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Review

Insect gut nucleases: a challenge for RNA interference mediated insect control strategies

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RNA interference (RNAi) is a post-transcriptional control echanism involving degradation of target mRNA which is mediated by small interfering RNAs (siRNAs). This phenomenon of gene silencing has now been considered as a potential strategy for the control of insect pests. The selection of the target gene and synthesis of double stranded RNA (dsRNA) comprise crucial component of application of this technology. Different methods for the delivery of dsRNA in to insect have been investigated in the recent years. However, there are many limitations for the application of RNAi as a potential strategy for insect control. Most of the studies on RNAi for insect control have been focused on the insect midgut as it is considered as most effective target for the RNAi based pest control. The delivery of dsRNA in to the midgut is affected by feeding as dietary component; however, the environment of midgut is hostile for the fed dsRNA, where the gut nucleases and pH plays a major role among other associated factors. The current developments have shown RNAi technology as an important tool for next generation insect control measure. To achieve the practical application of this technology in insect-pest control, further study on the protection of dsRNA in insect gut is needed. The present review focuses on the major threats for the integrity of dsRNA in the insect gut.

Key words: RNAi, insect control, midgut, nucleases.

Introduction

Gene silencing is a widespread phenomenon in animals, plants and microorganisms, which act as a defense mechanism against foreign DNA. RNA interference has now emerged as an important tool for the study of gene functions through silencing specific genes by degrading mRNA before it is translated. It is a post-transcriptional gene silencing mechanism which is initiated by the introduction of double-stranded RNA (dsRNA) into a cell. This gene knockdown mechanism by dsRNA is known as RNA interference (RNAi) in animals and post-transcriptional gene silencing in plants (Hannon, 2002; Baulcombe, 2004). After the advent of RNAi nearly 13 years ago by Fire et al. (1998), RNAi has been exploited in several plant and animal species for various applications ranging from study of functional genomics to gene knock down effect in the insects of economic importance. Gene knockdown by RNAi has now become a potential tool in genomics for ascertaining the functioning of various genes. The functional RNAi machinery has two major components, the core component inside the cells, which comprised of Dicer enzymes, RNA-binding factors and Argonaute protein, and systemic component that amplify the dsRNA signal and allow it to spread to other tissues within the animal (Siomi and Siomi, 2009). RNAi effects are mediated by the production of small interfering RNAs (siRNAs) from the dsRNA, which is cleaved by dsRNA-specific endonucleases referred to as DICERS (Figure 1). The siRNAs are generally 21 - 25 bp fragments of dsRNA and have two base extensions at the 3' end of each strand. These siRNAs are incorporated into a RNA-induced silencing complex (RISC), which is a ternary complex that consists of an Argonaute protein, Dicer and a dsRNA-binding protein. Argonaute proteins, the catalytic

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components of RISC, use siRNA as a template to recognize and degrade the complementary messenger RNA (mRNA) (Meister and Tuschel, 2004). Upon cleavage in to small fragments one strand of the siRNA duplex, which is known as the guide strand is loaded onto Argonaute protein at the core of RISC,. During loading of RNA, the other non-guide stand known as the passenger strand is cleaved by an Argonaute protein and ejected out of the complex. The Argonaute protein then uses the sequences of guide siRNA to associate with target RNAs that contain perfectly complementary sequence and then catalyses the slicing of these targets. The cleaved target RNA is released, and the RISC is recycled for another round of slicing. The sense strand is also known to recycle back and result in amplification of silencing effect (Sijan, 2001).

The majority of the research to unravel different mechanisms of RNAi technique was carried out in the nematode *Caenorhabditis elegans* and *Drosophila melanogaster* (Bischoff et al., 2006; Miller et al., 2008). Numerous studies involving RNAi are coming up in different categories of organisms including viruses, bacteria, nematodes and insects. In insects, further progression is becoming possible as more insect genomes are becoming available day by day. However, this technique has yet to deliver its practicality in the field for the crop defense against invading insect pests and there need to resolve the basic issues for success of this technology for the insect control. The dsRNA administration through feeding is most feasible approach for gene knockdown effects, however, the researchable issues related to fate of administered material inside midgut need to be resolved for expecting desirable RNAi effects.

RNAi and insect control

The crop plants are the major source of human nutrition, but the crop losses due to insects and insecticides cause huge economic losses every year. The chemical pesticides are still the major approach available for their control, but their application is associated with development of insecticide resistance along with the environmental concerns and health hazards in human and animals. This has continuously prompted for the development of durable and cost-effective alternative pest-control strategies. In the 21st century, the transgenic have emerged as a potent tool for the management of insect pests, which have given successful results for the control of some of the insect pests in the fields in the economically and agriculturally important crop plants. This is mostly attributed to *Bacillus thuringiensis* (*Bt*) toxin that has shown practical success in the protection of wide category of crops and to some extent, replacing chemical insecticides. However, all the crop plants cannot be covered under the *Bt* protection, and also there remains an prominent threat of at least some species developing *Bt* resistance. The phloem sap-sucking insects, such as aphids, whiteflies and plant bugs, have evolved as major pests from the minor pests of past. This is primarily due to no *Bt* toxin with adequate insecticidal effects on these category of pests (Gatehouse and Price, 2011). Therefore, there was a need for new approaches for the control of these insect types. In this context the gene knock down by RNAi is been seen as versatile technology to tackle this long pending issue of environment friendly control of insect pests. The success of this technology has been demonstrated in insects like *Drosophila melanogaster* (Bischoff et al., 2006; Miller et al., 2008), *Tribolium castaneum* (Miller et al., 2008), *Phyllotreta striolata* (Zhao et al., 2011), *Anopheles gambiae* (Zhang et al., 2010), *Nilaparvata lugens* (Chen et al., 2010), *Schistocerca gregaria* (Badisco et al., 2011), *Helicoverpa armigera* (Mao et al., 2007) , *Apis mellifera* (Nunes and Simo˜es, 2009) and *Bombyx mori* (Hossain et al., 2008) and some vectors of human diseases including *Rhodnius prolixus*, *Glossina morsitans morsitans* (Huvenne and Smagghe, 2010). The different insect pest catagories, where gene knockdown effect has been demonstrated are presented in Table 1. For achieving the gene knockdown effects by feeding, continuous supply of dsRNA is required in the insect midgut. Transgenic plants have turned out as an option for maintaining the continuous supply of dsRNA in the midgut. Plants producing dsRNAs directed against genes function in Lepidoptera, Coleoptera, and Hemiptera pests are becoming more common (Mao et al., 2007). The advantage of feeding transgenic plants expressing dsRNA is the generation of continuous and stable dsRNA material. Mao et al. (2011) generated dsRNA-expressing cotton *(Gossypium hirsutum*) plants and the bollworm larvae (*Helicoverpa armigera*) reared on these plants exhibited drastically retarded growth, and the transgenic plants were less damaged by bollworms than the control. Quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that the CYP6AE14 expression level was reduced in the larvae as early as 4 h after feeding on the transgenic plants. Chen et al. (2010) reported the successful feeding of TPS (trehalose-6-phosphate synthase for the synthesis of trehalose, main sugar reserve in haemolymph) dsRNA solutions to silence this gene thus proposing it as a useful pest control agent. However, achieving proper RNAi effects through feeding requires that the dsRNA remain protected inside the gut till its uptake and transport.

dsRNA inside insect gut

The application of this technology for the insect control through feeding require regular and autonomous take up the dsRNA by the organism, either added to the food or produced by transgenic plants as hairpin RNAs. In both

Table 1. RNAi mediated gene knockdown in different insects

Target insect	Mode of delivery of dsRNA	Target gene for knockdown	Reference
Alhalia rosae	Injection	Ar white gene	Sumitani et al., 2005
Anopheles gambiae	Feeding nanoparticles	*AgCHS1* and *AgCHS2*	Zhang et al., 2010
Apis mellifera	Mixed with natural diet	Vitellogenin gene	Nunes and Simoes, 2009
Bactericerca cockerelli	Injection/feeding	BC-actin	Wuriyanghan et al., 2011
Bactrocera dorsalis	Feeding	V-ATPase	Li et al., 2011
Blatella germanica	Injection	Hypertrehalosemic harmone	Hang and Lee, 2011
Bombyx mori	Transgenic	Ecdysis-triggering hormone gene *ETH*	Dai et al., 2008
Diabrotica virgifera virgifera	Artificial diet	Vacuolar ATPase Subunit A	Baum et al., 2007
Epiphyas postvittana	Droplet feeding	Larval gut carboxylase	Turner et al., 2006
Epiphyas postvittana	Feeding	Carboxylesterase gene (EposCXE1)	
Glossina morsitans morsitans	Feeding	*TsetseEP* gene and transferrin gene *2A192*	Walshe et al., 2009
Gryllus bimaculatus	Injection	Nitric oxide synthase gene *NOS*	Takahashi et al., 2009
Gryllus bimaculatus	Injection	Insulin receptor	Dabour et al., 2011
Helicoverpa armigera	Feeding	Acetylcholine esterase gene	Kumar et al., 2009
Helicoverpa armigera	Transgenic plant	Cytochrome P450 gene (CYP6AE14)	Mao et al., 2007
Monochamus alternatus	Injection	Laccase gene *MaLac2*	Niu et al., 2008
Nilaparvata lugens	Feeding	Trehalose phosphate synthase (*TPS*)	Chen et al., 2010
Nilaparvata lugens	Feeding	ATPsynthase subunit E	Li et al., 2011
Nilaparvata lugens	Feeding	Hexose transporter, carboxypeptidase	Zha et al., 2011
Oncoeltus fasciatus	Injection	Nubbin	Turchyn et al., 2011
Phyllotreta striolata	Feeding	Arginine kinase gene *AK*	Zhao et al., 2008
Reticulitermes flavipes	Artificial diet	Cellulose enzyme, hexamerin storage protein	Zhao et al., 2008
Rhodnius prolixus	Feeding	Salivary nitrophorin 2 gene *NP2*	Araujo et al., 2006
Schistocerca americana	Injection	Vermilion	Dong and Friedrich, 2005
Spodoptera frugiperda	Droplet feeding	Cytochrome P450 (CYP6BF1v4)	Griebler et al., 2008
Spodoptera littoralis	Injection	Circadian clock gene	Kotwica et al., 2009
Spodoptera litura	Soaking and in artificial diet	Aminopeptidase N	Rajagopal et al., 2002
Spodoptera litura	Injection (pupa)	Vitellogenin receptor	Shu et al., 2011
Tribolium castaneum	Injection	Chitinase-like proteins *TcCHT5*, *TcCHT10*, *TcCHT7*, and *TcIDGF4*	Zhu et al., 2008

the cases, dsRNA will first enter inside the insect gut The midgut , which is the second largest organ in the insect body, consists of a single layer of columnar cells with microvilli, endocrine cells, and stem cells at the base, grouped in the so-called nidi. It is designed so as to absorb nutrients from the lumen with its large absorption area created by the microvilli. The use of Cry toxins from *Bt* as an insecticide has targeted the use of midgut for insect control (Hakim et al., 2010). The unique characteristics of the midgut make this tissue very potential dsRNA uptake location. On the delivery of dsRNA in insect gut the two prominent trans-membrane proteins, SID-1 and SID-2 are known to be involved in the transport of dsRNA into cells *via* a passive uptake mechanism (Winston et al., 2007). SID-2 alone was not sufficient for dsRNA import from the intestinal lumen as *sid-1* mutants were unable for the uptake of environmental dsRNA. It was observed that SID-2 might modify or compliment SID-1 at the lumen or might function in series with, internalizing dsRNA for SID-1 transport across the membrane (Winston et al. 2007). Other than this the endocytic dsRNA uptake mechanism has also become known for the dsRNA uptake (Huvenne and smagghe, 2010). This pathway of dsRNA transport involving clathrin–mediated endocytosis has been proposed as a requirement for systemic RNA silencing (Saleh, 2006). In many insect species including *D. melanogaster* which apparently lack any SID-1 ortholog, the endocytosis appears to facilitate cell uptake, (Whyard et al., 2009). Apart from inhibiting RNAi effects, the pharmacological inhibition of endocytosis in *C. elegans* also results in knockdown of endocytotic pathway components and produced worms with a 'loss-of-RNAi-function' phenotype. This applies that dsRNA uptake into cultured *Drosophila* S2 cells does not involve a *sid-1* based mechanism and goes by more common receptor-mediated endocytosis (Saleh et al. 2006). Therefore, it seems like that receptor-mediated endocytosis is a widespread mechanism of dsRNA uptake and could be affirmative to the other classes of insects. However, prior to transport of dsRNA from the gut, it needs to be protected from the action of nucleases and pH extremes.

Insect gut nucleases and pH

The future practicality of insect control is visualized

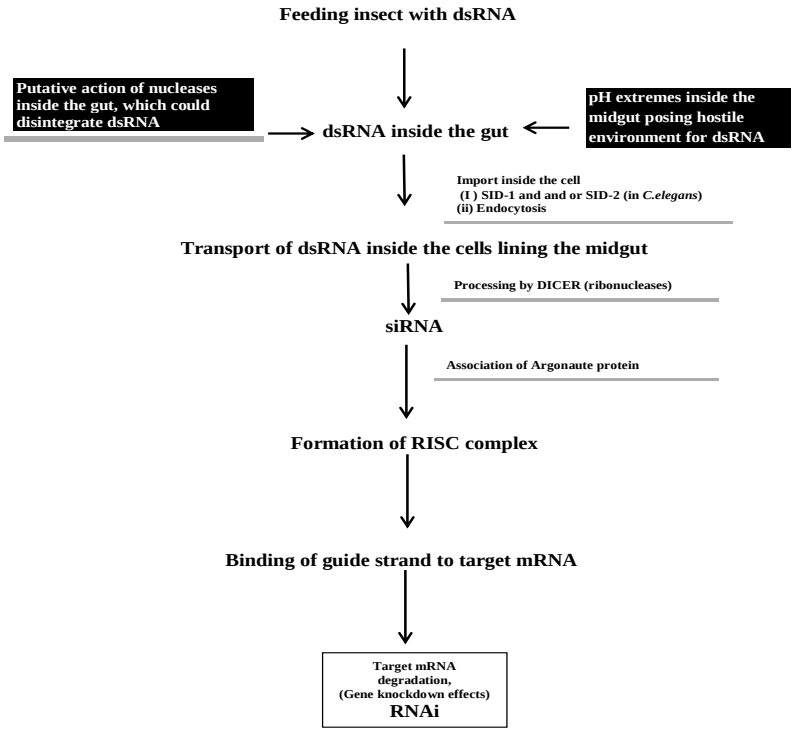

Figure 1. The mechanism of RNAi in insects. The dsRNA fed to the insect induces the degradation of mRNA transcripts after its processing in to 21–23 bp short interfering RNAs (siRNAs) by endonuclease known as DICER. The resulting siRNAs function direct an RNA-induced silencing complex (RISC) to degrade target mRNAs.

through feeding of dsRNA to insect. The targeted insect must be able to take up the dsRNA autonomously through feeding, which explains the whole process of non-cell-autonomous RNAi. For the regular supply of dsRNA for the RNAi effects, the transgenic plants expressing insect dsRNA have been proposed as a continuous source (Price and Gatehouse, 2008). Consequent upon feeding, there is uptake of the dsRNA from the gut lumen into the gut cells (environmental RNAi), which then spread to specific tissues beyond the gut (systemic RNAi). However, there remains the important concern for the stability of dsRNA in the gut lumen, the issue which has not been researched thoroughly. Till date, there is no concrete report regarding the fate of this double stranded RNA in the insect gut, where insect gut nucleases and pH play crucial role.

Gut nucleases

The action of nucleases on the dsRNA present in the insect gut is an important issue to be resolved for visualizing the field application of this technology for the control of insect pests. There have been successful leads with this technology for the knockdown of important genes in some of the insects of economic importance but, to our knowledge there is no thorough study on the fate of dsRNA in insect gut. This issue thereby need sensitization for further basic research in the area.

The insect midgut is the site for the exchange of materials between the hemolymph and gut contents. It contains different sets of enzymes which helps in the digestion of ingested material. In nature, the diet of the insects contains a good proportion of nucleic acids thereby nucleases *viz*., DNase and RNase plays important role in digestive process. The amount of nuclease however depends on the insect species and type of material they feed on. For example, the termites which particularly feed on the woody material will have the enzymes associated with the complex carbohydrate digestion mainly cellulases; however the other insects groups which feed on the material having considerable amounts of nucleic acids, the high proportions of nucleases are expected. Inside gut, the nucleic acids act as substrate for the digestive nucleases, therefore, protection of the ingested dsRNA from the action of nucleases become necessary to keep it intact until its uptake from the lumen of the gut in to the cells for the initiation of RNAi machinery (Figure 1). So far there are no conclusive reports explaining the fate of dsRNA inside the gut. The dsRNA may be a direct substrate for the RNases in the insect gut, which could disrupt the

functionality of whole RNAi mechanism. In this context the study on the insect nuclease is a novel area for diversion of present approach of research for the refinement of this technology. The action of gut nucleases poses a real challenge for effectiveness of RNAi technology in insect control.

Gut pH

The pH variation inside the gut is another constraint for RNAi effects through dsRNA feeding. The gut pH varies among different insect species ranging from strong acidic (like Coleoptera) to strongly alkaline (up to 10.5) in some species of Lepidoptera. Besides this, there is variation in pH in different parts of the gut and with the distance from the gut epithelium. The ingested dsRNA in the insect gut could be affected either by chemical hydrolysis or enzymes present in the gut or both (Hakim et al. 2010). In visualizing the practical feasibility of RNAi effects through feeding, the dsRNA should be functional till its transport through midgut epithelium. A method for coating the dsRNA for protection from gut nucleases and pH extremes need to be developed for increasing the efficiency of silencing. Huvenne and Smagghe (2010) have suggested for the protection of dsRNA by some sort of coating material for efficient RNAi in insects, Nevertheless, despite of such hostile conditions, the ingested dsRNA have been able to initiate RNAi in insect species (Terra and Cristofoletti, 1996), suggesting that there might been some associated factors for maintaining the ingested dsRNA sufficiently intact in the gut.

CONCLUSION

RNAi, or the post transcriptional gene silencing is a sequence specific technology for targeting a specific insect species and leaving other species unharmed. This method of insect control is also important for those insects, which are insensitive to *Bt* transgenic crops including sucking insects, aphids, planthoppers, and ticks and mites. Another attractive feature of this technique lies in resolving the issue of insecticide resistance, human and environmental safety. Although, gene knockdown mechanism mediated by RNA interference has a potential to be an alternate strategy for the control of insect pests in the agricultural crops, however, the key issues need to be resolved simultaneously. This method is unlikely to become an immediate plant protection strategy until the basic issues related to this technology are resolved. In order to achieve the practical applications of RNAi technology for the control of economically important insects, fundamental and the applied research is needed on the fate of dsRNA in the midgut of target insects for functionality of RNAi machinery.

Abbreviations: RNA, Ribonucleic acid; **dsRNA,** double stranded RNA; **RNAi-RNA interference,** siRNA-small interfering RNA; **RISC,** RNA-induced silencing complex.

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