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RNAi: A Promising Approach for Insect Management

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ABSTRACT

RNA interference (RNAi) in insects is a gene regulatory process that plays a vital role in the maintenance and in the regulation of host defenses against invading viruses. RNAi is the sequence-specific suppression of gene expression, offers great opportunities for insect science, especially to analyze gene function, manage pest populations and reduce disease pathogens.RNA interference (RNAi) is a highly conserved gene regulatory mechanism that controls the expression of genes at post transcriptional level. The efficacy of RNAi varies among different insect orders and it also depends upon various factors, such as target gene selection, method of dsRNAs delivery, expression of dsRNAs and presence of off-target effects.

Key words: *RNAi, dsRNA*

INTRODUCTION

Agriculture is the key development factor that had led to the rise of human civilization. The history of Indian agriculture dates back to ca. 9000 BC. Today, India ranks second worldwide in farm output. The unceasing struggle between man and the insect pests started even prior to the dawn of civilization. In spite of numerous advances made by man in evolving newer and deadlier weapons to fight the war against insects, he has not succeeded in eradicating even one of the thousands of serious pests which damage his food and other agricultural products, destroys his possessions and even attack himself and injures domestic animals. In a world of growing prosperity and agricultural abundance, about 800 million people still suffer from hunger and malnutrition. While Agricultural productivity has to be maintained, world has realized that it will not be at the cost of environment. Among many factors contributing to environmental hazards, pesticide remains at forefront Thus, at this juncture there is an urgent need to look for an eco-friendly, effective alternative to manage insect pests, where newer approaches like RNA interference (RNAi) poised to make a sea change in our approach in designing futuristic pest management strategies.

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In insects, RNAi has been developed to knockdown genes encoding proteins crucial for growth and development, or those involved in host–vector relationships. The laboratory experiments have been conducted in which dsRNA is injected directly in the organism, which is not applicable to control insect pests in the field. For efficient insect control, the organism should be able to autonomously take up the dsRNA, through feeding and digestion in the gut. RNAi is typically and has been successfully performed by injections of dsRNA into some insects including the following: Bombyx mori larvae[10,20,24] the milkweed bug, Oncopeltus fasciatus[11] a pea aphid, *Acytosiphon pisum*[12], the cricket, Gryllus bimaculatus[19] , the wasp, *Nasonia vitripennis* the Phlebotomine sand flies, *Lutzomyia lingipalpis*[22] and in the red flour beetle, *Tribolium castaneum*[1,26].

Overview of RNAi silencing pathway

Plant-mediated RNA interference (RNAi) shows great potential in crop protection. It is a highly sequence specific gene silencing mechanism that direct in eukaryotes primarily as nucleotide based defense maintaining the integrity of the genome. RNAi was first reported in plants (Co-suppression) and later by Fire *et al*.[7] in the nematode *Caenorhabditis elegans.* The introduction of the double stranded RNA (dsRNA) resulted in silencing of the cognate genes. This opened up a new approach in validation of the gene function and a novel strategy in pest and disease management. This process, RNA interference (RNAi) in animals[9] and post-transcriptional gene silencing in plants[3], is a type of highly specific defense reaction. PTGS in plants involves down-regulation of gene expression at the post transcriptional level, by targeting specific RNAs for degradation[25]. Plant PTGS is similar to other eukaryotic evidence such as suppression of transgenes and transposons, and cellular responses to double-stranded RNAs. It depends on the silencing specific pairing of Watson–Crick formed by the small RNAs that trigger gene silencing and their target mRNAs. mRNA destruction is mediated through the production of small interfering RNAs (siRNAs) from the long exogenous or endogenous dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as dicers a ribonuclease III (RNase III) enzyme. The siRNAs are ~21 nucleotides in length that carry two base extensions at 3' end of each strand. Further, one strand of the siRNAs acts as guide strand and is assembled into an RNA-induced silencing complex (RISC) in conjunction with the argonaute multi-domain protein, which contains an RNaseH-like domain responsible for target degradation (mRNA). The mature RISC associates with the cognate mRNA and cleaves it by endonuclease activity. Recently there are many successful examples of application of RNAi in the management of insect pests such as fruit flies, flour beetles, pea aphids, and tobacco hornworms were specifically killed when fed species-specific dsRNA targeting vATPase transcripts. Even closely related species can be selectively killed by designing dsRNAs that target the 3' or 5' UTRs of the genes. RNAi following ingestion of dsRNA in all of the species tested and the method offers promise of both higher through put RNAi screens and the development of a new generation of species-specific insecticides. RNAi has been exploited in plants for applications ranging from functional genomics to provision of valuable crop traits, such as resistance against viruses, bacteria and nematodes. Despite having been considered for many years, application of RNAi technology to give resistance to herbivorous insects has been realized.

RNAi can be divided into cell-autonomous and non-cell- autonomous RNAi. In cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is induced or expressed and encompasses the RNAi process within individual cells. In case of non-cell-autonomous RNAi process takes place in tissues/cells different from the location of application or production of the dsRNA. There are two different kinds of non-cell-autonomous RNAi: environmental RNAi and systemic RNAi. Environmental RNAi

describes all processes in which dsRNA is taken up by a cell from the environment. Therefore, this process can also be observed in unicellular organisms. In multi cellular organisms only Systemic RNAi take place because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another. In multicellular organisms, environmental RNAi can be followed by systemic RNAi and non-cell-autonomous RNAi will always be followed by cell-autonomous RNAi. Research on non-cell-autonomous RNAi is mainly conducted on plants and the model organism *Caenorhabditis elegans*.

Non-cell-autonomous RNAi is the promising application of RNAi in insect control. The dsRNAs have to be internalized through feeding in order to silence the target gene, which represents environmental RNAi. If the target gene is expressed in a tissue away from the gut, the silencing signal will also have to spread via cells and tissues, which is known as systemic RNAi. In insects, RNAi has been developed to knockdown genes encoding proteins crucial for growth and development or those involved in host–vector relationships. RNAi is typically and has been successfully performed by the injections of dsRNA into some insects including the following: *Bombyx mori* larvae, the milkweed bug, *Oncopeltus fasciatus*, a pea aphid, *Acytosiphon pisum* the cricket, *Gryllus bimaculatus* the wasp, *Nasonia vitripennis* the Phlebotomine sand flies, *Lutzomyia lingipalpis* and in the red flour beetle, *Tribolium castaneum*. Delivery of dsRNA through feeding is also capable of inducing RNAi of target genes as demonstrated in the following studies: dsRNA diet incorporated feeding to corn rootworms, *Diabrotica punctata* and *D. undecimpunctata howardii*, and the colorado potato beetle, *Leptinotarsa decemlineata* to silence genes involved in growth and survival such as vATPases. Droplet feeding to the light brown apple moth, *Epiphyas postvittana* to silence a gut carboxylesterase and a pheromone binding protein gene; and plants expressing short hairpin dsRNA feeding to *Helicoverpa armigera* to silence a cytochrome P450 gene involved in gossypol tolerance. The cytochrome P450 family of enzymes (P450s) is known to play significant roles in the metabolism of insecticides in many insect species. Transcriptional over expression of P450s is often thought to enhance the metabolism of insecticides and appears to be a common phenomenon in the evolution of resistance development in insects[5,16,17].

dsRNA uptake in insect gut

The insect gut is divided into three regions; foregut, midgut and hindgut. The first two are continuations of the 'outside' of the insect and are chitin-lined, so that their surfaces do not present areas of exposed cells (although receptors and transporters are present to allow processes such as taste recognition in the mouth cavity and water transfer in the hindgut to occur). The midgut region is the only part of the gut that contains surfaces of exposed cells, and it is the main site of exchange between the circulatory system and the gut contents. Nutrient absorption is also carried out by midgut , whereas excretion and water balance take place primarily in the Malpighian tubules attached to the hind end. RNAi effects occurring in insects as a result of oral delivery of dsRNA are presumably mediated by the midgut surfaces through exposure of cells of the midgut epithelium and the Malpighian tubules to dsRNA in the gut contents. Conditions in the gut vary considerably between insect orders. Gut pH is an important factor in insect digestion and can vary from predominantly acidic (coleopteran larvae) to strongly alkaline (up to pH 10.5 in some species of Lepidoptera). In addition, within a single insect the pH changes along the gut and with distance from the gut epithelium. The stability of ingested dsRNA in the insect gut could be affected both by chemical hydrolysis (which increases with increasing pH) and by enzymes present in the gut contents.

Six important factors largely influencing the silencing effect and therefore the efficiency of RNAi as insect control technique:

1. RNAi delivery

Efficacy of an RNAi experiment can be influenced strongly by the mode of delivery of the dsRNA. The most widely way to access RNAi in insects are injection into the hemolymph and feeding. Microinjection was used in the first successful application of RNAi in an insect, to obtain knockdown of frizzled in *Drosophila melanogaster* [13].

2. Concentration of dsRNA: For every target gene and organism an optimal concentration has to be determined to induce optimal silencing. It is not true that exceeding that optimal concentration results in more silencing[19,23].

3. Nucleotide sequence: The sequence used will determine possible off-target effects in the target organism, but also in other insects. Off-target silencing is reported in the triatomid bug *R. prolixus*: together with the targeted nitroporin 2, two highly homologous nitroporin genes were silenced[1]. Vacuolar H^+ ATPase dsRNA of the Colorado potato beetle (*Leptinotarsa decemlineata*) also silenced the ortholog gene in *D.virgifera*, although higher dsRNA concentrations were necessary for efficient RNAi in *D. virgifera* compared to the Colorado potato beetle[4].

4. Length of the dsRNA fragment: This is a determinant of uptake and silencing efficiency in intact organisms[18] and cell lines[21]. In feeding experiments most sequences range between 300 and 520 bp. However, there is a study using only one siRNA[14]. The choice to use siRNA is probably based on to the success of siRNA in clinical research[6,15]. In the case of S2 cells, Saleh *et al*.[21] reported that the length of the dsRNA should be minimally 211 bp.

5. Persistence of the silencing effect: the silencing effect on aquaporin in *A. pisum* persists for 5 days and is then reduced[23]. As reported by Turner *et al*.[27] this transient effect of dsRNA against the pheromone binding protein in the light brown apple moth (*Epiphyas postvittana*) may be correlated with the turnover rate of the target protein.

6. Life stage of the target organism: Although older life stages are more efficient for handling, the younger stages often show larger silencing effects. For instance, no silencing effect was observed after treating 4th instars of *R. prolixus* with nitropin 2 dsRNA compared to 42% silencing when using 2nd instars[2]. Also in the case of the fall armyworm (*Spodoptera frugiperda*) a stronger silencing effect was observed in 5th instar larvae compared to adult moths[8].

Applications of RNAi technology:

Functional genomics and RNAi

A surprising discovery has recently revolutionized *C.elegans* "reverse genetics"- the process by which scientists begin with a known gene sequence and attempt to define its biological function by disrupting its activity in vivo.A large-scale functional analysis of ≈19,427 predicted genes of *C. elegans* was carried out with RNA interference. Similarly, in *D. melanogaster*, RNAi technology has been successfully applied to identify genes with essential roles in biochemical signaling cascades, embryonic development, and other basic cellular process. In plants, gene knockdown-related functional studies are being carried out efficiently when transgenes are present in the form of hairpin (or RNAi) constructs.

Virus induced gene silencing (VIGS)

Replication of plant viruses, many of which produce dsRNA replication intermediates, very effectively cause a type of RNA silencing called VIGS (Virus induced gene silencing).When viruses or transgenes are introduced into plants, they trigger a post transcriptional gene silencing response in which double stranded RNA molecules, which may be generated by replicative intermediates of viral RNAs or by aberrant transgene coded RNAs.

PTGS is believed to be an anti-viral response. Viral RNAs not only trigger PTGS, but they also serve as targets. Cleavage of viral RNA results in reduces virus titers in local and distant leaves and a plant recovery phenotype. Induction of PTGS was visualized if the cauliflower mosaic virus infection and subsequent recovery were followed up in a transgenic *B. napus* expressing a p35S-GUS (β-glucuronidase) transgene.

RNA interference – a defense against jumping genes

Transposons also known as jumping genes are DNA sequences that can move around in the genome. They are present in all organisms and can cause damage if they end up in the wrong place. Many transposons operate by copying their DNA to RNA, which is then reverse-transcribed back to DNA and inserted at another site in the genome. Part of this RNA molecule is often double-stranded and can be targeted by RNA interference. In this way, RNA interference protects the genome against transposons.

4. Therapeutic potential of RNAi

The strategy used was to silence the main structural protein in the virus, p24, and the human protein CD4, which the virus needs to enter the cells. This impairs the virus in infected cells and limits its spread into healthy cells, siRNAs have been shown to inhibit infection by human immunodeficiency virus, poliovirus, and hepatitis C virus in cultured cell lines. McCaffrey and Song demonstrated effective targeting of a sequence from hepatitis C virus and the fas gene by RNA interference in mouse liver (199).

5. Modification of plant height via RNAi suppression of OsGA20ox2 gene in rice:

GA 20-oxidase (GA20ox) is a regulatory enzyme for the syntheses of biologically active GAs in plants. The loss-of-function mutations in OsGA20ox2 of rice (*Oryza sativa L.*) generate the well known Green Revolution gene sd-1, which cause the semi-dwarfism phenotype. In this investigation, semi-dwarf plants were generated from a taller rice variety QX1 by RNAi suppression on the expression of OsGA20ox2. The 531 bp-fragment of OxGA20ox2 was amplified by PCR from genomic DNA of QX1 and used to construct the hairpin RNAi vector pCQK2. The wild type QX1 was transformed with pCQK2 by Agrobacterium-mediated transformation and some independent transgenic RNAi lines exhibited semi-dwarfism. RT-PCR and Northern blot analysed showed that the expression of OsGA20ox2 was specifically suppressed in the RNAi semi-dwarflines. Endogenous GA assays reveals GA19' GA20 and the down-stream biologically active GA, were drastically reduced in the RNAi semi-dwarf limes. It was shown that the RNAi semi-dwarf lines could be restored to normal plant height by applying exogenous GA3.

6. Using RNAi to improve plant nutritional value

Caffeine content in coffee plants has been markedly reduced by RNAi-mediated suppression of the caffeine synthase gene RNAi has been successfully used to generate a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein, a protein that is poor in lysine content RNAi mediated by a hairpin RNA has been used in cotton to down regulate two key fatty acid desaturase genes encoding stearoyl-acyl-carrier protein D9-desaturase and oleoyl phosphatidyl choline u6-desaturase[17]. Knockdown of these two genes in cotton leads to an increase in nutritionally improved high-oleic and high-stearic cottonseed oils, which are essential fatty acids for health of the human heart.

7. Technology reduces Gossypol in cotton seed

It's possible to significantly reduce gossypol levels within cottonseed and not reduce the levels of gossypol and related compounds in the foliage. The presence of these compounds in the foliage helps protect the plant from attack by insects. In addition, U.S. Consumers craving a new and nutritious snack food could soon be reaching for crunchy "TAMU nuts," which were developed at Texas A&M over 20 years ago. Reduced gossypol cotton seeds have a nutty flavor and crunch.

CONCLUSION

The discovery of RNAi has been a central key enabling greater understanding of gene expression and their functions carried out in range of eukaryotic organisms, as well as for the development of research in several fields at the biological, medical and biotechnological level. This technology has helped in identification of a new set of targets which can be used in the control of pests. Because these

targets have previously not been identified, there should be no acquired resistance in the pest species. The successful use of RNA interference with insects also requires the identification of best targets for RNAi and selection of the best interfering RNAs (siRNAs, piRNAs, miRNAs or dsRNAs).

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