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PLANT MEDIATED RNAi : A NEW LINE OF DEFENSE AGAINST INSECT PESTS

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Abstract- RNA interference (RNAi) is a potent tool in silencing of genes with high specificity in both invertebrates and vertebrates, thus, it has been widely applied to analyze gene function in various organisms including the insects. This high specificity of gene silencing has potential applications in insect pest management. Recent studies, however, have indicated that specific inhibition of gene expression in insects can also be attained by feeding and topical application of double stranded RNA (dsRNA) in certain insect species. The specific gene silencing using RNAi with feeding and *in vivo* dsRNA delivery methods holds outstanding promise of application of RNAi for controlling both agriculturally and medically important insect pests. Indeed, transgenic plants expressing dsRNA of specific genes have already been demonstrated for plant resistance against insect pests. This manuscript highlights the improvements and vistas of RNAi technologies in insect pest management, which in turn provides methods for producing transgenic plants express the dsRNA molecules, as well as pesticide agents and commodity products produced by the inventive plants.

Keywords- dsRNA, gene expression, RNAi, Transgenic plants

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Introduction

RNAi is as a tool has applications in virtually all spheres of the molecular biology to consider and in the characterization of gene function. The mechanism can be initiated by the introduction of the dsRNA into the system. Until 1980s, RNAi was unknown, but afterwards this genetic modification tool was called as gene silencing and today popularly known as RNA interference (RNAi). The history of RNAi began when Jeorgenson conducted an experiment to over express *Chalcone synthase* (*CHS-a*) gene in petunia plant to intensify its flower color to purplish, which resulted in a white flower due to suppression of *CHS-a* gene and they called it as 'Co suppression' [1]. Thereafter, Fire and Mello in 1998 demonstrated and elucidated details of the RNAi mechanism by the introduction of dsRNA into the *Caenorhabditis elegans*. After that a spate of publications were appeared, including those of mechanism of dsRNA uptake in insects [2], structural components and function of DICER and RISC etc. [3-5]. Due to its high sequence specificity, it is widely employed in many areas of biology, especially in crop improvement, metabolic engineering, functional genomics, and pest control [6].

Plant pests are the major biotic stress factors decreasing crop productivity both qualitatively and quantitatively to a great extent, control measure using chemical insecticides has resulted in escalation of production cost along with issues of residues leading to and water pollution and more importantly contributed the environmental issues as well as health hazards. The repeated use of chemical pesticide has broken out pest resistance [7]. The evolution of the *Bacillus thuringiensis* (*Bt*) toxin transgenic has been utilized for the past one decade in the pest management. The *Bt* transgenic plants scored a spectacular success in managing many of insect orders of agricultural importance *viz.,* Coleopterans, Lepidopterans, etc. but due to continuous *Bt* cultivation it has resulted in the development of resistance [8]. Lu, et al. [9] described the outbreak of pest the myriad bug and the *Bt* technology is met with limited success/ ineffective against some of sap sucking pests *viz*., white fly, aphid, grasshopper. So adding RNAi technology with *Bt* transgenic either separately or in combination will have added advantages in enhancing the plant protection against insects.

RNAi in Insects

The discovery of RNA interference was first studied in *Caenorhabditis elegans* [10]. The soaking of nematode in dsRNA solution demonstrated the degradation of the target mRNA and repression of the gene expression. The method of dsRNA application was varied in animals, especially in insects. In *C. elegans*, soaking and

microinjection methods were preferred and in insects all the methods, including the incorporation of dsRNA synthesizing bacteria in the artificial diet were used, the effect of RNAi was systemic and transitive, the amplification and spread of the RNAi pathway signal occurs through RNA-dependent RNA polymerase [11]. But in insects presence of RDRP is yet to be ascertained, recently RDRP orthologous were characterized in *D. melonogaster*. In *C. elaegans* turn over of the siRNA molecules occurs through RDRP however, it doesn't require RNAi core elements in the production of siRNA and also siRNA amplification are not necessary for the systemic spread of RNAi. Recently in *C. elegans* two types of dsRNA mechanism had characterized, one is the transmembrane channel mediated dsRNA uptake mechanism, in this mechanism two types of proteins, namely SID1 (Systemic RNAi Defective) and SID2 are involved, SID1 is required for the systemic spread of RNAi signal and SID-2 is found in the gut tissues and in cooperation with SID1 enhances the environmental RNAi [12-15]. In *Drosophila* endocytosis mediated dsRNA mechanism has been characterized, the *Drosophila* cells don't have neither robust systemic RNAi nor SID orthologs but S2 cells when soaked in dsRNA solution showed environmental RNAi, which indicated the possible existence of other alternative mechanism i.e., endocytosis mediated dsRNA uptake [16].

So far in more than 32 insects target genes have been screened for their potential for use in gene silencing mediated by *in vivo* synthesized DsRNA, recently reviewed by Zhang, et al. [17]. In other insects the RNAi response varied considerably. This was due to the intrinsic nature of the target gene, its size and tissue type and expression of the gene/s being used for silencing [18]. The RNAi response in several less studied insect species was robust and was inheritable to the next generation [19,20], whereas in other insect species it is stubborn. For example, Lepidoptera showed a variable silencing response when dsRNA was injected [21]. A number of factors are supporting to the susceptibility of insect species to RNAi, apart from DICER, a dicer like DNA/RNAse an extracellular enzyme digests dsRNA, these are secreted in various tissues. Other factors include the element of stability/half life of the dsRNA after the entrance into the hemocoel [22-24]. In *Blattella germanica* the dsRNA persisted for a longer period and in *Drosophila* and Lepidoptera microinjection of the dsRNA into the hemolymph enhanced the susceptibility to RNAi. The sequence specificity of RNAi was demonstrated in four different insects using E-subunit of V-ATPase, which demonstrated the high sequence selectivity of RNAi. E-subunit of V-ATPase *D. melanogaster, Maduca sexta, Tribolium castaneum* Herbst and *Acyrthosiphon Pisum Harris,* when each of the specific dsRNA fed individually to all four insects resulted in the selective silencing and death of the respective host insect without affecting other three insects [25].

Plant Mediated-RNAi (PM-RNAi)

The enormous success of RNAi experiments invited plant biotechnologists to utilize the insect functional genes as an insecticidal ingestible dsRNA produced by the transgenic plants. The era of PM -RNAi as new line of defense against insects was began [26-28]. Today there are eleven reports that have been published and are summarized in the below [Table-1].

The first outcome of the experimental validation of the insect gene in the form of insecticidal dsRNA was achieved in various insect orders *viz.,* Coleptera, Lepidoptera and in Hemiptera. The first evidence for insect proof plants of RNAi technology demonstrated by Mao, et al. [28] and Baum, et al. [26] against cotton boll worm, *Helicoverpa armigera* and corn worm, they opinioned that transgenic plants expressing ingestible dsRNA will emerge as a potential and alternative tool in insect pest management like *Bt* transgenics.

A PM-RNAi response to root knot nematode (RKN) was successfully established. The RKN i.e, *Meloidogyne* species, which severely deplete the harvest in a number of food and fiber crops. It infects more than 1700 host plants. Very few resistance crops against to RKN have been identified and in other crops resistant gene loci have been not identified. In cognizance of this problem they identified secretary parasitism protein, i.e., a peptide 16D10, which plays a vital role in the early signaling of RKN-host interactions. 16D10 expressed in sub ventral esophageal gland cells. This protein after infection to the roots, directly act with transcription factor domain of SCARECROW [27] establishes its colony and affects root growth. The *in vitro* studies conducted revealed that a full length dsRNA16D10 led to a reduction of 93-97% of transcript in the *M. incognita* and 16D10 peptide of 65-69%, further inoculation of the same dsRNA experimented *M. incognita* on *Arabidopsis* roots hindered its reproductive ability and reduced galling formation in the roots both in size and in number. To extend this outcome, two fragments of 16D10 i.e., 41 bp and 271bp used in the engineering of *A.thaliana* plant to silence four species of *Meloidogyne* RKN species (*M.incognita, M.Javanica, M. arenaria* and *M. hapla*), where the peptide shared nucleotide sequence 95-98% homology. The transgenic plants expressing the above fragments showed good growth of roots and showed abundant siRNA accumulation in northern blotting, thereby cut off the parasitic process by silencing the 16D10 transcript of *M.incognita.* This experiment could be employed for the development of transgenic plants where, natural resistance genes do not exist.

Baum, et al. [26], selected 290 genes from the Western Corn Root warm (WCR) cDNA library based on the functional role of the gene in the insect life cycle, these genes are called 'targets', the depletion of any one of target genes in the WCR insect may lead to death. The selected genes were screened and determined the larval competence by incorporating the corresponding dsRNA through artificial diet. After seven days most of the larvae had stunted growth and later died. These observation were recorded for two doses of dsRNA concentration, i.e., 520 ng/cm^2 and 720 ng/cm^2. Further, they extended their research by reducing the dsRNA concentration to 52 ng/cm^2 and 5.2 ng/cm^2 to determine lethal concentration, at this concentration RNAi response in the form of death and stunted growth was recorded for 125 bp and 69 bp dsRNA's target genes respectively, 14 potential genes for the RNAi in WCR were characterized. Among the selected 14 genes V-ATPase A, D, E and α-Tubulin were showed immediate RNAi response under low dose condition within 24 hours of dsRNA delivery. Due to the rapid RNAi response the above genes were tested for the other Coleopteran member pests, Southern corn rootworm (SCR; *Diabrotica undecimpunctata* Howardii), Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) and cotton boll weevil (*Anthonomus grandis* Boheman) to determine the minimized non target effect. Where nucleotide sequence similarities between WCR and CPB were compared which showed the similarities of only 83% and 79% for V-ATPase A and V-ATPase E, respectively. It was apparent that the RNAi response of WCR dsRNA directed to CPB exhibited same results. The success of insecticide V-ATPase dsRNA in insects applied to the cogent evidence of principle in the maize plant by pressing out the V-ATPase 246 bp gene in the sense and antisense

orientation, the maize plant on exposure to WCR larvae showed reduced root damage and significantly protected the plants.

Recently *Mi-Rpn7* dsRNA producing target gene of *M.incognita* was introduced into the soyabean plant through hairy root culture for the nematode pest management [29], *Rpn7* gene maintains the intactness of the 26S proteasome and for cleanup of the short lived proteins that are produced in the regulation of cell cycle and other cell related processes like, metabolic regulation, signal transduction and apoptosis, the group initially repressed *Rpn7* gene by soaking with 408 bp ds*Rpn7* solution it broke up the motility and reduced the infectivity, when the *Mi-Rpn7* was transformed to the soyabean plants through hairy root culture. The above results were repeated and also affected the reproduction. However, the transgenic plants developed were not fully tolerant to *M. incognita.*

The PM-RNAi has been efficiently utilized in the control of a sucking pest like the green peach aphid *Myzus persicae*, it is one of the major agricultural sap sucking pest that feeds over 40 different plant families and transmits more than 100 types of plant viruses. Pitino, et al. [29] selected *Rack*1 (*Receptor of Activated Kinase C*) and *C002* genes. *Rack*1 based on the earlier gene silencing results was carried out in the *C. elegans* [30,31] and *Heterorhabditis bacteriophora* [32]. Where knockdown of this gene in *M. persicae* resulted in faulty phenotypes, like lethality in an early stage of development, growth retardation and reduced fecundity and caused sterility. *Rack*1 is the multifunctional receptor protein and one of the internal components of the circadian clock binds to the various proteins and initiate signal transduction cascades, it also functions in the actin organization. Another target gene of *M. persicae* Mp*C002* a homologous gene of *COO*2 play a role in the plant host interaction and is expressed predominantly in salivary glands, knockdown *COO*2 gene showed mortality and improved tolerance to peach aphid in transgenic tobacco plants. Pitino, et al. [29] first investigated by expressing both the dsRNA genes transiently in tobacco plants which resulted in a decreases in both the gene transcript levels in insects. Latter they developed stably expressing transgenic *Arabidopsis* lines by the floral dip method. The transgenic plant showed tolerance to *M. persicae,* by reducing survival rate and also resulted in a production of fewer nymps.

Similarly, Mao, et al. [28] silenced the gossypol detoxifying gene of cotton bollworm in *in vivo* experiment using artificial diet incorporated dsRNA of *CYP450 monooxygenease* gene, before the engineering of *CYP450 monooxygenease* gene in the model plant *A.thaliana* and *Nicotina tabaccum,* first they confirmed toxicity effects of gossypol on larvae using different concentration of gossypol and observed that gossypol at higher concentration (3 mg/L) led to larval growth retardation. Based on this they anticipated that down regulation of the *CYP6AE14* gene could cause gossypol toxicity on larvae due to excessive accumulation of gossypol. The *CYP6AE14* expression was high in midgut and was low in Malpighian tubules, transgenic plants imparted susceptibility to cotton bollworm to gossypol. After validating the *CYP6AE14* target gene as a potential target in silencing cotton bollworm in model plants *A.thaliana* and *N.tobacum*. Mao, et al. [33] extended their work to express dsRNA of *CYP6AE14* in cotton plants. Their work significantly proved that the expression of dsRNA of *CYP6AE14* in cotton plants showed resistance to bollworm. In their experimental proof of principle the ds*CYP6AE14* ingestion of the transgenic leaves arrested the growth of the second instar larvae, the larval weight loss was two folds compared to control larvae after injection of the transgenic leaves and control leaves respectively and also higher gossypol accumulation was recorded in the larvae fed on transgenic leaf. The down regulation of the *CYP6AE14* gene in the midgut of bollworm caused gossypol toxification, which resulted in growth retardation and reduced activities.

Mao, et al. [34] further extended their studies and developed a new cotton plant harboring both membrane permeability enhancer gene *35S:GhCP1* and Cytochrome 450 monoxygenease down regulating genes (*35S:dsCYP6AE14)*. *GhCP1* is the serine protease of the cotton plant and play an important role in plant-insect interactions. The entry of *GhCP1* increases easier food absorption in the insect midgut. This newly developed cotton plant showed more tolerance over earlier developed RNAi cotton plant (*dsCYP6AE14*). The cotton plant developed with cysteine protease *GhCP1* allowed the entry of larger food molecules into the midgut of *H. armigera*, as a result more accumulation of gossypol had occurred in the midgut which retarded the growth due to the arrest of Cytochrome 450 monoxygenease gene (*35S:dsCYP6AE14*), also further enhance the susceptibility to *H. armigera* to virus *DpCPV.* The result of their experiment proves the potential of RNAi in bollworm management and urges the scientific community to develop double constructs in the efficient utilization of if RNAi in the pest management using different targets.

To elicit RNAi mediated protection to tobacco plants against *H. armigera*, Zhu, et al. [35] used 20-Hydroxyecdysone gene (*Ha*EcR), EcR is a steroid hormone required for the growth and development, particularly for insect molting and metamorphosis, the initiation of the metamorphosis occurs by 20-Hydroxyecdysone *via* the 20E-EcR-USP receptor complex and triggers primary and secondary transcriptional genes for molting and metamorphosis. Repression of the 20-hydroxyecdysone caused deformities in the molting and metamorphosis and lead to death, the experimental group first screened in *H. armigera, Bombax mori*, and in *Spodaptera exigua* by bacterially expressing the *EcR*, *USP* (Ultraspiracle) in HT115-cells contained L4440 and orally delivered *dsRNA-USP* (*S. exigua and B.mori*) [36,37] they found the successful RNAi response and observed increased rate above said deformities. Latter *EcR* was transformed into the tobacco plants. The tobacco plants expressing *dsEcR* gene showed improved resistance to Lepidopteran *insect H. armiger* and *S. exigua*, where the *EcR* sequence homology was high among them. The transgenic plants expressing *dsEcR* leaves on ingestion larvae showed a decreased transcript level after 2 and 4 days, the decrease in the mRNA level interrupted and affected larval molting, pupation and adult emergence.

In another study Zha, et al. [38] demonstrated efficient knockdown of the Hemipteran member *Nilaparvata lugens* by expressing the midgut genes, hexose transporter, trypsin like serine protease and carboxypeptidase. Their experiment successfully demonstrated the utility of RNAi in the control of phloem sap sucking insect *N. lugens* and conveyed the usage of such genes in the control of other sucking pests *viz.,* aphids, whiteflies, plant hoppers and plant bugs. Where, the effect of *Bt* toxin on these sap sucking pests is limited [39]. *N.lugens* is one of the major sap-sucking pests of rice crop, it sucks the sap from the phloem through its mouth stylet and affects the crop growth by reducing the vigor and tiller number/grains and nutritional quality. In this regard, Zha, et al. [38] first identified elements of the RNAi core machinery *Nlsid-1* and *Nlaub* gene, where Nlsid-*1* is needed for the dsRNA uptake and RNAi spread between the tissues. [40,41] where as *Nlaub* is argonate protein required for

the binding of the small RNAs and for the breakdown of the mRNA. The above midgut genes were cloned and transformed into rice plants. All the target genes of dsRNA were produced and were partially processed into siRNA in the transgenic plants, on ingesting *N lugens* nymph showed depletion in the transcript, highest level of gene silencing was recorded for *Nltry* (61%) and for *NlHT1* (59.3%) and moderate depletion was observed in trypsine like serine protease (41%). Although gene down regulation occurred in the insect, but no mortality was observed.

By using virus based production of dsRNA in plants, a new type of approach was developed for RNAi in the pest management by *M. sexta* [30]. They named it as 'plant virus based dsRNA producing system' (VDPS). Originally described and used against roundworms in the presentation of the transient RNAi response in tobacco plants using tobacco rattle virus [42,43]. To target *M. sexta* they used VDPS approach. They selected nicotine detoxifying *cyp* genes which were up regulated during nicotine detoxification, particularly, *CYP4B46, CYP4M1 and CYP4M3*. They selected above three *cyp* targets based on the sequence homology where *CYP4B46* had 85% to *CYP4B45*, the other two *CYP4M1* and *CYP4M3* had 53% similarity. They transformed the entire fragment individually into tobacco plants using VDPS method and *CYP4B46* fragment alone transformed into the plants using *Agrobacterium* mediated transformation method. Interestingly the *Agrobacterium* mediated gene transformation of *CYP4B46* did not have any effect on larval mass gain except transcript repression, similarly they had the same effect in the VDPS agro-infiltrated tobacco leaves with *CYP4B46, CYP4M1* and *CYP4M3* but VDPS-*CYP4M3* ingested larvae had comparatively low mass to the other two fragments.

In another study, successful control of *H. armigera* was achieved by knock-down of the Hormonal regulating transcription factor *HR*-3 [44], studied the effect of dsRNA on the larvae by providing different fragment of *dsRNAHaHR*-3 in the artificial diet, this bacterially expressed dsRNA could cause significant mortality in the *in vitro* studies, It is evidenced that within 3-7 days all the four types of dsRNA synthesized *in vitro* successfully silenced the gene, among the fragment *HaHR*-3-1 and *HaHR*-3-3 were dominant in silencing effect than *HaHR*-3-2 and *HaHR*-3-4, the repression of this protein negatively affected the development of the *H. armigera* which caused the incomplete larval molt and no shedding of the cuticle had occurred, apart from this dsRNA ingested larvae lost its weight. The fragment *HaHR*-3-1 was resulted as most effective target, employing this fragment a transgenic tobacco plants was engineered to produce *HaHR*-3-3 dsRNA. Tobacco leaf ingested larvae decreased the transcript level and within seven days larvae were dead with developmental deformities. Overall, these results either by transient or stable mode of gene transformation demonstrate that transgenic plants expressing dsRNA do provide protection against insect pests. The gene silencing technique may be a potential strategy for field insect pest control, but suitable insect target genes must be strictly selected when designing dsRNA transgenic plants [38].

Table 1- List of Plant mediated RNA interference transgenic plants

Target Insect	Insect order	Target Gene for Gene silencing	Fragment size used	Intron used for vector construct	Binary vector type	RNAi Transgenic Plant	Effect of PM-RNAi on Insect	References
Helicoverpa armigera		*Cytochrome 450 monoxygenease CYP6AE14*	448 BP	*Arabidopsis RTM gene*	pBI121	*Arabidopsis thaliana,*	Enhanced gossypol toxicity in larvae, decreased weight & size of the larvae.	Mao, et al [28]
Helicoverpa armigera		*GST*- Glutathione - s-transferase	448 BP	*Arabidopsis RTM gene*	pBI121	Tobacco	Depletion in the transcript level	Mao, et al [28]
Helicoverpa armigera		*Cytochrome 450 monoxygenease CYP6AE14*	448 BP	*Arabidopsis RTM gene*	pBI121	Cotton	Larval growth retarded, insect displayed reduced feeding	Mao, et al [33]
Western root corn worm	Lepidoptera	*VATPase -A*	246 BP	-	pMON94805	Maize	Reduced feeding, stunted larval growth.	Baum, et al [26]
Helicoverpa armigera, Spodeptera exigua		20-Hydroxyecdysone	482 BP	*Arabidopsis RTM gene*	pBI121	Tobacco	Partial lethality to H. armigera and S. exigua, affected larval moulting, pupation and adult emergence.	Zhu, et al [35]
Manduca sexta		*MsCYP6B46*	312 BP	-	pSOL8	Tobacco	Depletion in the transcript level	Kumar, et al [30]
Helicoverpa armigera		*HaHR-3*	>300 BP	-	pCAMBIA2300	Tobacco	Incomplete moulting, Loss of weight	Xiong, et al [44]
Meloidogyne incognita	Nematodes	*Rpn7*	408 BP	*CHS- a intron*	p3301 and pSAT6	Soybean	Reduced motility and infestation of host	Niu, et al [49]
Nilaparvata lugens	Hemiptera	Hexose transporter gene, Carboxypepti dase, Trypsin like serine protease	<600 BP	-	pKANNIBAL	Rice	Depletion in the transcript level	Zha, et al [38]
Myzus persicae		*Rack 1*	309 bp	-	pJawohl8-RNAi	*Arabidopsis thaliana,*	Adversely affected survival rate and decreased fecundity	Pitino, et al [29]
		MpC002	710 bp	-	pJawohl8-RNAi			

Efficacy of PM-RNAi

The efficacy of the RNAi again depends on increasing the availability of longer dsRNA of insects [28,30]. The plant expressed dsRNA targeted insect genes in the plants were partially processed into small RNA in the plants before it is being available to the insects. Two groups have shown the efficiency of gene silencing in insects by increasing the duration of the ingestible dsRNA by silencing the plant DICERs [28,30].

Four types of plant DICERs have been characterized in *Arabidopsis* and all of the DICERS have overlapping functions. *dcl*1 generates siRNA and miRNA by cleaving long dsRNA and other three involved in the siRNA production. To test the availability of long dsRNA to the insect from the plant factory, Mao, et al. [28] employed *Arabidopsis* triple *dcl* deficit plant (i.e. *dcl*2, *dcl*3 and *dcl*4) and

transformed the *dsCYPAE14* gene. The transformed plant accumulated longer *AtdsCYPAE14* transcript and depleted siRNA transcript in the mutant plant as evidenced in northern blotting, further the leaf ingested *H. armigera* larvae showed much depletion the *CYPAE14* as compared to normal *AtdsCYPAE14* plant. Further Kumar, et al. [30] also examined the similar role of triple *dcl* mutant in maximization of the silencing efficiency in insect by delivering the long intact dsRNA. In their transient expression, the group got 16 combinations of four *Ntdcl* constructs and agro-infiltrated with VIGS construct to the *NtdsCYP6B46* plant, they could establish that two *Ntdcl* combinations, i.e. *Ntdcl 1, 3* and *4* and *Ntdcl 2, 3* and *4* silenced plants doubled the accumulation of intact *dsCYP6B46* and also the *dsCYP6B46* ingested larvae reduced the *CYP6B46* transcript.

Future Prospects for PM-RNAi

Although much advancement has been made in the area of RNAi over the past few years, its potential is not fully utilized in the crop improvement for trait of interest. The complexities of RNAi pathway, the molecular machineries, and how it links up to plant development are yet to be clearly elucidated. Employing RNAi, it would be possible to target multiple genes for silencing using a thoroughly designed single transformation constructs. In the future, RNAi can be applied in the management of several insects pest, although it is a prerequisite to determine the chronological succession of high-specificity target genes. It is also essential to a firm the safety of transgenic crops to other non-target organisms including human beings before utilization.

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