel gene functions were efficiently discovered. In 1998, Kennerdell and Carthew were the first to use RNAi *in vivo* to study the genes *Frizzled* and *Frizzled-2* in *Drosophila melanogaster* [3]. The tremendous success of RNAi in model organisms has prompted its use for research in other insect species as well. In genomics and post-genomics era with the availability of a large amount of sequence information, RNAi further provides an opportunity to investigate the vital functions and crucial interactions that are of importance in both biomedical research and agriculture [4–6]. RNAi studies on insects of economic importance would provide new insights into unravelling the molecular interactions between various disease vectors and ultimately helping in the discovery of novel vaccine and drug targets. Disease vectors such as mosquito, ticks, mites, lice and others were studied extensively using RNAi [7]. These insects cause many serious diseases in humans and animals.

Among other applications, this genetic tool is also gaining popularity as a promising technology in controlling a wide array of agricultural pests. There is a substantial amount of literature available which documents the success of RNAi as a feasible and sustainable strategy in managing the agricultural pests [8–11]. The core RNAi machinery being present in all the insects makes it possible to silence a wide variety of target genes to produce diverse physiological, developmental and reproductive restrains. The sequence specificity of endogenous RNAi pathway allows the targeted suppression of genes essential for insect survival and thus offers the development of a specific, logical and sustainable strategy to combat against insect pests.

Agricultural pests are notorious and they cannot be efficiently managed by employing a single control agent or technique. Most commonly, the integrated pest management (IPM) strategies are utilised for combating the diversity of insect pests in the agro-ecosystem [12]. Along with mechanical, cultural, biological and chemical methods, the transgenic technology should also be embraced in the IPM regime. In this regard, RNAi can play an important role along with the available insecticidal molecules. Among various transgenic approaches to manage insect pests, *Bacillus thuringiensis* (Bt) toxin has shown spectacular success [13]; however, many important insect pests (primarily sap-sucking pests) are not amenable to Bt protection [14]. RNAi can be harnessed to defend crops against insect pests. Successful application of RNAi technology in agricultural pest management requires (i) suitable candidate gene where its silencing can cause mortality of the insect and (ii) an effective method of dsRNA delivery. Nevertheless, prior to field application, many aspects of this multi-faceted technology including safety and possible risks to environment need to be evaluated in detail.

In this chapter, we discussed the potential of this technology in gene silencing experiments to study the gene function as well as on opportunity to combat against agricultural pests and other disease vectors. Further, the factors responsible for a successful RNAi experiment, the

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link with immune response and viral infections have been discussed, highlighting the possible shortcomings of this strategy.

2. RNAi in insects

RNAi offers species-specific molecules that can be flexibly manipulated and used in understanding various complicated biochemical pathways. The research application of RNAi in entomology has elucidated the functions of several genes. Decrease in the mRNA levels of a candidate gene due to introduction of a complementary dsRNA fragment, and the study of the corresponding phenotype, illuminates a gene function. RNAi has been used to study various mechanisms related to insect development (embryonic and post-embryonic), reproduction, behaviour and other complicated biosynthetic pathways [15].

Various insect orders have demonstrated amenability to RNAi-mediated silencing. Species of orders Coleoptera, Lepidoptera, Diptera, Hemiptera, Orthoptera, Blattodea and Hymenoptera have been studied for various aspects using RNAi technique [15]. The silencing efficiency ranges from 0% to 100% in different insects. A large majority of the target genes were gut-specific genes; however, genes from salivary glands, brain and antennae have also been targeted [16]. RNAi-based studies can be carried out by either *in vivo* or *in vitro* studies. The former method is much easier and involves incubating the cells with the dsRNA added to the medium. However, the *in vivo* approach is more useful in the field of functional genomics, especially in case of non-model organisms. Here, the dsRNA dosage and developmental stage of insect can be specified. In addition, RNAi can be very helpful in identifying the mutant genes that are fatal to the organism,. The significance and compilation of various categories of RNAi experiments in entomology are summarised in Table 1 [17–45].

Experiments	Insect	Gene and function
RNAi in developmental biology		
Parental RNAi	*Tribolium castaneum*	Zygotic genes [16]
Parental RNAi is an important method for analysing early embryogenesis. It is crucial for many insects whose eggs are not accessible or do not survive after microinjection.	*Oncopeltus fasciatus*	Determination of the role of the gap genes, *hunchback* and *Krüppel* [17,18]
	Gryllus bimaculatus	Antenna and appendages [19], *hedgehog*, *wingles*s and *dpp* in the initiation of proximodistal axis formation during the regeneration of insect legs [20]
	Nasonia vitripennis	Various genes in demonstration of parental RNAi [21]
	Tetranychus urticae	*Distal-less* was used, resulting in phenotypes with canonical limb truncation as well as the fusion of leg segments [22]
Embryonic RNAi	*T. castaneum*	Role of *wingless* (*wg*) in leg development [23]

Experiments	Insect	Gene and function
	N. vitripennis	Role of *bicoid* gene in the structural pattern of the anterior body region. Also in the absence of *bicoid* gene, *orthodenticle*, *hunchback* and *giant* genes are responsible for proper head and thorax formation [24]
	Oplegnathus. fasciatus	*Hox* genes and genes involved in segmentation and segment specification [25]
Larval/nymphal/pupal RNAi	*T. castaneum* (larval RNAi)	To study the molecular basis of adult morphological diversity in various organs [26]
	T. castaneum (larval RNAi)	*Ubx*/*Utx* during hindwing/elytron development [27]
	T. castaneum (larval RNAi)	*Laccase* 2 [28]
	Bombyx mori (pupal RNAi)	Fatty acid transport protein (Bm'FATP) [29]
	Schistocerca Americana (Nymphal RNAi)	The eye colour gene of first-instar nymphs triggered a suppression of ommochrome formation in the eye [30]
	S. Americana	Importance of early retinal genes *eyes absent (eya)* or *sine oculis (so)* in eye development [31]
	Blattella germanica (nymphal RNAi)	RXR/USP, along with EcR, of the heterodimeric nuclear receptor of 20-hydroxyecdysone (20E) [32]
	G. bimaculatus	Mechanisms of leg regeneration [33]
	G. bimaculatus	*period* (*per*)gene for circadian-dependent locomotor activity rhythm [34]
	G. bimaculatus	Genes responsible for certain human disorders: *fragile X mental retardation 1* (*fmr1*) and *Dopamine receptor* (*DopR*) [35]
Regeneration-dependent RNAi	*G. bimaculatus*	Insect leg regeneration [36]
	G. bimaculatus	Orthologs of *Drosophila hedgehog* (*Gb'hh*), *wingless* (*Gb'wg*) and *decapentaplegic* (*Gb'dpp*) are expressed during leg regeneration and play essential roles in the establishment of the proximal–distal axis [37]
RNAi in behavioural biology	*Rhodnius prolixus*	Nitrophorin 2 a decrease of anticoagulant activity and less efficient feeding behaviour [38]
	Anopheles gambiae	Apyrase AgApy in the salivary glands shows important role in host probing behaviour [39]
	D. melanogaster	3-Hydroxy-3-methylglutaryl CoA reductase has been identified for the control of

Experiments	Insect	Gene and function
		sexual dimorphism of locomotor activity in [40]]
	B. germanica	Neuropeptide pigment dispersing factor in the regulation of locomotor circadian rhythms [41]
Mechanism of insecticidal action	Mosquitoes	NADPH cytochrome P450 reductase led to increased sensitivity of mosquitoes to pyrethroids [42]
	Spodoptera litura and *Helicoverpa armigera*	Aminopeptidase M led to decreased sensitivity to BT toxin [43]
Understanding the biosynthetic pathway	*T. castaneum*	Chitin synthases *CHS1* and *CHS2* are crucialexoskeleton and the midgut peritrophic matrix [28]
	Bombyx mori	*Bombykol* is the main component of sexual pheromone, as well as pheromone-binding proteins and the receptor of the pheromone biosynthesis activator neuropeptide [44]
	Epiphyas postvittana	Silencing of pheromone-binding protein of the antennae [45]

Table 1. RNAi in the study of gene function in insects.

Apart from deciphering the function of genes involved in various metabolic pathways, RNAi also finds relevance in other aspects of insect science. It is quite beneficial in maintaining the beneficial insects and saving them from various parasites and pathogens. Certainly, this is useful in case of those parasites and pathogens, which have operative RNAi machinery. A successful study in this regard shows the control of honey bee parasite *Nosema ceranae*. When the gene related to energy metabolism was silenced, it was observed that the honey bee population had reduced infestation of *Nosema*, and lower mortality [46]. In another study, multiple genes of an ectoparasite of honey bee *Varroa destructor* were targeted [47]. It poses a great threat to the health of bees, and its control is of utmost importance for the rearing industry. It is a blood-sucking parasite, so the bees were fed on a meal containing dsRNA against the genes of *Varroa*. The RNAi-mediated control decreased the mite population by 50%, causing no evident damage to the bees. RNAi has also been useful in elucidating the importance of various immunological pathways in *D. melanogaster* [48]. Host–parasite relationships such as that of *Anopheles–Plasmodium* have also been studied by using RNAi. Early research was conducted on *defensin* and it was shown to be important for protecting mosquitoes against infections of Gram-positive bacteria [49]. Later, the same group demonstrated how the development of Plasmodium is affected by *Anopheles gambiae* immune genes [50]. Similarly, in *Manduca sexta* haemocytes, knockdown of haemolin (a bacterial recognition protein) decreased the ability of insects to clear *Escherichia coli* from the haemolymph. This eventually reduced their ability to engulf bacteria and highlighted the role of haemolin in the *M. sexta* immune response [51].

3. RNAi in pest control

Plants are damaged by a plethora of insect pests. The losses due to these pests and expenditure on the chemical pesticides amount to billions of dollars. In an attempt to reduce these losses, many reports have been published which demonstrate the successful application of RNAi technique in crop protection. Huvenne and Smagghe [52] have summarised the reports on insects in which RNAi has been applied through feeding, and they discussed several factors that influence the success of RNAi on target insects, such as the concentration of dsRNA, the nucleotide sequence, the length of the dsRNA fragment and the life stage of the target insects. The downregulation of expression of critical genes, caused by dsRNA/siRNA, eventually leads to death/growth retardation of the insect and forms the basis of pest control. In this view, the efficient delivery and uptake of the dsRNA trigger is of prime importance. In contrast to the study of gene function, pest control strategies cannot depend on injection of the molecule. The deployment of pest controlling siRNA/miRNA molecules would involve oral exposure, either as transgenic plants or as sprays. Most of the nutrients from gut lumen are absorbed in the midgut tissue of the insect; therefore, this tissue is an attractive target for RNAi. After ingestion, the dsRNA enters into the gut lumen of the insect. Insect gut is divided into foregut, midgut and hindgut. While both foregut and hindgut are covered with chitin, it is only the midgut that has exposed cell surfaces. It is the site of nutrient exchange between the haemolymph and the gut contents. Therefore, midgut epithelium is an attractive target as it is the primary tissue exposed to dsRNA in the gut lumen. The stability of dsRNA molecule and the efficiency of the silencing process (discussed in section 5) is determined by the gut pH and nucleases.

A breakthrough research in RNAi-mediated pest control was published in 2007 on the western corn rootworm, *Diabrotica virgifera virgifera* (WCRW) [53], and cotton bollworm *Helicoverpa armigera* (CBW) [54]. In the former study, a candidate gene was screened based on the complete cDNA library. Out of a total of 290 dsRNAs, *Vacoular ATPase (V-ATPase)* subunit A was finally selected for the development of transgenic corn plants. The larvae reared on transformed plants caused much less damage to the roots and also showed the reduced expression of the target gene. The other study of Mao et al. [9] targeted the pesticide detoxifying gene *Cytochrome P450* (*CYP6AE14*), which provides gossypol tolerance to the insect. Transgenic plants expressing dsRNA corresponding to *CYP6AE14* levels of this transcript in insect body was decreased and the larval growth was retarded. Following these two studies, many research groups started to consider RNAi as a feasible technique which could be employed in the transgenic approaches to manage insects. As we know from the various studies conducted, the Bt (*Bacillus thuringiensis*) toxins are effective on lepidopteran and coleopteran pests but fail to work against hemipteran pests like aphids, whiteflies, phyllids, etc. [55–58].

RNAi-engineered plants can be more useful in case of these phloem-feeding hemipteran pests, which are notorious not only because of feeding damages but also because of their ability to transmit plant viruses [59]. The midgut genes of *Nilaparvata lugens* were downregulated by feeding on transgenic rice expressing dsRNA against three separate genes, but no lethal phenotype was detected in this case [60]. Pitino et al. [61] demonstrated RNAi in aphid *Myzus persicae* directed towards the receptor of activated kinase (*Rack-1*) gene by transgenic expres-

sion in *Arabidopsis thaliana*. Another important pest, the whitefly, is also now amenable to RNAi [62]. Its control was demonstrated both by feeding on artificial diet and by feeding on transgenic tobacco plants expressing dsRNA of *V-ATPase A* gene [11, 63]. RNAi experiments conducted on agricultural pests as well as on insect vectors of several human diseases are summarised in Table 2 [53–118].

Insect pests	**Mode of delivery**	**Gene target**
		Order Coleopteran
Diabrotica virgifera virgifera	Artificial diet and transgenic plant	*a*-Tubulin, vacuolar ATPase subunit A [53]
Phyllotreta striolata	Plant tissue	Arginine kinase [64]
Leptinotarsa decemlineata	Artificial diet	Vacuolar ATPase subunit A and E [53]
Diabrotica undecimpunctata howardi	Artificial diet	Vacuolar ATPase subunit A and E, *a*-tubulin [53]
Monochamus alternates	Injection	*Laccase* gene [65]
		Order Diptera
Aedes aegypti	Blood meal Artificial diet Feeding	Inverted repeat (IR) RNA derived from the premembrane protein coding region of the DENV-2 RNA genome [66] V-ATPase A [67] ATP-dependent efflux pump [68]
Anopheles gambiae	Feeding	Chitin synthase [69]
Glossina morsitans morsitans	Feeding	Midgut protein Tsetse EP; transferrin [70]
Bactrocera dorsalis	Feeding and injection	Rpl19; V-ATPase D subunit; fatty acid elongase Noa; small GTPase Rab11 [71]
		Order Hemiptera
Acyrthosiphon pisum	Injection Feeding	Calreticulin, cathepsin-L [72] C002 [73] Hunchback [74] V-ATPase [75] aquaporin [76]
Cimex lectularius	Injection	Cpr gene[77]
Diaphorina citri	Topical In planta (virus induced)	Abnormal wing disc [78] Abnormal wing disc [79]
Laodelphax striatellus	Feeding	Disembodied [80]
Rhodnius prolixus	Injection	Nitrophorins1–4 [81] *a*-Glucosidase [82] Gap gene giant [83] Phospholipase A2 [84]

Insect pests	Mode of delivery	Gene target
Sitobion avenae	Feeding In planta	Catalase [85] CbE E4 [86]
Lygus lineolaris	Injection Injection	Inhibitor of apoptosis gene (IAP) [87] Polygalacturonase (PG) [88]
Oncopeltus fasciatus	Injection	Hunchback [89]
Riptortus pedestris	Injection Injection and feeding	Circadian clock gene, mammalian-type cryptochrome; Bla [90] Gland nitrophorin 2 (*NP2*) [91]
Nilaparvata lugens	Feeding and transgenic Feeding Feeding and injection	Hexose transporter; carboxypeptidase; trypsin-like serine protease [92] ATP synthase subunit [93] Trehalose phosphate synthase [94] Cathepsin B-like protease; nicotinic acetylcholine receptors [95]
Myzus persicae	Transgenic plant	*MpC002* and *Rack-1* [96]
Bemisia tabaci	Injection Artificial diet Transgenic plant	Snap, Chickadee, CG5885, GATAd [62] Actin; ADP/ATP translocase; α-tubulin; ribosomal protein L9; V-ATPase [63] V-ATPase subunit A [11]
Bactericerca cockerelli	Injection/feeding	Actin, V-ATPase [97]
	Order Hymenoptera	
Athalia rosae	Injection	*Ar white gene*
	Order Isoptera	
Reticulitermes flavipes	Feeding	Endogenous digestive cellulase enzyme, hexamerin storage protein [98]
	Order Lepidoptera	
Ostrinia furnacalis	Spraying	LIM protein 1; myosin 3 light chain; chymotrypsin-like serine protease; chymotrypsin-like protease C1; chymortypsin-like serine protease C3; hydroxybutyrate dehydrogenase; Kazal-type serine proteinase inhibitor 1; fatty acid binding protein 1; unknown; caboxypeptidse 4 [99]
Ostrinia nubilalis	Spraying	Chitinase (*OnCht*); chitin synthase (*OnCHS2*) [100]
Epiphyas postvittana	Feeding	Carboxylesterase gene (*EposCXE1*); pheromone binding protein [101]

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Insect pests	Mode of delivery	Gene target
Helicoverpa armigera	Feeding Transgenic plant Transgenic plant Feeding and transgenic plant	Acetylcholinesterase (*AChE*) [102] Cytochrome P450; gluthatione-S-transferase[9, 103] Ecdysone receptor EcR [104] HaHR3 moulting factor [105]
Hyalophora cecropia	Injection	*Haemolin* [106]
Manduca sexta	Injection	*Cadherin* [107]
Spodoptera litura	Injection	Vitellogenin receptor [108]
Spodoptera exigua	Feeding	Chitin synthase gene A [109]
Spodoptera littoralis	Injection	β-Actin gene [110]
Spodoptera frugiperda	Allatostatin C; allototropin 2; cytochrome	Feeding [111]
Plutella xylostella	Feeding	Rieske iron–sulphur protein (RISP) [112]
	Order Orthtoptera	
Gryllus bimaculatus	Injection Injection Injection Injection Injection	Delta; Notch [113] Insulin receptor; insulin receptor substrate; phosphatase and tensin homolog; target of rapamycin; PRS6-p70-protein kinase; forkhead box O; epidermal growth factor receptor [114] Nitric oxide synthase gene *NOS* [115] Circadian clock gene *per* [116] Sulfakinins [117]
Schistocerca americana		Eye colour gene vermilion [118]

Table 2. Different insect pests targeted by RNAi.

The choice of a suitable target gene is central to pest control strategy. The gene selection approaches can be based on choosing the gene with known function such as detoxification enzymes, cell synthesis, nutrition, metabolism and cytoskeleton structure. These types of genes can be selected as insect pest control targets. For this, expressed sequence tag (EST) library of European corn borer (*Ostrinia nubilalis*) was screened to find out that a *chitinase* gene (*OnCht*) and a *chitin synthase* gene (*OnCHS2*), which are very important in regulating the growth and development of this insect [119]. Likewise, the EST library of *Bemisia tabaci* was also used to screen out few important genes for RNAi-mediated control [63]. The cDNA library screening approach was also used. Mao et al. [9] constructed a cDNA library from RNAs expressed in the midgut of fifth-instar larvae exposed to gossypol. Several cDNA libraries of WCR (*D. virgifera virgifera*) were prepared and considered upon the underlying principle that genes encoding proteins with essential functions would be the best RNAi targets for causing lethality [8].

As an extension of the cDNA library screening approach, the next-generation sequencing (NGS) technologies have led to novel opportunities for expression profiling in organisms lacking any genome or transcriptome sequence information. It enables the direct sequencing of cDNA generated from mRNA (RNA-seq) [120, 121]. Hence, it provides the *de novo* generation of the transcriptome for a non-model organism, including various pests. Wang et al. [99] adopted Illumina's RNA-seq and digital gene expression tag profile (DGE-tag) to screen optimal RNAi targets from Asian corn borer (ACB; *Ostrinia furnacalis*). The same technique has been used for the grain aphid, *Sitobion avenae* and *Spodoptera litura* [122, 123]. It seems likely that the combination of DGE-tag with RNA-seq is a rapid, high-throughput, cost-effective and easy way to select for candidate target genes for RNAi, which may not be only limited to the midgut tissue but can also be selected from the whole insect.

The convenience of RNAi to target specific pests while not harming other species is a perfect method of pest management. However, target gene selection and efficient delivery methods are the two major cornerstones of pest management by RNAi. The candidate gene for RNAi can be tailored to be species specific or they can also have a broad spectrum. Specificity can be achieved by designing dsRNAs that target the more variable regions of genes, such as untranslated regions (UTRs). It was first demonstrated in *Drosophila* where UTR of the *gamma-tubulin* gene was targeted and even closely related species could be targeted selectively [75]. However, it can also be possible to target multiple organisms with a single gene. One such example is of *V-ATPase* gene, which is an effective target in *B. tabaci*, *D. virgifera* and *B. dorsalis* [8, 63, 124]; all of these insects belong to different orders. In this case, either a conserved region can be selected which could affect closely related species or a mixture of dsRNA fragments from different genes belonging to different species can be selected.

Delivery methods that ensure continuous supply of dsRNA/siRNA will be applicable in the fields. A more reliable and verified method would be transgenic plants as the dsRNA can be applied as bait, sprays, or supplied through irrigation systems [125, 126]. The application approach by spray could be quite practical like the spray of chemical pesticides. Gan et al. [127] have also demonstrated the control of viral infection using dsRNA spraying. Similar results were obtained with Asian corn borer, *Ostrinia furnacalis*. This study showed that larval lethality or developmental disorders can be achieved by gene-specific RNAi, and spraying can be an efficient method for continuous supply of dsRNA [99]. The coating of dsRNA molecule with liposomes is used for delivering siRNA to mammalian cells, specific tissues and some insects [128, 75]. This coating prevents the degradation of the molecule and enhances its uptake ability; spraying may also be explored for such particles. Zhang et al. [129] used the chitosan nanoparticle based RNAi technology to suppress the expression of two chitin synthase genes (*AgCHS1* and *AgCHS2*) in African malaria mosquito (*A. gambiae*) larvae. Bacterial expression or chemical synthesis allows large-scale production of dsRNA at efficient costs [75, 130, 131].

4. RNAi: Link with immunity and viral infections

The RNAi mechanisms evolved primarily as a defence mechanism against viruses and transposons [132]. Research has established that RNAi pathway also contributes to the innate

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immunity of the insects against the viruses having either dsRNA genome or such replicative intermediates. It was demonstrated that the *Drosophila* S2 cells utilise the endocytosis-mediated pathway involving the pattern recognition scavenger receptors for the uptake of dsRNA from the surroundings. These receptors are key players in the innate immune responses of the cell [133, 134]. Saleh et al. [135] demonstrated the strong link of dsRNA uptake pathway and the activation of the immune response in the infected cells. The normal cells used dsRNA uptake pathway to internalise viral dsRNA and subsequently showed manifestation of antiviral response in these cells. On the contrary, the mutant cells (defective genes used for dsRNA uptake) did not show activation of any antiviral response. Mutants in the core siRNA components Dicer-2, AGO-2 and R2D2 are more susceptible to viral infections [140].

Further, it was also reported that receptors such as Sr-CI and Eater, which contribute to majority of dsRNA uptake in the *Drosophila* S2 cells, were significantly down-regulated after pathogenic virus treatments, and significant changes in phagocytic activity were observed. The role of RNAi in antiviral defence has also been firmly established in mosquitoes [136]. Viruses can also affect the availability of RNAi machinery for other candidate dsRNA molecules. They can saturate the RNAi machinery and affect the efficiency of RNAi mechanism. Many viruses are known to produce viral suppressors of RNA silencing (VSRs), which bind to the key elements of the RNAi pathway rendering it unavailable. Many of the viral proteins (viz. B2 protein from Flockhouse virus, 1A proteins from *Drosophila* C virus and cricket paralysis virus) are known to interfere in the siRNA pathway of RNA-mediated silencing [137–140]. These viral proteins may affect the biogenesis of the trigger molecule by binding to important enzymes such as dicer, which generate the siRNA from long dsRNA or affect the target cleavage by binding to RISC. The viral proteins may also sequester the dsRNA signal molecule or form complexes with the replicative intermediates of the siRNA pathway.

Viruses also produce large amount of RNAs and small RNAs that accumulate in the infected cells. It has also been hypothesised that the occurrence of alternative and effective antiviral pathway may become important in controlling the viral infections and may supersede the RNAi pathway. Few of these possible pathways have been worked upon. Goic et al. [141] have reported the potential interaction of nucleic acid-based acquired immunity with the core RNAi machinery in the study of persistent infection of S2 cells by Flock House Virus (FHV). The insects also protect themselves from foreign nucleic acids by becoming refractory to RNAi. In the oriental fruit fly, *Bactrocera dorsalis*, orally administered dsRNA-targeting endogenous genes, resistance to RNAi was seen due to a blockade in the dsRNA uptake pathway. A very interesting hypothesis is presented by Swevers et al. [142] about the possible impact of persistent viral infection in the insects. In their work, the authors have analysed various factors that determine the response to exogenous dsRNA in the background of viral infection.

5. Efficiency of RNAi

Though RNAi is a conserved mechanism in eukaryotes, its efficiency is governed by various factors. The response of different insect species towards this mechanism of gene silencing is

imperative for successful implementation in the study of gene function and more importantly in the pest management programs. Also, the efficacy is governed by many factors which are not intrinsic to the organism such as the delivery, dosage and choice of the candidate gene. Comprehending these factors will provide a better insight into designing the experiments for successful application of RNAi. The available reports indicate that the lower *Insecta* species such as that of *Blatella* show a much robust and persistent RNAi response, while higher *Insecta* species belonging to the orders Lepidoptera and Diptera are non-compliant [15]. The sensitivity could vary within and among the orders. For instance, many Lepidopteran insects are resistant to RNAi. Terenius et al. [143] have reviewed various factors that may be contributing to the poor responsiveness of these insects. The efficiency can vary with insect species, target gene, developmental stage of the organism, expression of RNAi machinery, method of delivery, stability of dsRNA, etc. A few factors that may be crucial in determining the efficiency of RNAi are discussed below.

5.1. The RNAi machinery

RNAi evolved in organisms as a defence mechanism against viral infections at the cellular level [144, 145]. The differences in the expression of core RNAi machinery can be a prime reason affecting the adequacy of RNAi mechanism. The systemic RNA-interference-deficient 1 (sid-1) protein forms a gated channel which is selective for dsRNA molecule. Its role is well established in the systemic spread of the RNAi signal in the model organism *Caenorhabditis elegans* [146]. The presence of *SID-1*gene orthologs in insects varies with the insect orders [52]. The dipterans lack this gene completely. The mosquito *Culex quinquefasciatus* also lacks sid-1 ortholog but shows the systemic spread of the dsRNA trigger [147]. On the one hand, honey bee (*Apis mellifera*) showed an increase in the expression of *SID-1* during the RNAi experiments, indicating its role in the uptake pathway [148]. On the other hand, in *Tribolium castaneum*, the silencing of all three orthologs of *SID-1* casted no influence on the efficiency of RNAi [149].

In *Bombyx mori*, three orthologs are present but no significant success has been found in this lepidopteran, while mosquitoes show systemic RNAi despite the absence of *sid-1* in several species [149–152]. R2D2, a cofactor of dicer-2 enzyme which cleaves long dsRNA into siRNA for loading into the RISC, is absent in *B. mori* making the insect very insensitive to RNAi. Another important enzyme RNA-dependent RNA polymerase (RdRP), which amplifies the primary siRNA signal in *C. elegans*, is entirely not reported in the insects [149]. *T. castaneum* showed a robust systemic RNAi, but a wide survey of RNAi related genes did not show any traces of RdRP [149]. However, RdRP-like activity was substituted in *Drosophila* cell lines by certain other enzymatic pathways. In many cases, the absence of certain well-known genes of the RNAi pathway is directly responsible for the poor response of the organism while in several other examples, the absence is compensated by other genes/pathways which play key roles of their counterparts.

5.2. The RNAi molecule

The exogenous dsRNA molecule is the trigger for initiating the RNAi pathway. These molecules are delivered in the form of dsRNA, siRNA or hairpin RNA. Apart from sequence

specificity, other parameters are also crucial in determining the efficiency of RNAi experiments. The study on the administration of dsRNA (feeding by means of artificial diet, natural diet, droplet method, blood meal, transgenic plants, etc.) in insects has used varied length of dsRNA molecule. The nucleotide length used in these reports ranges from 134 to 1842 bp, while most of the studies used 300–500 bp as the optimal length [153]. However, silencing effects have also been observed in the case of single siRNA synthesised chemically (administration in *H. armigera*) or a cocktail of siRNA (obtained by using dicer enzyme to chop the dsRNA molecule) [154]. In the cell line experiments done on the *Drosophila* S2 cell line, 211 bp was found to be the optimum length of dsRNA that could be absorbed by the cells [135]. Not only the length but also the specificity of the RNAi molecule is a concern. It can be understood by the reports on *Drosophila* that feeding specific sequence of *V-ATPase* dsRNA caused no silencing in non-target species [75], which implies the specificity of the process. On the contrary, various other studies report non-specific silencing. Off-target effects were reported in *Rhodnius prolixus* [81] and Colorado potato beetle (*Leptinotarsa decemlineata*) [8]. Single mismatches are known to impair the RNAi effect in the mammalian cell lines [155]. Further studies will clarify whether single mismatches show similar impact in insects as well. In case of pest management programs, long dsRNAs (>200 bp) are generally used which generate many probable siRNA maximising the RNAi response [153]. The dosage of the dsRNA molecule also plays an important role in determining the efficiency of the process. Higher doses are required in case of feeding experiments as compared to injection. The silencing effect in *R. prolixus* was enhanced by multiple doses [38]. Therefore, it follows that doses and types of administration (oral or injectable) also need to be optimised according to the life stage of the organism and the target tissue.

5.3. Delivery of the molecule/uptake of silencing signal

One of the most decisive factors for inducing RNAi is the efficient delivery of the dsRNA molecule. The common methods of delivery are by microinjection, soaking, oral delivery and transgenic technique [156]. Microinjection-based delivery is the most commonly used technique in studying gene functions. It has proven to work well for *Tribolium, Drosophila* and many other lepidopteran insects. Although it works well for larger insects, success with smaller insects is limited due to the invasive nature of this technique. The survival of aphids after microinjection procedure is highly dependent on the injected volume [157].

Further, factors such as needle choice, optimal volume and place of injection are very crucial considerations and tend to vary with organisms and laboratories. Feeding-based experiments involve either *in vitro* synthesised or bacterially expressed dsRNA molecules. The success of oral delivery methods indicates the possible employment of RNAi technique for target pest control. However, the stability of the molecule will always be a concern in the gut lumen. Artificial diet mixed with dsRNA could not induce RNAi in *Drosophila* spp. Ingested dsRNA against a gut-specific *aminopeptidase N* gene also failed to develop RNAi response in *Spodoptera litura* [43]. Therefore, it can be suggested that oral delivery is not equally suitable for all species. Another convenient method of delivery is soaking. Nevertheless, this method is more applicable for cell line experiments rather than whole insects.

After the delivery of the molecules, the next step is the uptake of the molecules by insects. Huvenne and Smagghe [52] have elaborately reviewed the basic mechanisms involved in the uptake of dsRNA in the insects. The spreading of the RNAi signal, i.e., systemic RNAi, is an important determinant of the efficiency of RNAi. In cases of functional genetics, cell autonomous RNAi has been successfully employed to study the function of genes; however, for implementation in the pest control programs, non-cell autonomous RNAi is important. The systemic spread of the silencing signal is absent in the most studied model insect *Drosophila*. In contrast, the most studied insect *Tribolium* shows a powerful systemic silencing effect [149].

5.4. Potency of the silencing signal

The manifestation of the RNAi effect also depends on the stability and persistence of the dsRNA molecule. In *Acyrthosiphon pisum*, the silencing effect on the aquaporin gene began to reduce after five days [157]. The early stability of dsRNA molecule may be disrupted by the non-specific nucleases as reported in many of the lepidopteran insects [143]. These are extracellular enzymes different from dicer and digest the trigger molecule, thereby preventing the RNAi cascade. In certain cases, the activity of dsRNA degrading enzymes have been studied and their levels were measured in different stages, which was found related to the developmental stage. The dsRNase activity is also found in the digestive juices of *Bombyx mori*, saliva of *Linus lineolaris* and in haemolymph of *Manduca sexta* [87, 158, 159]. The existence/stability/mode of action of these enzymes are not sufficiently studied and future research in this direction needs to be carried out to comprehend the stability of dsRNA molecule in the *in vivo* studies. The choice of gene can also decrease the strength of the silencing signal. Ideally, the protein whose function is to be silenced should have a short half-life, whilst the mRNA turnover number should be high. The stability of protein explained the weak RNAi response in both *D. melanogaster* and *T. casteneum* [160]. However, such studies have not been conducted for the majority of the genes and therefore it can be concluded that expression of RNAi is limited by many uncovered phenomena.

6. Conclusions

The advent of RNA interference has been a crucial phase of the modern day science. The wide array of applications in the entomological research has led to many momentous findings. The functionality of many genes has been understood by this technique. Its implication in functional genomics is not only restricted to the study of a given set of genes but is also used to unveil the interaction of different genes in a particular metabolic pathway. The rapid pace of RNAi-based research suggests that it would soon facilitate better understanding of evolution, circadian rhythms, behavioural pattern, reproductive biology and interaction between host and parasites/pathogens. However, successful manifestation of RNAi is dependent on several factors. The insect species might lack the basic RNAi machinery [161] or may rapidly degrade alien dsRNA. Such factors could be intrinsic to the concerned tissue or gene. The gene might have high transcription rate and could evade the effect of RNAi or the target mRNA may be too transient.

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As happens with every phenomenon, this mechanism can also undergo selection pressure. Viruliferous insects that also have RNAi suppressors would be able to thrive on RNAi-protected crops. Furthermore, single nucleotide polymorphisms (SNPs) that result in lower effectiveness of the RNAi could potentially be selected for and lead to the evolution of resistance [153]. Genetic variations among insect species are already a challenge for RNAi. Therefore, parallel research must be carried out to develop strategies, which would minimise the resistance development and selective pressures.

RNAi has proved its utility as a futuristic tool of insect pest management. However, there are several issues that need to be addressed before the implementation of this technology in fields. The knowledge gaps underlying large-scale implications of pesticidal RNAi-based crops on the environment should be identified and bridged. The off-target gene silencing is a serious concern where unintended organisms are adversely affected [162]. The non-target effects can be categorised as off-target gene silencing, silencing the target gene in non-target organisms, immune stimulation and saturation of the RNAi machinery [163]. A balanced approach should be taken with maximum effects on the target pests with minimal effects on non-target organisms.

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