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RNA interference: A potential method of crop pest management: A review

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Abstract
Suppression of genes that code for specific proteins through RNA interference is an alternative strategy for insect pest management. RNA interference is a powerful tool that exemplifies the gene function by making changes in post transcriptional gene silencing phenomenon. This ability of suppressing a particular gene expression helps in obtaining a new trait by elimination or accumulating particular plant trait that are desired which are phenotypic or biological changes which are not present in non-transgenic plants. Clear evidence is obtained from the recent studies that RNAi has potential application in controlling insect pests. However environment and non target organism and risk resistance is yet to be determined.

Keywords: Gene silencing, insect pest management, RNAi, RNAi based insecticides

1. Introduction
RNA interference is a method of regulating or manipulating the process of gene silencing to disturb a particular gene expression and in turn their function. This method was first discovered in *Cenorhabditis elegans* [1] and *Drosophila melanogaster* Megnin [2] which was published back in 1998. The RNAi machinery utilises a particular sequence of information in dsRNA and produces a RNA-protein complex that ultimately degrade the corresponding mRNA [3-5]. This is a highly specific process where genes sharing a complimentary sequence with dsRNA are affected. Both in plants and animals the very most important function of RNAi is to maintain integrity of genome, which is possible only by subduing the moment of transposons and the accumulation of repetitive DNA in germline [6-9]. Since then this method was constantly being explored in a much deeper manner to find the functioning of host genes and to generate and enhance the plant traits that correspond to stress tolerance, qualitative and quantitative yield characteristics [10]. Apart from regulating host genes RNA interference has also been successfully employed for making changes in the genes of insect pests and pathogen carrying vectors which pose a critical threat for crop production [11-13]. Thus it is much important to understand RNA interference technique exemplifying core RNA interference mechanism, players involved, new developments regarding dsRNA uptake, current RNAi based insecticides and lastly we also discus recent studies of using RNAi transgenic plants for controlling agriculturally important insect pests.

2. Players involved in RNAi
Many compounds serving as initiators, effectors, an amplifier, transmitters has been identified. It is hopefully expected that in future many other components and their interrelations may be unveiled. Here we discuss some of the components known so far.

2.1 Dicer
Dicer belongs to RNase III family are among the few nucleases that show specificity for dsRNAs [7]. Dicer cleaves dsRNA into short ssRNA approximately of 20-25 base pairs long with 3' overhangs of 2-3 nucleotides of 5' phosphate and 3' hydroxyl termini. Usually some species specific RNase III like enzymes called dicer act upon these long dsRNAs resulting in small dsRNA now these short RNAs gets binded to RISC complex and thus helps in finding mRNAs that are to be blocked for translation in post transcriptional silencing [14, 15].

2.2 RNA and DNA helicases
Small interfering RNAs of 21-25 nt have a very prominent role in post transcriptional

gene silencing phenomenon as they bind to the complementary portion of target mRNA and tag it for degradation. To block this process it just take a single base pair difference between target mRNA and siRNA template.

2.3 RNA-induced silencing complex (RISC)

RISC is a ribonucleic protein in a multiprotein complex that plays an important role in RNAi. Its main function is to introduce ssRNA fragments such as (si RNA) or short interfering RNA (dsRNA) which acts as a complementary strand to messenger RNA that is ought to be blocked or degraded [16]. When this binding is completed, a protein called agronaute2 (AGO2) which is a catalytic compound and an endonuclease capable of degrading mRNA.

3. Mechanism of gene silencing

Gene silencing mechanism occurs in two major steps. One is an initiation step followed by effector step. In the first step i.e, initiation step the large double stranded RNA molecule usually 1000 bp long is diced in to very small fragment of 21-25 bp called siRNA (small interfering RNA). In the effector step these siRNAs unwind in to two individual sense and antisense strand strands the later strand now get coupled to RISC and carry it to target mRNA. The siRNA strand which has a complementary sequence to the mRNA in to dsRNA itself, which then becomes substrate for dicer cleavage activity thus get degraded and leads to formation of new siRNAs. This step further amplifies the RNAi response and creates a self replicating cycle of degenerative polymerase chain reaction which remains continued till no target mRNA remains. This basic core process explicates RNAi response as one of the most discerning and efficient biochemical mechanism in nature.

4. Uptake of dsRNA in to target sites

A well efficient method for the delivery of siRNA for producing an efficient silencing effect in yet to be discovered so it is essential to select a target sequence that is complementary to the mRNA and also quantify their possible effects in mRNA or target protein. The very first method developed for successful delivery of siRNA invivo is high pressure injection. Electroporation, a method of using electric current to make pores in organ surface and small RNA gets directly absorbed through these pores was also successful. Delivery of siRNA to the target tissues is quite problematic because after reaching target organ it has to enter the cytoplasm of the organ also. Systemic delivery of RNA is not very efficient as RNA cannot penetrate cellular membrane so systemic delivery of siRNA is unlikely to be successful. Moreover the action of RNase activity in serum quickly degrades the RNA and although siRNAs are chemically modified to be more stable and yet has a half life of only few hours mostly. Considering all these possible drawbacks the following methods of delivering siRNA to target cells was devised and was efficiently used at micro levels.

4.1 Micro injection

It is one of the most effective method for delivering systemic RNA. It involves direct injection of dsRNA in to insect body. Usually the short dsRNA showed maximum success rate in this mechanism [17]. It reported that gene silencing was much better with phosphorylated 5' end [18]. The major advantage of using microinjection method is its higher efficiency in inhibiting gene expression. One of the major limitations of this method is invitro production and storage of dsRNA is quite complicated and involves in high production costs. Additionally the wounds generated due to injection pressure also limit its application.

4.2 Feeding of artificial diet

Feeding dsRNA is more convenient and easy to manipulate method a it is one of the natural method of inducing dsRNA in to the body of insects, without causing any physical injury to the target organism. For small insects which are difficult to handle through micro injection method can be operated easily by feeding artificial diet. A pheromone binding protein *EposPBP1* in antenna of adult *Epiphyas postvittana* successfully inhibited when their larvae are fed with dsRNA, along with inhibition of carboxyl esterase gene *EposCXEL* in larval midgut [19]. Successful attempts were also reported in orders like hemiptera, coleopteran, and lepidoptera [20, 21]. The main constrain remains that silencing has been shown to be incomplete and also need a large amount of material for delivery [22].

4.3 Soaking

In an experiment, when embryos of *D. Melanogaster* were soaked in dsRNA solution the results for inhibition of gene expression were much efficient. Moreover the amount of solution with dsRNAs in this method is less when compared with injection method which requires a higher concentration of dsRNA [23]. Soaking method is limited to few insects and life stages which can readily absorb dsRNA from solution.

4.4 Transgenic crop technique

Transgenic crop technique is one method that was found much better than direct feeding wioth dsRNA [20, 21]. This method has an advantage of producing dsRNA continuously. Studies showed that gene silencing was not successful in *D. melanogaster* when fed with dsRNAs produced from genetically engineered yeast strand [24]. However dsRNA produced by bacteria were effective when fed to *C. elegans* [25]. Since then bacteria is widely used for production of dsRNA in RNAi.

4.5 Virus-mediated uptake

In virus mediated RNAi host organisms get infected with dsRNA which are produced during the replication of virus. The target gene of interest into host organism is degraded by small interfering RNA produced from dicing dsRNA. For example when *Bombyx mori* cells were infested with recombinant Sindbis virus the dsRNA then produced inhibits BR-C gene expression which has lead to the poor pupation and defects in adult insects [26]. Though pathogenecity and rapid spreading ability of virus in host population in high virus mediated RNA studies are still rare.

5. Current RNAi-based insecticides

An extensive research on the response of the RNA interference in western corn root worm *Diabrotica vergifera vergifera* [20, 27-30] showed very sensitive results. The oral administration of dsRNA targeting some specific genes of interest showed a significant positive interference with gene function [20]. Based on these positive results United States environment protection agency waved green signal for developing a first RNAi based insecticides to control insect pest. Monsanto and Dowagro sciences has made a proposal for making an RNAi based insecticide which will later be

called as SmartStax pro® a plant incorporated protectant (PIP) pyramiding several Bt proteins, dsRNA targeting western corn rootworm Snf7 gene [31]. Bt proteins in this insecticide may act on gut epithelium leading to gut paralysis and death of insect [32]. Besides down regulation of Snf 7 gene leads to protein trafficking which leads to insect's mortality [33]. So this combined strategy would not only result in immediate mortality of insect but also prevent from resistance development in insects against this PIP. It would only be a question of time before SmartStax Pro® or other yet to be discovered insecticidal strategies will appear in the market with such a great pace in RNAi technology.

6. RNAi studies for controlling agriculturally important insect pests

Table 1: Summary of HI-RNAi for the control of insect pests.

Target organism	Target gene	Function of target gene	Intron	Binary Vector	RNAi plant M	Effect of HI- RNAs on target insect	References
Bemisia tabaci	Acetylcholinesterase (AChE) and ecdysone receptor (EcR)	1. Required in signaling 2. Role in molting and metamorphosis	--	pJIT163+pCam bia2300	Tobacco	Silencing of targets gene in chimeric construct produced detrimental effect on survival (appox. 80% mortality) with decrease in target genes transcripts	Malik *et al*. (2016)
Helicoverpa armigera	Chitinase	Role in molting and metamorphosis	Chalcone synthase *(ChaS)*gene intron	pMVR-hp	Tobacco and tomato	Detrimental effects on larval growth and survival, decrease in larval and pupal weight, pupation and adult emergence with significant reduction in target gene transcript level	Mamta *et al*. (2016)
Helicoverpa armigera	Arginine kinase	Play role in role in cellular energymetabolism	GUS linker fragment	pANDA35HK	*Arabidopsis thaliana*	Detrimental effects on larval growth and survival, decrease in target gene transcripts	Liu *et al*. (2015)
Helicoverpa armigera	1. Chitin synthase 2. Cytochrome P450 monooxygenase 3. V-ATPase	1. Synthesis of chitin 2. Required for tolerance against gossypol 3. Role in cellular energy production	9 bp loop	pLD	Tobacco	Reduction in target gene transcript level and reduction in weight, growth and pupation rate	Jin *et al*. (2015)
Helicoverpa armigera	Arginine kinase	Play role in role in cellular energymetabolism	GUS linker fragment	pANDA35HK	*Arabidopsis thaliana*	Detrimental effects on larval growth and survival, decrease in target gene transcripts	Liu *et al*. (2015)
Helicoverpa armigera	1. Chitin synthase 2. Cytochrome P450 monooxygenase 3. V-ATPase	1. Synthesis of chitin 2. Required for tolerance against gossypol 3. Role in cellular energy production	1. Trachea and epidermis and midgut 2. Midgut 3. Whole insect body	pLD	Tobacco	Reduction in target gene transcript level and reduction in weight, growth and pupation rate	Jin *et al*. (2015)
Myzus persicae	hunchback(hb)	Play role in insect axial patterning		pUCCRNAi	Tobacco	Reduced Mphb mRNA level in the fed aphids and inhibited insect	Mao and Zeng (2014)
Bemisia tabaci	v-ATPase	Encode for v-ATPase subunit A, role in cellular energy production	*Arabidopsis RTM* gene	pBI101	Tobacco	Depletion in the transcript level and affected insect survival	Thakur *et al*. (2014)
Nilaparvata Lugens	0-Hydroxyecdysone	Role in molting and metamorphosis	*pSK*-int vector	p*CanG-HA*	Rice	Depletion in the transcript level, reduction in fecundity	Yu *et al*. (2014)

Source: adopted from Mamta and Rajam. (2014)

7. Challenges for successful RNAi in insects

Though there has been an enormous utility of RNAi as a promising technique for control of crop pests there is still a long way awaiting to be explored to decide do's and dont's in this technique before establishing it as a long term effective pest control method in the field. There are still many challenges in adopting this potential technique; here we discuss a few of those challenges.

7.1 Digestion of dsRNA

The very first challenge is to protect these ingested dsRNA which triggers the whole process of RNAi. These dsRNA has threat from nucleic acid degrading enzymes in the insect gut which awaits for their degradation, but clearly very less is known about fate of these dsRNA inside insect gut after the ingestion of plant material by insect. For example when polygalacturonase dsRNAs were injected to tarnish plant bug (*Lygus lineolus*) an immediate down regulation of gene was observed where as when these dsRNA was ingested by feeding approach did not show any response this maybe due to the presence of nucleases in insect saliva and midgut [34-36].

7.2 Chemical hydrolysis of dsRNA
Preventing the breakdown of dsRNA antagonistic environment of insect gut nucleases and pH variation can be done by coating dsRNAs was observed that degradation of dsRNA is quit faster in case of lepidopteran than coleopteran insects this maybe due to differences in haemolymph composition, uptake and processing methods of dsRNA [37-39]. In certain cases a very robust and positive RNAi response was observed even in hostile environmental conditions of insect gut which may be due to additional factors that gives stability to dsRNA in insect gut [40-42].

7.3 Quantity of dsRNA molecules
The optimal dose of dsRNA is influenced by many external and internal factors like insect species, their life stage, method of delivery of dsRNA and also the amount of target gene transcripts in addition, ability of spreading dsRNA molecules also play a major role. For example in *D. melanogaster* dsRNA can be induced only by haemocytes as they do not have the ability to uptake dsRNA through extracellular injection in to the tissues repeated injection of dsRNA repeated injections of dsRNA can reduce or enhance the RNAi efficiency [43-46].

7.4 Length of dsRNA molecules
Uptake of dsRNA and gene silencing efficacy in organisms usually depends on length of the dsRNA which later get diced to produce siRNA molecules [21, 47]. It was reported that longer DNA usually greater than 200 bp produces more siRNA through dicer activity. But reports have shown that even a single chemically synthesized siRNA was also successful in producing silencing effect in *Helicoverpa armigera* and tse-tsefly [48, 49]. This shows that both long and short dsRNA is effective in producing silencing effect conditioned target gene or pest.

7.5 Life stage of insects
Younger stages of organisms show much greater efficacy in gene silencing due to their small size or less developed body. In an experiment when a gene nitropin-2 was silenced in both second and fourth instar stage with same dose of dsRNA promising gene silencing effects were observed in second instar larva in comparison to fourth instar the difference in physiological and genetic characteristics of insect might be reason for difference in RNAi efficacy [50].

8. Future prospects and Conclusion
RNAi has been a promising technology in controlling crop pest by enhancing resistance traits in plants. Moreover this mechanism do not depend on the plants ability to produce foreign protein that would be toxic to invading pests, which makes this technique much widely acceptable than transgenic approaches, but one cannot solely rely on this particular technique for complete eradication of crop pest but can substantially reduce the application of toxic synthetic pesticides. Effect of RNAi on human and other mammals health is still unfound further additional studies should be determined to test the safety of transgenic dsRNA PIPs which are ought be used in food and feed products, further a potential research is required for understanding the effect of dsRNA on environment and their potential for uptake by target organisms.

However, development of effective delivery mechanisms and creation of transgenic plants producing dsRNA still remains lacunae in adopting such large scale projects. Overtime, the use of transgenic insects will also lead to more efficient pest control. Therefore all the potential risks associated with RNAi technology needs to be evaluated in order to have a successful pest control strategy.

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