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Mini Review

RNA interference: Applications and advances in insect toxicology and insect pest management

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ABSTRACT

Since its discovery, RNA interference (RNAi) has revolutionized functional genomic studies due to its sequence-specific nature of post-transcriptional gene silencing. In this paper, we provide a comprehensive review of the recent literature and summarize the current knowledge and advances in the applications of RNAi technologies in the field of insect toxicology and insect pest management. Many recent studies have focused on identification and validation of the genes encoding insecticide target proteins, such as acetylcholinesterases, ion channels, *Bacillus thuringiensis* receptors, and other receptors in the nervous system. RNAi technologies have also been widely applied to reveal the role of genes encoding cytochrome P450 monooxygenases, carboxylesterases, and glutathione *S*-transferases in insecticide detoxification and resistance. More recently, studies have focused on understanding the mechanism of insecticide-mediated up-regulation of detoxification genes in insects. As RNAi has already shown great potentials for insect pest management, many recent studies have also focused on host-induced gene silencing, in which several RNAi-based transgenic plants have been developed and tested as proof of concept for insect pest management. These studies indicate that RNAi is a valuable tool to address various fundamental questions in insect toxicology and may soon become an effective strategy for insect pest management.

1. Introduction

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process elicited by double stranded RNA (dsRNA) that occurs widely among plants, animals, and microorganisms [1,2]. Since its discovery in 1998, RNAi has revolutionized functional genomics due to its relatively easy use and its power as a reverse genetic tool [2–6]. RNAi-high throughput screening is being used in fully genome-sequenced organisms to elucidate gene function related to numerous medically and agriculturally relevant topics [7]. In addition, RNAi can be applied to organisms lacking genetic tools as it does not require transformative methods, and it can be performed *in vivo*, allowing for the study of tissue specific and life-stage specific phenotypes associated with gene expression, as well as complex phenotypes that cannot be modeled in cell-based assays [7,8].

Detailed descriptions of the history, universal applications, and mechanisms of RNAi have been reviewed elsewhere [1,7,9,10]. In this review, we focus on recent applications and advances of RNAi in the field of insect toxicology and host-induced gene silencing as it is related to insect pest management. Specifically, our review summarizes the current knowledge and progress in the applications of various RNAi technologies to: 1) identify and validate the genes encoding major insecticide target enzymes or proteins; 2) reveal the role of the genes encoding detoxification enzymes and transporters; 3) elucidate the mechanism of insecticide-induced up-regulation of detoxification genes; and 4) develop RNAi-based transgenic plants as proof of concept for insect pest management [11].

2. Applications in insect toxicology

2.1. Identification or validation of insecticide target genes

RNAi technology has been widely applied for identification or validation of the genes encoding insecticide target proteins (Table 1). Many recent studies have focused on acetylcholinesterase (AChE, EC 3.1.1.7), an essential enzyme at the cholinergic synapses and neuromuscular junctions of most invertebrates and vertebrates [24], and the target of organophosphate (OP) and carbamate (CB) insecticides. Reduced sensitivity of AChE has also been reported as one of the major insecticide resistance mechanisms against OPs and CBs. Two different *ace* genes (*ace1* encoding AChE1, *ace2* encoding AChE2)

Abbreviations: AChE, acetylcholinesterase; APN, aminopeptidase N; *APP*, aminopeptidase P-like gene; Cad, cadherin; CarE, carboxylesterase; CB, carbamate; CPR, NADPH-dependent cytochrome P450 reductase; dsRNA, double-stranded RNA; GABA-R, γ-aminobutyric acid receptor; GST, glutathione *S*-transferase; HIGS, host-induced gene silencing; nAChR, nicotinic acetylcholine receptor; OP, organophosphate; RNAi, RNA interference; RyR, ryanodine receptor.

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Table 1
Summary of studies to identify or validate insecticide target genes by RNAi.

Insecticide target	Insect	Suppression of transcript (%)	Insecticide treatment	Remarks	Reference
AChE 1 & 2	*Plutella xylostella*	7–34	NT*	AChE1 has major effects on larval growth.	[12]
AChE 1 & 2	*Helicoverpa armigera*	NA[a]	NT	siRNA is designed at a conserved region of the two AChE genes. High mortality, growth inhibition.	[13]
AChE 1 & 2	*Tribolium castaneum*	92–95	Carbaryl, carbofuran, dichlorvos, malathion	AChE1 is a major target of OPs and CBs. AChE2 plays non-cholinergic role.	[14]
AChE 1 & 2	*Blattella germanica*	95–97	Chlorpyrifos, lambda-cyalothrin	AChE1 is the predominant enzyme and major target of OPs.	[15]
AChE 1 & 2	*Chilo suppressalis*	50–70	NT	AChE1 has major effects on larval growth.	[16]
nAChR-α6	*Tribolium castaneum*	Approx. 50	Spinosad	Reduced expression of nAChR-α6 by RNAi is insufficient to alter spinosad susceptibility.	[17]
nAChR-α6	*Drosophila melanogaster*	25–44	Spinosad	Reduced expression of nAChR-α6 by RNAi is insufficient to alter spinosad susceptibility.	[17]
$GABA_A$-R	*Drosophila melanogaster*	50	NT	GABAA receptors negatively modulate olfactory associative learning.	[18]
RyR 1 & 2	*Leptinotarsa decemlineata*	35–55	Chlorantraniliprole	RyR is a target of chlorantraniliprole.	[19]
RyR 1 & 2	*Sogatella furcifera*	78–82	Chlorantraniliprole	RyR is a target of chlorantraniliprole.	[20]
APP	*Ostrinia nubilalis*	38	Cry1Ab	APP is associated with *Bt* resistance.	[21]
APN	*Spodoptera litura*	95	Cry1C	APN is a receptor of Cry1C.	[22]
Cad	*Spodoptera exigua*	Approx. 80	Cry1Ca	Cad is a receptor of Cry1Ca.	[23]

* Insects were not treated with insecticides.
[a] Information not available.

have been characterized in various insect species [25–31]. AChE1 is proposed to be the major catalytic enzyme and target of OPs and CBs, because AChE1 generally has higher expression levels and shows higher frequencies of point mutations associated with insecticide resistance than those of AChE2 [25,26,29,30,32–36].

Lu et al. [14] successfully suppressed *ace1* and *ace2* transcripts by injection of corresponding dsRNA in *Tribolium castaneum*. They observed 100% mortality after adult eclosion when larvae were treated with *ace1* dsRNA; however, the injection of *ace2* dsRNA did not lead to a significant mortality. The effects of exposure to OPs and CBs were then investigated in larvae injected with *ace1* or *ace2* dsRNA. Larval susceptibility to each of the four insecticides increased significantly when *ace1* transcript level was suppressed by RNAi but did not differ significantly from the control when the *ace2* transcript level was suppressed. If an *ace* gene encodes the AChE targeted by these insecticides, suppression of its transcript level by RNAi followed by insecticide exposures would be expected to increase the insect susceptibility to the insecticides. Thus, these results indicate that AChE is a major target of OP and CB insecticides. Although the RNAi of *ace* gene acts at the transcriptional level whereas the inhibition of AChE by insecticides acts at the enzymatic level, both ultimately reduce the amount of AChE for insects to function normally. Thus, RNAi of an *ace* gene encoding AChE targeted by these insecticides followed by exposures of the insects to the insecticides is expected to work additively to reduce the amount of AChE, leading to increased insect mortalities (Fig. 1A).

In *Blattella germanica*, 65–75% of total AChE activity was reduced following *ace1* dsRNA treatment. Moreover, a significant increase in susceptibility to chlorpyrifos was observed after *ace1* dsRNA injection [15]. These findings strongly support that AChE1 plays a major role in cholinergic functions, and is the major target of anticholinesterase insecticides. However, more recent studies revealed that some insects express AChE2 as the major catalytic enzyme, rather than AChE1 [28,37,38]. Although the ability of AChE2 to function as a major catalytic enzyme does not necessarily indicate either its major function in cholinergic neurotransmission or as a major target for OP and CB insecticides, it would be interesting to investigate if AChE2 is a major enzyme responsible for cholinergic neurotransmission and serves as a major target for these insecticides by RNAi.

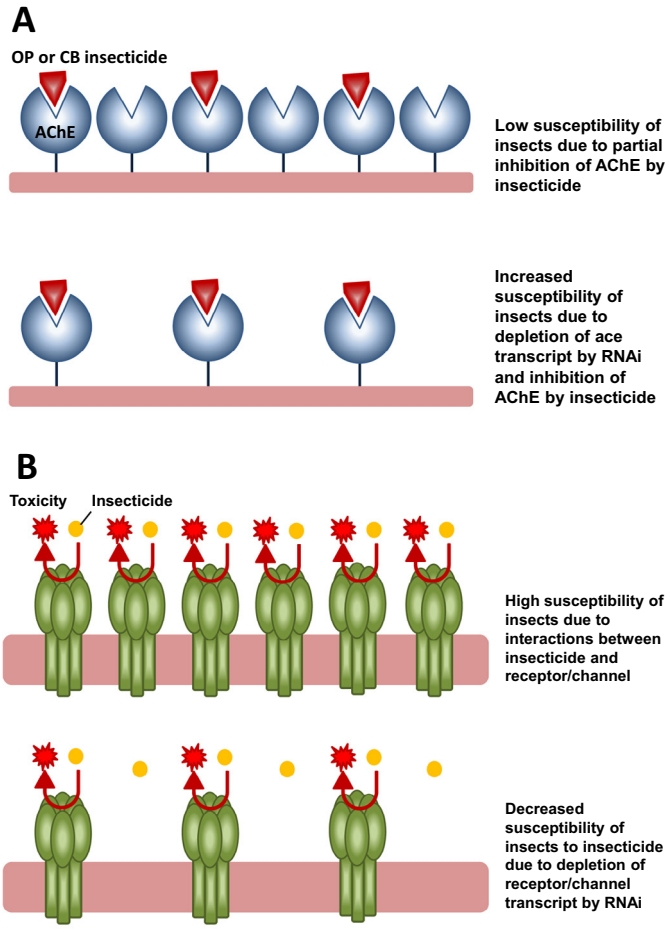

Fig. 1. Schematic illustrations of (A) increased insect susceptibility to an organophosphate or carbamate insecticide as a result of additive-like effects of the RNAi-mediated depletion of the *ace* transcript and the insecticide-mediated inhibition of AChE encoded by the same *ace* gene, and (B) decreased insect susceptibility to an insecticide as a result of the RNAi-mediated depletion of the transcript encoding a receptor or ion channel protein that serves as a target of an insecticide.

Some studies have demonstrated RNAi-mediated silencing of *ace* genes in *Chilo suppressalis* [16], *Plutella xylostella* [12], *Helicoverpa armigera* [13] and *Bombyx mori* ovary cell lines [38]. Although changes of the insecticide susceptibility in these insects were not evaluated after RNAi, these studies suggest that RNAi can be used to determine the target gene for OP and CB insecticides in a wide range of insect species possessing both *ace1* and *ace2* genes.

Other popular applications of RNAi are to elucidate or verify neurotransmitter- and voltage-gated ion channels that mediate fast excitatory or inhibitory synaptic transmission in the insect nervous system as insecticide targets. For example, nicotinic acetylcholine receptors (nAChRs) mediate fast actions by becoming transiently permeable to cations following the binding of acetylcholine. L-glutamate and γ-aminobutyric acid (GABA) are also fast-acting chemical neurotransmitters in insects. L-glutamate channels mediate excitatory neurotransmission at insect neuromuscular junctions, whereas GABA is an inhibitory neurotransmitter in both the insect nervous system and neuromuscular junctions. In contrast to neurotransmitter-gated ion channels, voltage-gated sodium channels open transiently for a few milliseconds in response to membrane depolarization (activation), then the channel pore is occluded [39]. As insecticide targets, nAChRs are attacked by many different insecticides including cartap, imidacloprid, and spinosad; GABA and L-glutamate receptors are attacked by fipronil and avermectins; and sodium channels are targeted by pyrethroids, dihydropyrazoles, and oxadiazines [39].

RNAi has been used to identify or confirm genes encoding receptor proteins and ion channels that are insecticide targets. If a receptor or ion channel protein serves as a target of an insecticide, silencing its gene by RNAi would be likely to reduce the susceptibility of insects when they are exposed to the insecticide. This is because RNAi-mediated suppression of the target transcript can presumably reduce the density of the receptor or ion channel protein. The reduced density of the insecticide target protein will diminish the insecticidal effect, leading to reduced toxicity of the insecticide to the test insects, which should be interpreted as reduced susceptibility of the insects to the insecticide (Fig. 1B).

The Dα6 gene encoding a nAChR subunit has been suggested as a major target site of spinosad because a null mutation of this gene was found to be associated with approximately 1200-fold resistance to spinosad in *Drosophila* [40]. Rinkevich and Scott [17] were able to successfully suppress the transcript level of Dα6 by 60–75% using RNAi; however, the suppression of the Dα6 transcript level did not change the susceptibility of either *Drosophila* or *T. castaneum* to spinosad. The authors suggested that lack of changes in spinosad susceptibility could be due to incomplete silencing of the Dα6 gene, high stability of Dα6 protein synthesized prior to RNAi, difficulties in dsRNA uptake in the nervous system, or other biological factors associated with the insects [41]. In the case of GABA-R, although no study has attempted to validate it as an insecticide target site, physiological function of the receptor was confirmed by RNAi. Liu et al. [18] showed that suppression of the $GABA_A$-R transcript level by RNAi enhanced memory acquisition and calcium responses, which confirms that $GABA_A$-R negatively modulates olfactory associative learning by gating the input of olfactory information into the mushroom bodies of *Drosophila*.

In addition to the receptors and ion channels in the insect nervous system, ryanodine receptors (RyRs) have recently been proposed to be a target site of a novel class of insecticides including phthalic acid diamides and anthranilic diamides [41]. RyRs are a distinct class of intracellular calcium release channels that regulate entry of calcium into the cytosol from intracellular organelles. Several studies have shown that RNAi-mediated silencing of RyR genes can lead to decreased susceptibility of *Leptinotarsa decemlineata* [19] and *Sogatella furcifera* [20] to chlorantraniliprole, suggesting that RyRs are target molecules for chlorantraniliprole.

Cry toxins from *Bacillus thuringiensis* (*Bt*) have been extensively used as sprayable formulations and in transgenic plants for insect pest control. Cry toxin receptors have been widely studied to understand the mode of action for *Bt* in various insect species. Khajuria et al. [21] found that feeding *Ostrinia nubilalis* larvae artificial diet containing dsRNA of the aminopeptidase P-like (*APP*) gene reduced the transcript level of APP by 38% and the susceptibility of the insect to Cry1Ab by 25%, suggesting that APP could be a receptor molecule of Cry1Ab. In *Spodoptera litura*, when dsRNA of aminopeptidase N (APN) gene was injected in larvae, both the expression level of the APN gene and the susceptibility of the insect to Cry1C were significantly reduced, suggesting APN as a receptor for Cry1C [22]. In *Spodoptera exigua*, feeding of *Cad1b* dsRNA resulted in an 80% reduction in the expression of the target gene cadherin [23]. Consequently, reduced *Cad1b* transcript level in actively feeding larvae overcame the negative effect on larval survival and growth caused by the intoxication of Cry1Ca. These results strongly suggest that *Cad1b* is a functional receptor of Cry1Ca in *S. exigua*.

2.2. Identifications of genes involved in insecticide detoxification and resistance

Common mechanisms of insecticide resistance in insect populations include increased detoxification and reduced sensitivity of insecticide target sites. Increased detoxifications are commonly caused by increased expression of detoxification genes or increased activity of detoxification enzymes including cytochrome P450 monooxygenases (CYPs), carboxylesterases (CarEs) and glutathione *S*-transferases (GSTs). In contrast, the reduced sensitivity of target-sites is often caused by point mutations of a target gene, which lead to reduced binding affinity of the target proteins to the insecticide molecules [42]. Several recent studies have shown the possibility of using RNAi to elucidate resistance mechanisms due to increased insecticide detoxification in insects (Table 2). In contrast, it is not feasible to elucidate insecticide resistance due to point mutation(s) of the insecticide target genes by RNAi because RNAi affects only the gene transcript level.

The CYP enzymes are heme-containing proteins found in various organisms and are involved in the detoxification of a wide range of xenobiotics, such as insecticides and phytotoxins, as well as in other physiological processes of insects [55]. These enzymes attack functional groups of various insecticides, which often lead to detoxification of insecticides and contribute to insecticide resistance in insect populations. Several CYP genes have been successfully silenced in insects and their roles in insecticide detoxification revealed. In *H. armigera*, expression levels of genes encoding components of the P450 enzyme system, including CYP6B7, NADPH-dependent cytochrome P450 reductase (CPR), and cytochrome b_5 (Cyt-b_5), decreased after injection of their corresponding dsRNAs in a fenvalerate-resistant strain. The dsRNA treated larvae also became more susceptible to the insecticide, suggesting that CYP6B7, CPR, and Cyt-b_5 collaboratively participate in the metabolism of fenvalerate and serve as a resistance mechanism to fenvalerate in *H. armigera* [56]. Similarly, RNAi of CPR [57] and each of four CYPs [58] resulted in reduced resistance in deltamethrin-resistant *Cimex lectularius*. In a deltamethrin-resistant strain of *T. castaneum*, expression of *CYP6BQ9* was 200-fold higher than that of a susceptible stain. After the expression of *CYP6BQ9* was suppressed by RNAi, the resistance level was significantly reduced [59]. The role of the *T. castaneum CYP6BQ9* in deltamethrin resistance was further confirmed through transgenic expression in *Drosophila melanogaster*, and with kinetic studies of its enzyme heterologously expressed in a baculovirus expression system [44].

Another line of research focuses on CarEs, which play a significant role in metabolism of specific hormones, and in detoxification

Table 2
Summary of studies to reveal roles of the genes in insecticide detoxification and resistance by RNAi.

Target gene	Insect	dsRNA delivery method	Suppression of transcript (%)	Insecticide treatment	Remarks	Reference
CYP321E1	*Plutella xylostella*	Injection	13–54	Chlorantraniliprole	Increased susceptibility	[43]
CarEA1 & A2	*Locusta migratoria*	Injection	86–97	Chlorpyrifos	Increased susceptibility	[44]
CYP409A1 & CYP408B1	*Locusta migratoria*	Injection	99	Deltamethrin	Increased susceptibility	[45]
CYP6AE14	*Helicoverpa armigera*	Transgenetic plant	NA*	Gossypol	Increased susceptibility	[46]
CYP6BG1	*Plutella xylostella*	Feeding	44–69	Permethrin	Increased susceptibility	[47]
CarE E4	*Sitobion avenae*	Transgenetic plant	30–60	Phoxim	Increased susceptibility	[48]
CarE	*Aphis gossypii*	Feeding	33	Omethoate	Reduced resistance	[49]
CarE9 & E25	*Locusta migratoria manilensis*	Injection	NA	Malathion	Increased susceptibility	[50]
GSTe1 & m2	*Nilaparvata lugens*	Injection	60–90	Chlorpyrifos	Increased susceptibility	[51]
GSTs5 & u1	*Locusta migratoria*	Injection	NA	Carbaryl, malathion chlorpyrifos	Increased susceptibility	[52]
GSTs3	*Locusta migratoria*	Injection	NA	Carbaryl	Increased susceptibility	[53]
CYP6AA5	*Aedes aegypti*	Injection, feeding	39–78	Cypermethrin	Increased susceptibility	[54]

* Information not available.

of dietary and environmental xenobiotics in insects. Gong et al. [49] reported reduced CarE activity associated with increased susceptibility to OPs after oral delivery of a CarE dsRNA to an OP-resistant strain of *Aphis gossypii* overexpressing CarE. These results demonstrate that the overexpression of CarE plays an important role in OP resistance in this aphid species. Additionally, dsRNA targeting CarE1 and CarE2 led to the increased mortality in *Locusta migratoria* (20.9 and 14.5%, respectively) when chlorpyrifos was applied to this insect [44]. Furthermore, *L. migratoria* nymphs injected with CarE9 and CarE25 dsRNAs followed by malathion exposures increased the mortality from 34 to 65% and 54%, respectively [50]. Similarly, in omethoate-resistant *A. gossypii*, ingestion of dsRNA targeting a CarE led to a 33% reduction of target gene expression, as well as increased susceptibility to omethoate [49]. Moreover, silencing of an aphid CarE (E4) gene with plant-mediated RNAi impaired *Sitobion avenae* tolerance of phoxim [48]. These results indicated significant roles of CarEs as revealed by RNAi in insecticide detoxification and resistance in different insect species.

GSTs belong to one of the three major enzyme systems involved in the detoxification of endogenous and xenobiotic compounds; however, very few studies have used RNAi to reveal their roles in insecticide detoxification and resistance. The primary role of GSTs is to protect cells against cytotoxic or genotoxic compounds, such as reactive oxygen species. In insects, GSTs are also involved in detoxification of various classes of insecticides. In *Nilaparvata lugens*, RNAi experiments were carried out to target two GST genes, *GSTe1* and *GSTm2* [51]. Results showed a maximum of 90% reduction in the transcript levels of the two genes. Subsequent exposures of dsRNA-injected nymphs to chlorpyrifos led to a significant increase in susceptibility to this insecticide, but not to β-cypermethrin. These observations suggest the involvement of *GSTe1* and *GSTm2* in detoxification of chlorpyrifos, but not β-cypermethrin.

Similarly, RNAi experiments in *L. migratoria* nymphs resulted in significant decreases in transcript levels of *GSTs5* and *GSTu1* [52]. Insecticide bioassays showed that dsRNA-injected nymphs become more susceptible to carbaryl, as indicated by increases in mortality by 28 and 12%, respectively, after *GSTs5* and *GSTu1* were silenced. Mortality of nymphs in response to malathion treatment increased from 14 to 40% after *GSTs5* was silenced, whereas mortality of nymphs after chlorpyrifos treatment increased from 39 to 58% when *GSTu1* was silenced. Furthermore, the injection of *GSTs3* dsRNA in *L. migratoria* nymphs resulted in a significant decrease of *GSTs3* transcript and an increased mortality of the insects after exposure to carbaryl. Because the insect colonies used in these studies did not implicate any insecticide resistance, it would not be appropriate to extrapolate the roles of these GST genes in insecticide resistance. Nevertheless, these studies demonstrated the role of these GST genes in the detoxification of OP and CB insecticides [53].

Xu et al. [48] took a different approach entirely and targeted a CarE gene implicated in insecticide tolerance. The authors used transgenic wheat expressing dsRNA to suppress the CarE gene in *S. avenae* and observed retardation of larval growth. Moreover, hydrolysis of phoxim solution with aphid extracts was reduced by half when the aphids were reared on transgenic plants as compared with those on the control plants, suggesting that metabolic detoxification mechanisms against insecticides in insects could be addressed using this strategy.

The ATP-binding cassette (ABC) transporters, which are known to play important roles in translocation of pigment in the eyes, transfer of attractants for germ cell migration, and modulation of molting hormone signaling in tissues, have been implicated in insect resistance to chemical insecticides. Zhu et al. [58] applied RNAi to suppress the expression of an ABC transporter gene in a pyrethroid-resistant bed bug (*C. lectularius*) strain and found an increased susceptibility of the insect to β-cyfluthrin. This study suggests that this ABC transporter gene is likely involved in pyrethroid resistance,

probably through an increased ability to extrude the insecticide out of the insect.

All the studies reviewed in this section clearly indicate that RNAi is a powerful technique to reveal the role of metabolic (i.e., CYPs, GSTs and CarEs) and ABC transporter genes in insecticide detoxification and resistance in insects. However, one of the greatest challenges in using RNAi to reveal the role of these genes in insecticide detoxification and resistance is the design of the dsRNA specific to a target gene. This is particularly true because each of these detoxification enzyme systems and ABC transporter proteins represents large gene families or superfamilies. Within each gene family or superfamily, there are several dozens or over one hundred genes with various levels of sequence similarity. There is a probability that dsRNA designed to suppress a specific gene may unintendedly suppress other closely related gene(s). Consequently, the observed RNAi effect or phenotype may not be solely due to the suppression of the target. Therefore, care must be taken in the design of a dsRNA to avoid off-target gene suppression. The other challenge in revealing the role of these genes in detoxification of insecticides is the versatile nature of these enzymes or proteins. Several enzymes or transporter proteins within each family or superfamily may be able to detoxify the same chemical. Thus, silencing just one gene may not display a significant increase in insect susceptibility to the insecticide, thereby hindering the role of the gene in insecticide detoxification and resistance.

2.3. Reveals of mechanisms in up-regulation of detoxification genes

The biochemical basis of insecticide resistance has been extensively studied in various insects over the last few decades. Efforts toward unraveling the underlying mechanisms of resistance development have provided advancements crucial for insect pest control programs. Among the aforementioned resistance mechanisms, enhanced detoxification is of great importance. In insects, detoxification of insecticides and phytotoxins is often enhanced by both qualitative and quantitative changes in detoxification enzymes. Despite the fact that up-regulation of detoxification genes has been found in many insect species, little is known about the regulatory mechanisms leading to such changes. In mammals, the up-regulation of CYP genes following an exposure to phenobarbital is known to involve the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR); both are members of the nuclear receptor family [60–62]. Upon binding of xenobiotics to the nuclear receptors, the complex migrates to the nucleus where it binds to a specific DNA sequence called xenobiotic response element (XRE) upstream of the CYP gene. However, very few studies have directly examined putative nuclear receptors that may act as regulators to mediate the up-regulation of detoxification genes in insects.

In *Aedes aegypti*, several CYP genes have been found to be up-regulated in adult mosquitoes by pyrethroid insecticides, including permethrin (*CYP6AL1, CYP4J16B*), cypermethrin (*CYP6AL1, CYP9J32, CYP4J16A* and *CYP4J16B*) and deltamethrin (*CYP6AA5, CYP4J16A*, and *CYP4J16B*) [54]. In order to evaluate the possible role of the nuclear receptor gene *HR96* in insecticide-mediated up-regulations of the CYP genes, RNAi was performed to silence the *HR96* gene in adult mosquitoes followed by monitoring the change in expression of CYP genes. Results showed a decrease of the cypermethrin-mediated up-regulation of *CYP4J16B* from approximately 15-fold to 5-fold after *HR96* was silenced by injection of *HR96* dsRNA in *A. aegypti* adults followed by exposure of the mosquitoes to cypermethrin for 24 h. The significant diminishing of the cypermethrin-mediated up-regulation of *CYP4J16B* after RNAi of *HR96* clearly indicates an important role of *HR96* in the up-regulation of *CYP4J16B* in the mosquitoes (Fig. 2). In contrast, the up-regulation of the remaining CYP genes or transcript (*CYP4J16A*) by pyrethroids did not seem to be regulated by *HR96* because silencing the *HR96* gene with RNAi did not diminish the up-regulation of these genes or transcripts.

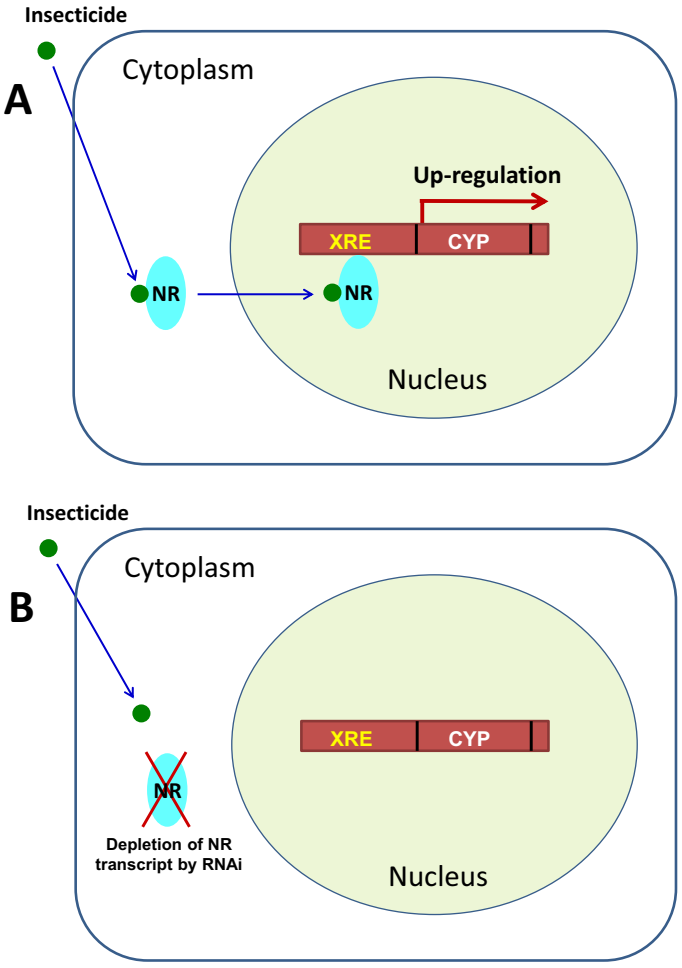

Fig. 2. Schematic illustrations of using RNAi techniques to reveal the possible role of a nuclear receptor (NR) in insecticide-mediated up-regulation of CYP genes. (A) In control, binding of an insecticide molecule to a nuclear receptor (NR) or an NR associated with a nuclear receptor dimeric partner allows the complex to migrate into the nucleus of a cell. The binding of the complex to a xenobioitic response element (XRE) upstream of the CYP gene leads to the transcription of the CYP gene. (B) In RNAi-treated insects, the depletion of the NR transcript abolishes or diminishes the insecticide-mediated up-regulation of the CYP gene.

2.4. Advances of host induced gene silencing for insect pest management

RNAi-mediated crop protection [2] which utilizes host induced gene silencing (HIGS) [11] is considered a potential next generation pest management strategy with benefits over chemical-based pesticides and traditional biological control because the sequence-specific nature of RNAi allows for individual species [63,64] and potentially specific orders of pests to be selectively targeted [2,3]. In addition, insect susceptibility to chemical pesticides and/or phytotoxins can be manipulated so less chemical pesticides are required to achieve lethality [2,47]. Moreover, the possibility of using RNAi to control insect populations through the disruption of oviposition or pheromone receptors also exists [65,66]. Recently, many advances have been made in HIGS technology which utilizes transgenic plants expressing dsRNA targeting a pest species [11]. One day HIGS technology may be commercialized for use in a similar manner to *Bt*-based transgenic crops (Table 3).

Baum et al. [69] were the first to demonstrate proof of concept for HIGS against insect pests with the development of maize plants expressing dsRNA containing a region of the *Diabrotica virgifera virgifera* V-ATPase A transcript. Diet feeding of this construct and

Table 3
Summary of studies to attack insect pests by employing *in planta* RNAi.

Target gene	Insect	Delivery method	Remarks	Reference
Salivary gland gene (*C002*) and intracellular receptor gene (*Rack1*)	*Myzus persicae*	Leaf disks of *Nicotiana benthamiana* and transgenic *Arabidopsis thaliana*	30–60% reduction in target gene expression, progeny reduced, no other phenotypic effects	[67]
Serine protease	*Myzus persicae*	Transgenic *Arabidopsis thaliana*	Significant reduction in target gene expression, 31% decrease in protease activity, progeny reduced, pathogenic population reduced, no other phenotypic effects	[68]
V-ATPase A and *α-tubulin*	*Diabrotica virgifera virgifera, D. undecimpunctata howardii, Leptinotarsa decemlineata*	Feeding and transgenic maize	Significant reduction in target gene expression, damage to host plant reduced	[69]
CYP6AE14 and *GST1*	*Helicoverpa armigera*	Transgenic *Nicotiana tabacum*	Significant reduction in target gene expression, larval weight loss, GST activity in larval midguts reduced	[46]
CYP6AE14	*Helicovverpa armigera*	Transgenic *Gossypium hirstum*	Significant reduction in larval damage, significant reduction in target gene and protein expression, larval growth retardation, increased gossypol accumulation in the gut	[70]
CYP9A14	*Helicovverpa armigera*	Transgenic *Gossypium hirstum*	Gossypol consumption confers resistance to deltamethrin through gut P450 activity, resistance to deltamethrin reduced when *CYP9A14* suppressed, larval growth reduced	[71]
CYP6AE1	*Helicovverpa armigera*	Transgenic *Gossypium hirsutum* and *Arabidopsis thaliana*	Overexpression of cysteine proteases (*GhCP1* and *AtCP2*) disrupts the peritrophic matrix, allows for greater accumulation of gossypol in the larval gut, increased susceptibility to infection by the dsRNA virus *Dendrolimus punctatus*, and enhanced response to dsRNA treatment (increased target gene suppression, larval growth retarded, and plant damage reduced)	[72]
Nuclear receptor complex of 20-hydroxyecdysone (*HaEcR*)	*Helicovverpa armigera* and *Spodoptera exigua*	Transgenic *Nicotiana tabacum*	Significant target gene suppression, molting defects, larval lethality and damage to host plant reduced	[57]
Molt-regulating transcription factor gene (*HR3*)	*Helicovverpa armigera*	Transgenic *Nicotiana tabacum* and *Escherichia coli*	Developmental deformities and larval lethality, more detrimental effects when transgenic bacteria are used	[73]
Dicer genes (*NaDCL1-4*) and midgut *CYP6B46*	*Manduca sexta*	Transgenic *Nicotiana attenuata*	Three fold reduction in larval target gene expression prior to dicer knockdown, no effect on larval mass gain, knockdown *in planta* doubled larval target gene suppression	[63]
Hexose transporter gene (*NlHT1*), carboxypeptidase gene (*car*), and trypsin-like serine protease gene (*try*)	*Nilaparvata lugens*	Transgenic *Oryza sativa*	42.7–59.3% reduction in NlHT1 expression, 42.1–43.3% reduction in Nlcar expression, and 61–73.3% reduction in Nltry, no significant lethal phenotype observed	[74]
Hunchback (*hb*)	*Myzus persicae*	Transgenic *Nicotiana tabacum*	Target gene suppressed 32.4%, no lethal phenotype in adults, inhibited reproduction	[75]
(*CbE E4*)	*Sitobion avenae*	Transgenic *Triticum aesticum*	Target gene suppressed 30–60%, retardation of larval growth, reduced hydrolosys of Phoxim solution *in vitro*, and reduced tolerance of Phoxim solution *in vivo*	[48]

similar constructs was shown to trigger RNAi in *D. v. virgifera*, *Diabrotica undecimpunctata howardii*, and *L. decemlineata*, and resulted in significant larval stunting and mortality. Maize lines with the highest expression of V-ATPase A dsRNA received significantly less *D. v. virgifera* damage in growth chamber bioassays compared to non-transgenic control plants and other transgenic lines with lower levels of dsRNA expression, suggesting that this technique has commercial potential for the control of coleopteran pests, for which *Bt*-based control is limited [69].

Similarly, Mao et al. [46] reduced the tolerance of *H. armigera* larvae, to the phytotoxin gossypol through feeding of genetically modified tobacco leaves expressing dsRNA containing a region of a *H. armigera*-derived CYP gene, which is involved in detoxification. Feeding of third instar larvae on transgenic material prior to feeding on artificial diet containing gossypol resulted in significant down regulation of the target gene and larval weight loss when compared to larvae moved to diet with no additives, diet containing tannic acid, or to cotton leaves with gossypol. This study is an excellent example of the need for appropriate control constructs, as feeding on transgenic tobacco expressing dsGFP prior to transfer to the secondary diets was sufficient to cause developmental arrest despite having no effect on gene expression [46]. Mao et al. [70] went on to create transgenic cotton plants expressing a *H. armigera*-derived CYP gene, and a significant reduction in larval damage compared to control plants was documented, in conjunction with retarded larval growth associated with suppression of the target gene and protein. In addition to participating in phytotoxin detoxification, CYP genes were demonstrated to also be involved in insecticide metabolism [71]. Enhancing the expression of these genes with gossypol conferred increased tolerance of the insect to insecticides. However, feeding of larvae on the transgenic cotton expressing *H. armigera*-derived CYP dsRNA increased larval susceptibility to deltamethrin, suggesting that RNAi based suppression of CYP genes could be used to decrease required dosages of chemical insecticides [71].

Most recently, Mao et al. [72] demonstrated that uptake of cotton-derived and *Arabidopsis*-derived cysteine proteases overexpressed in either tobacco or *Arabidopsis* by *H. armigera* disrupts the peritrophic matrix, allowing for higher accumulation of gossypol, increased susceptibility to infection by the dsRNA virus *Dendrolimus punctatus*, and most importantly, an enhanced response to dsRNA treatment. Transgenic cotton plants expressing both cysteine proteases and dsRNA against the *H. armigera*-derived CYP gene received less

damage from larval *H. armigera* than either transgenic plants expressing the constructs individually or wild type cotton plants.

Kumar et al. [63] used a similar dual target approach to increase the efficiency of midgut gene suppression in *Manduca sexta* larvae. They created tobacco plants transiently expressing dsRNA targeted against larval midgut genes and the plant's dicer genes. The combination resulted in plants that contained higher concentrations of long, undiced *CYP6B46* dsRNA targeted against the insect. These longer constructs increased target gene knockdown in the larvae, suggesting that expression of longer dsRNAs is more effective for gene suppression in insects. Duel-target strategies of this nature have important applications for increasing the effectiveness of HIGS technologies, which is of extreme importance because these RNAi-mediated pest management strategies must be able to reduce pest populations as fast or faster than traditional methods before they will be accepted [2]. In addition, Kumar et al. described a viral vector based method for quick and reliable transient expression of dsRNA *in planta* that can be used for high throughput screening of dsRNA constructs to facilitate target gene selection [63].

Two additional studies by other groups have explored the effects of alternative target genes for control of *H. armigera*. Zhu et al. [57] demonstrated increased tolerance to larval feeding from both *H. armigera* and *S. exigua* with transgenic tobacco plants expressing dsRNA derived from the *H. armigera*–nuclear receptor complex of a steroid hormone involved in molting. In another study, Xiong et al. [73] used hairpin RNA expressed in both *Escherichia coli* and transgenic tobacco plants to decrease mRNA and protein levels of the *H. armigera*-derived molt-regulating transcription factor in larval *H. armigera*, which resulted in developmental deformity and larval lethality. The success of these studies attests to the functionality of the RNAi procedures that have been developed for this system.

Another important line of research is focused on creating HIGS technologies for the control of piercing–sucking insects for which *Bt*-crops are not effective and reliance on chemical pesticides are undesirable due to resistance [76]. Zha et al. [74] published the first attempt in 2011, in which they developed transgenic rice expressing dsRNA targeting three midgut genes in *N. lugens*. Expression levels of the target genes were reduced; however, lethal effects were not observed. The same year, Pitino et al. [67] also published similar results for gene suppression in an aphid species using transgenic tobacco and *Arabidopsis* plants expressing dsRNA for genes expressed in salivary glands and gut tissue of *Myzus persicae*. Suppression of a homologous salivary gene to one of the target genes in *Acyrthosiphon pisum* had previously been shown to be crucial for survival on a host plant but not on synthetic diet [77,78]. The other target gene, an intracellular receptor that binds activated protein kinase C that is primarily involved in signal transduction cascades, had not been previously knocked down in aphids, but had been shown to be an effective target in *Caenorhabditis elegans* [67]. Nonetheless, lethal effects were not observed with HIGS, despite confirmed suppression of the targets by as much as 60%. Progeny numbers, however, were reduced. Then in 2012, Bhatia et al. again achieved similar results when they expressed dsRNA of the *M. persicae* serine protease gene in transgenic *Arabidopsis* [68]. Despite significant reduction in target gene expression and a corresponding decline in gut protease activity, no lethal effects were documented, but fecundity was again reduced.

Recently, Mao and Zhang [75] targeted the gap gene *hunchback* in *M. persicae*, which had previously been shown to be an effective target in *A. pisum* when fed in artificial diet. Feeding of transgenic tobacco expressing dsRNA targeting *hunchback* of *M. persicae*, however, elicited different results. The target gene was suppressed to a lesser degree and no lethal phenotype was observed, but reproduction was successfully reduced. The authors speculate that the lack of adult phenotype was linked to an inadequate dose of dsRNA received from the plant, and that increased expression of dsRNA could enhance the effectiveness. These results are promising and deserve further optimization.

All the studies reviewed in this section indicate great potentials of RNAi-based insect pest management. In addition, several studies also demonstrated that using HIGS for hemipteran control is feasible, but only when appropriate target genes have been identified or a sufficient level of RNAi-mediated silencing of the target gene has been achieved. Unfortunately, achieving lethal effects with HIGS has not proved to be straightforward, even when target genes that have been documented to be lethal in related species are used. Apparently, different levels of RNAi-mediated suppression of a target gene can significantly affect the outcome. Even for the same target gene, a low level of suppression of its transcript in one study or in an insect with low RNAi efficiency may not display a lethal effect, but a high level of suppression in other study or in an insect with robust RNAi response may result in a strong lethal effect [79,80]. Although it has been less well documented, different dsRNA delivery methods for the same target gene may also lead to different outcomes due to possible differences in cellular uptake and degradation of dsRNA, or due to differential expression of genes involved in the RNAi pathway in different tissues of an insect species.

3. Conclusion

Current studies indicate that RNAi is a valuable tool to address various fundamental questions in insect toxicology and has great potentials for insect pest management. Despite these recent advances, many practical, financial, technical, regulatory, and safety concerns still exist for the use of RNAi in pest management strategies. However, thanks to the devotion and enthusiasm of many scientists worldwide, advances are being made to overcome the current limitations of RNAi. Variation in RNAi efficiency at the species, tissue, and gene levels has limited the development of effective RNAi protocols for a number of insect species, especially Lepidoptera [4,81]. Uptake into cells and systemic spread are suspected to be the most limiting factors for RNAi [4,5], and are likely linked to observed differences in the effectiveness of various dsRNA delivery methods and differing phenotypic effects of individual target gene suppression. Much research is currently focusing on uncovering the molecular and physiological mechanisms governing cellular uptake and spread of dsRNA, as well as function of the RNAi machinery [2–5,82–84]. Novel methods for target gene selection [2,85,86], improved dsRNA construct designs [4,8], transfection reagents [4,5,64], and stability-enhancing technologies such as chitosan/dsRNA nanoparticles [64,79] are being used to enhance RNAi efficiency in many species. Together, these efforts and numerous other toxicology studies utilizing RNAi, many of which are described in this review, are advancing the development of new RNAi-based tools for better management of insect pests.

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