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Chapter 18

Management of Insect Pest by RNAi — A New Tool for Crop Protection

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Abstract

The fast-growing human population requires the development of new agricultural technologies to meet consumers′ demand, while minimizing environmental impacts. Insect pests are one of the main causes for losses in agriculture production, and current control technologies based on pesticide application or the use of transgenic crops expressing *Bacillus thuringiensis* toxin proteins are facing efficacy challenges. Novel approaches to control pests are urgently necessary. RNA interference (RNAi) is a gene silencing mechanism triggered by providing double-stranded RNA (dsRNA), that when ingested into insects can lead to death or affect the viability of the target pest. Transgenic plants expressing dsRNA version of insect specific target genes are the new generation of resistant plants. However, the RNAi mechanism is not conserved among insect orders, and its elucidation is the key to develop commercial RNAi crops. In this chapter, we review the core RNAi pathway in insects and the dsRNA uptake, amplification, and spread of systemic silencing signals in some key insect species. We also highlight some of the experimental steps before developing an insect-pest-resistant "RNAi plant". Lastly, we review some of the most recent development studies to control agricultural insect pests by RNAi transgenic plants.

Keywords: Biotechnology, dsRNA, Entomology, Gene silencing, Insect control, RNA interference

1. Introduction

Agriculture has to continually adapt to rising environmental concerns in conjunction with meeting the increasing consumers′ demand. The fast-growing human population creates the need for the sustainable intensification of agriculture throughout the world which can be acomplished by adopting mechanization and new technologies to close yield gaps while

minimizing environmental impacts. In the past few decades, insect pest control has been mainly conducted by the application of chemical pesticides because of the low cost and efficacy; but their indiscriminate use has caused escalating problems with the evolution of insect resistance to the pesticides together with secondary pest outbreaks. The development of new biotechnological approaches, with the introduction of transgenic crops expressing *Bacillus thuringiensis* (Bt) Cry toxin proteins, also known as insect-resistant transgenic Bt-plants, decreased pesticide utilization in certain key crops, such as cotton and maize, and brought economical and environmental benefits [1-3]. But once again, insect resistance has arisen, now against the Bt toxins, and outbreaks of nontarget pests have emerged [1, 4], which makes necessary the development of novel approaches to control selected agriculture pests.

RNA interference (RNAi) is a gene silencing mechanism at the cellular level triggered by double-stranded RNA (dsRNA) and is likely to be the new approach underlying the next generation of insect-resistant transgenic plants. In some studies, successful delivery of dsRNA molecules to insects by ingestion resulted in the expected essential gene target silencing [5, 6], which led to death or affected the viability of the target insect, resulting in control of the pest.

In general, long dsRNAs are processed by species-specific RNase-III-like enzymes, resulting in smaller double-stranded molecules. These shorter RNAs are loaded into RNA complexes as a guide for finding target mRNAs that are either cleaved or blocked for translation in posttranscriptional silencing, or inducing histone modifications when involved in transcriptional silencing response [7, 8]. However, the RNAi systemic spreading mechanism is not conserved across organisms, and its elucidation is an essential step in developing an efficient method to control agricultural pests by RNAi technology.

In this chapter, we review how the RNAi mechanism occurs in insects, highlighting the core RNAi pathway and components, and new developments regarding dsRNA uptake, amplification, and the spread of systemic silencing signals in some key insect species. We also discuss the critical experimental steps before developing an "RNAi plant" protected against a specific insect pest, with consideration to application. Lastly, we review some of the most recent published studies to control agriculturally important insect pests based on RNAi transgenic plants.

2. RNAi mechanism

RNAi is an important and natural antiviral defense mechanism, protecting organisms from RNA viruses, or even avoiding the random integration of transposons [9]. Over time, with the discovery of some aspects of the mechanism, RNAi has become a widely used tool to knock down and analyze the function of genes. Most of the RNAi pathways have dsRNA as the precursor triggering molecule that vary in length and origin [7, 8]. In addition, the RNAi pathways differ not only in the RNA precursor molecule, but also in genes, enzymes, and effector complexes involved throughout the process. However, some key steps are conserved. Briefly, RNA duplexes are processed into short RNA duplexes, which are then used to guide the recognition of their target, either to cleave a complementary mRNA, or to repress their

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target translation at a posttranscriptional silencing level, or to modify the chromatin structure at the transcriptional level [7, 8].

The RNA precursor molecules from the RNAi pathways, all of which are already identified in insects, are small RNAs, categorized into three classes [8, 10]: the first two classes are small interfering RNAs (siRNAs; 20–25 nucleotides) and microRNAs (miRNAs; 21–24 nucleotides) [7] (Figure 1). Both miRNAs and siRNAs share a common RNase-III processing enzyme, Dicer, and closely related effector complexes and both can regulate gene expression at the posttranscriptional level [8, 10, 11]. Conversely, the third class of small RNAs, the PIWI-interacting RNAs (piRNAs; 24–30 nucleotides), are generated independent of the Dicer activity [12]. piRNAs have been reported to play an essential role in germ-line development, stem cell renewal, transposon silencing, and epigenetic regulation [13-16]. These piRNAs originate from a diversity of sequences, including repetitive DNA and transposons, and they seem to act both at the posttranscriptional and chromatin levels [10]. The mechanism that generates and amplifies piRNAs is not well-understood, but involves slicer activities (Argonaute proteins associated with cleaving activity) [8, 10, 17]. Considered a specialized subclass of piRNAs, **r**epeat-**a**ssociated **si**RNAs (rasiRNAs; 25-29 nucleotides) were identified in the *Drosophila* genome [18], and suggested by Meister and Tuschl [7] to be involved in guiding chromatin modification in this insect species (Figure 1).

The most recognized RNAi pathways are the siRNA and miRNA; despite being triggered by different molecules, both precursors are long double-stranded RNAs (dsRNAs). Naturally in a cell, long dsRNAs can derive from RNA virus replication, from the transcription of convergent cellular genes or mobile genetic elements, or from self-annealing cellular transcripts [8]. In the siRNA pathway, these long dsRNA are processed by Dicer into siRNA duplexes. By contrast, in the miRNA pathway, miRNAs are generated from endogenous transcripts (primary miRNAs; pri-miRNAs) that form stem–loop structures [19, 20]. In the nucleus, these hairpin regions are recognized and cleaved into precursor mirRNAs (pre-miRNAs) by Drosha, another RNase-III-family enzyme, and the pre-miRNAs are transported to the cytoplasm through the nuclear export receptor Exportin-5 (Expo-5) [19, 20]. Subsequently, the pre-miRNA undergoes another endonucleolytic cleavage, now catalyzed by Dicer, generating an miRNA duplex [19, 20] (Figure 1).

The siRNA and miRNAs duplex containing ribonucleoprotein particles (RNPs) are subsequently rearranged into effector complexes. Although it is difficult to assign distinct functional labels, an siRNA-containing effector complex is referred to as an "RNA-induced silencing complex" (RISC), and an miRNA-containing effector complex is referred to as an miRNP [7]. In these complexes, the regulation is at a posttranscriptional level and every RISC or miRNP contains a member of the Argonaute (Ago) protein family [7]. For the regulation at the transcriptional level as guided by rasiRNAs, a specialized nuclear Argonaute-containing complex, known as the RNA-Induced Transcriptional Silencing complex (RITS) mediates gene silencing [10]. In general, one strand of the short-RNA duplex (the guide strand) is loaded onto an Argonaute protein at the core of the effector complexes. During loading, the nonguide strand is cleaved by an Argonaute protein and ejected. The Argonaute protein then uses the guide RNA to associate with target RNAs that contain a perfectly complementary sequence

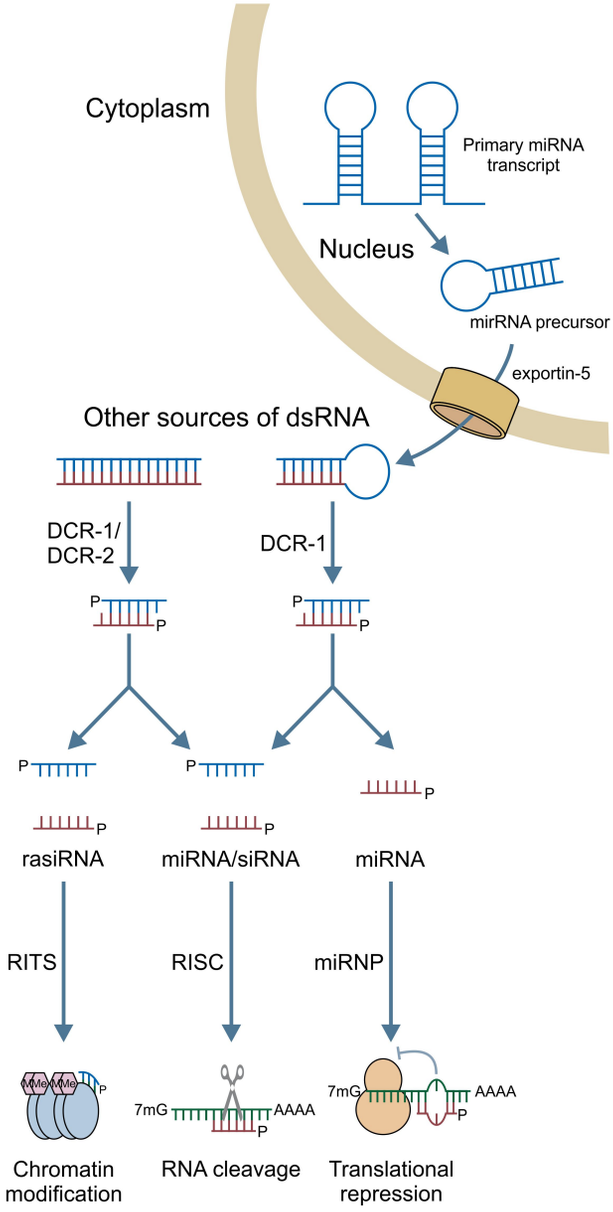

Figure 1. RNA-mediated gene silencing pathways. In the nucleus, primary miRNA transcripts (pri-miRNAs) are processed to mirRNA precursors (pre-miRNA) by the RNase-III-like enzyme Drosha. The pre-miRNA is exported through the export receptor Exportin-5 (Exp-5) to the cytoplasm, and then processed by Dicer to microRNA (miRNA). These miRNAs are unwound and assembled into miRNP (miRNA containing effector complex of RiboNucleo Protein particles) or RISC (RNA Induced Silencing Complex), triggering silencing responses by translational repression of target mRNAs or mRNA-target degradation, respectively. In the cytoplasm, long dsRNA are processed to siRNA (small interfering RNA) by the RNase-III-like enzyme Dicer. The short dsRNAs are unwound and assembled into RISC or RITS (RNA-Induced Transcriptional Silencing) complexes. The siRNA guides the RNA cleavage by the RISC complex, while rasiRNA (repeat-associated short interfering RNA) guides the condensation of heterochromatin by the RITS complex. 7mG: 7-methyl guanine; AAAA: poly-adenosine tail; Me: methyl group; P: 5′- phosphate. (Adapted from [7])

and then catalyzes the slicing of these targets, either to be cleaved by RISC, to be blocked for translation in miRNP or by inducing histone modifications in RITS [7] (Figure 1). The mechanism of miRNA-guided translational regulation is not as well-understood in the case of target-

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RNA cleavage, and to make things more complicated, miRNAs can act as siRNAs, and siRNAs can act as miRNAs [7].

Dicer is one of the enzymes involved in RNAi mechanism that is encoded by a variable number of genes and presents distinct specificity among organisms [21]. For instance, mammals and *Caenorhabditis elegans*, the best characterized animal for RNAi, have each a single Dicer responsible for functions in both siRNA and miRNA pathways [12], while *Drosophila melanogaster* has two paralogues: Dicer-1 (Dcr-1) that preferentially processes miRNA precursors, and Dicer-2 (Dcr-2) required to process long dsRNA into siRNAs [22]. However, in *Tribolium castaneum*, a model organism among insects for systemic silencing by RNAi, Dcr-2 showed an important role at the RNAi pathway, whereas Dcr-1 is suggested to have an involvement in wing development, most likely through the miRNA pathway [23].

Another gene family involved in RNAi pathways is the Argonaute proteins (Ago). Ago is a central protein component of silencing complexes (RISC, RITS, miRNP) that acts in mediating target recognition and silencing [24]. Argonaute proteins contain two domains: a PAZ domain involved in dsRNA binding, and a PIWI-domain responsible for RNase activity [23]. In *Drosophila*, Ago-1 is involved in the miRNA pathway; Ago-2 in the siRNA pathway; while Piwi, Aubergine (Aub), and Ago-3 are associated in transcriptional silencing [13, 25-27]. In *Tribolium*, a single class of Argonaute was identified (Tc-Ago-1) in the miRNA pathway, while two classes of Ago-2 paralogues (Tc-Ago-2a and Tc-Ago-2b) were found in the siRNA pathway, probably deriving from gene duplication in the beetle lineage [23]. This duplicated Tc-Ago-2 might lead to higher amounts of Ago-2 protein, potentially with the enhancement of the RNAi response [23]. In the silkmoth *Bombyx mori*, a Lepidoptera species in which the RNAi response is considered much less robust [28, 29], *AGO* genes from all three main RNAi pathways were identified (*BmAGO-1* – miRNA; *BmAGO-2* – siRNA; *BmAGO-3* – piRNA), which were shown to be involved in the RNAi response in Bm5 cells [30]. Taken together, these findings, and other reviews [29], support the idea of a function overlap of the three main RNAi pathways in *B. mori* [29].

2.1. Systemic RNAi

Systemic RNAi is described as a silencing signal transmitted widely throughout a treated organism [5, 31]. The knowledge about the systemic RNAi mechanism in insects is important as it may affect the approaches adopted to develop "RNAi-mediated pest control" because the systemic mechanism is not conserved among those organisms. Systemic RNAi has two important steps to be considered: the uptake of dsRNA by the cells and the systemic spreading of the signals. Some of the main genes involved in systemic RNAi are presented below and discussed for the model organisms.

2.1.1. dsRNA uptake

In insects, two types of dsRNA uptake mechanisms have been identified [32]. The first one involves a multi-transmembrane domain protein, Systemic Interference Defective (Sid). In *C. elegans*, Sid-1 is essential and sufficient to mediate uptake and systemic spread of RNAi signal

in both somatic and germ-line cells [33, 34]; conversely, in insects, Sid-1-like (Sil) proteins appear to be variable across orders [32]. For instance, Diptera do not present *SIL* genes, while *Tribolium* and *B. mori* presented three *SIL* homologues [23, 35]. However, these *Tribolium* genes share more identity with another *C. elegans* gene, *TAG-130*, not required for systemic RNAi in *C. elegans* compared to that with *SID-1* [23]. The second dsRNA uptake mechanism involves endocytosis, specific for dsRNAs acquired from the environment, known as environmental RNAi [5, 31]. First discovered in *Drosophila* S2 cells and later in *C. elegans*, this uptake of dsRNA by endocytosis appears to be evolutionary-conserved [36-38]. However, we should be cautious to conclude that all organisms have a dsRNA uptake mechanism based on endocytosis. For instance, Ulvila and colleagues [38] working with *Drosophila* S2 cells, which are hemocyte-like, described high rates of endocytosis as compared to the majority of other cell types in this species [39].

Other important proteins for systemic RNAi were identified in *C. elegans* but are specific only to germ-line cells, such as Rsd-2, Rsd-3, and Rsd-6 [40]. The Rsd-2 protein contains no particular known motifs but interacts with Rsd-6 that has a Tudor domain, suggesting that these two proteins act together [40]. The *RSD-3* gene encodes a protein that contains an epsin amino-terminal homology (ENTH) domain, found in proteins involved in vesicle trafficking, suggesting the involvement of endocytosis in systemic RNAi [40]. In *Tribolium*, a homologue for *RSD-3* (*Tc-RSD3*) has been found, but in *Drosophila*, which does not exhibit a systemic silencing response, a homologous Rsd-3 protein (Epsin-like) was identified [23]. So, the presence of Rsd-3 does not seems to determine whether or not systemic RNAi occurs in insects, and it is possible that the expression level and/or tissue specificity of this gene may affect the degree of RNAi efficiency and the dsRNA uptake from the environment [23].

2.1.2. RNAi systemic spreading: amplification and maintenance of dsRNA

Once the dsRNA overcomes all the uptake barriers, the silencing signal should be transported from treated cell to other cells, and spread to other tissues. Further, dsRNA should be constantly produced, e.g., either by the amplification of dsRNA by an RNA-dependent RNA polymerase (RdRP), and/or constantly acquired for the maintenance of the silencing responses.

In *C. elegans*, primary siRNAs processed by Dicer are used as a template for an RdRP activity to produce secondary dsRNAs [41]. The RdRP activity is key for the RNAi signal amplification. However, so far in insects, no RdRP-related protein has been found [21, 23], suggesting that strong RNAi response in insects does not rely on amplification of the trigger dsRNA, and it must be based on a different mechanism yet to be identified. Alternatively, constant supply of dsRNA may be provided by RNAi-plants to provide continuous effects.

The presence of the main genes involved in systemic RNAi and amplification in *Drosophila* and *Tribolium* fail to explain the respective absence and presence of systemic RNAi [23]. Nevertheless, the several differences in the number of these core component genes found between species suggests an interesting avenue for further investigation [23]. For instance, Ago protein that have already been shown to determine RNAi efficiency [42], was found to be duplicated in the *Tribolium* genome, while *Drosophila* carries only a copy of *AGO-2* gene, suggesting a relationship between number of *AGO* copies and RNAi response [23].

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Another component that might affect RNAi efficiency in different insects are the proteins containing the dsRNA-binding motif (dsRBM), which help small molecules to properly load inside of the silencing complexes [23]. These proteins act together with Dicer, and seem to be responsible for determining Dicer specificity in *Drosophila* [23]. In the *T. castaneum* genome, two *R2D2-like* genes (a particular dsRBM) were found (*TcR2D2* and *TcC3PO*), which might help *Tribolium* to be hypersensitive to dsRNA molecule uptake by the cells [23]. However, in the *Anthonomus grandis* transcriptome, only an *R2D2* contig was identified [21]. The presence of an additional R2D2-like protein in *Tribolium* might also allow a longer-lasting RNAi effect, once dsRBM proteins are known to bind to dsRNAs, and might be involved in the maintenance of dsRNA in cells [23].

3. Factors affecting the silencing effect and RNAi efficiency as an insect control method

The RNAi approach to control insect pests had been considered for many years, but application of this technology was just realized after it was shown that ingestion of dsRNA would trigger RNAi. The concept of RNAi-plant mediated pest control was demonstrated in 2007 by the development of transgenic plants producing dsRNAs against specific insect genes, with the consequent effect on the target species [43, 44]. The main prerequisites to generate successful RNAi insect-resistant transgenic plants are: (i) identification of a specific gene with an essential function in the insect that can cause developmental deformities and/or larval lethality when knocked down or knocked out; and (ii) dsRNA delivery by oral ingestion that must be uptaken by the insect cells, and spread systemically.

The insect must uptake the dsRNA version of a target gene region by feeding. To silence the target gene, this specific dsRNA must be taken up from the gut lumen into the gut cells as what is considered as "environmental RNAi." If the target gene is expressed in a tissue distinct from the digestive system, the silencing signal should successfully spread via cells and tissues as a systemic RNAi. Both environmental and systemic RNAi are considered noncell-autonomous RNAi, which means that the interfering effect takes places in tissues/cells different from the location of application or production of the dsRNA. Conversely, in the cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced [5]. However, the mechanism of ingested dsRNA uptake and systemic spreading of the silencing signal in the insect have yet to be fully characterized and understood.

Some factors can affect the efficiency of the dsRNA uptake and systemic silencing spread in different insects. Here, we highlight important points that must be considered in developing an RNAi approach against insect pests.

3.1. Target gene

The choice of the target gene should be carefully considered. Each gene requires particular effort to be silenced. Terenius and colleagues [28] reviewed more than 150 RNAi experimental

results from RNAi of lepidopterans involving 130 genes, from which only 38% were silenced at a satisfactory level, while 48% failed to be silenced, and 14% were silenced at insufficient levels. Among the target genes, those involved in immunity were more effectively silenced, and, in contrast, genes expressed in epidermal tissues seem to be most difficult. Differences for RNAi sensitivity among genes in the same tissue was described in [28].

3.2. dsRNA design

The design of the dsRNA determines the one particular target gene to be silenced, but off-target effects can occur if siRNAs have some sequence similarity with unintended genes. Tobacco plants expressing *Helicoverpa armigera* ecdysone receptor (*EcR*) dsRNA improved resistance to another insect, *Spodoptera frugiperda*, due to the high identity shared between the nucleotide sequence of *HaEcR* and *SeEcR g*enes [45]. Although this result implies that an RNAi-plant can control two or even more lepidopteran pests, this can also affect nontarget insects, becoming a biosafety issue.

3.3. dsRNA length

The length of the dsRNA fragments plays an essential role in the effectiveness of molecular uptake in insects, which is directly involved in the success of the target gene silencing. In most of the RNAi experiments, the insects are fed with long dsRNAs [5]. Some experiments showed that long dsRNAs are more efficiently uptaken than siRNAs [37, 46]. This may be due to the fact that a long dsRNA, with 100% match of the target mRNA, after processing into siRNA will provide a greater diversity of siRNAs available to cause specific suppression of target gene and increase the desired effect, and, additionally, reduce the likelihood of developing resistance [47]. In contrast, other studies reported suppression of genes in different insects via incorporation of siRNA in diet instead of dsRNA [48, 49].

3.4. dsRNA concentration

Optimal concentration of dsRNA delivered to the insect is required to induce sufficient gene target silencing. It is noteworthy to mention that exceeding the optimal dsRNA concentration may not result in more silencing [50, 51]. However, higher concentration of dsRNA decreased the duration of dsRNA exposure to reach 50% mortality of *Diabrotica virgifera virgifera* [46], suggesting an inverse relationship between dsRNA concentration and duration of exposure. In cricket (*Gryllus bimaculatus*), the highest concentrations of dsRNA yielded more efficient gene knockdown [52]. The amount of dsRNA sufficient to significantly reduce mRNA levels of *PER* and *CLK* genes were one and two μM, respectively. These concentrations reduced the expression level of the targets, but a higher concentration (20 μM) for the C*YC* gene was required, suggesting that the sensitivity to dsRNAs also depends specifically on the gene [52].

3.5. Controls

Empty vector, empty cassette, buffer only, irrelevant or nonspecific control (such as dsGFP – Green Fluorescent Protein gene region), or any other kind of negative control are essential to

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discriminate specific gene silencing from the simple induction of siRNA processing machinery by exposure to a dsRNA. Mainly, a negative control should demonstrate the specificity of the dsRNA designed for a target, not interfering in specific target expression, and even unspecific effects. Also, any control should have similar size and concentration of the used dsRNA [53].

3.6. Molecular silencing confirmation

An efficient molecular confirmation of the RNAi silencing should be conducted, which includes target RNA expression, and analyses of protein amount and/or enzyme activity. In RNA analysis, additional care should be taken for expression analysis. The method of choice for RNA expression analysis is the quantitative amplification of reversed transcripts or RT-qPCR, considered a very sensitive and accurate method. To provide precision in RT-qPCR, some essential care is required, such as the choice of appropriate stable reference genes and primer pair design with sufficient amplification efficiency. The reference genes should exhibit stable expression among experimental conditions, providing reliable estimate of gene expression results [54]. Additionally, primers should be designed flanking the region used to design the dsRNA to ensure that the initial cleavage of the mRNA could be detected, thus avoiding false-positives [55]. Conventional care of RT–qPCR reactions defined by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines must be strictly followed [56].

3.7. Protein stability and phenotype analysis

Proteins can exhibit a long half-life and interfere with the phenotypic changes. However, phenotype changes are not totally related to small decrease of protein levels; haplo-sufficient genes produce proteins capable of performing the biological processes normally, even at half of the protein levels [53]. Phenotype changes could be more difficult to be observed in RNAi responses if the protein product of the target gene has a long half-life. For example, the reduction of *Da6* (nicotinic acetylcholine receptor subunit) expression in both *D. melanogaster* and *T. castaneum* exhibited weak phenotype responses [57], which may be explained based on the long stability of the nicotinic acetylcholine receptors (nAChRs) [58]. Nevertheless, for most of the genes, mRNA turnover and protein half-life are unknown and this lack of information presents one of the principal challenges for the RNAi experiments [39].

3.8. Insect issues, life stage, nucleases, and gut pH

Some insect characteristics should also be considered before starting an RNAi experiment including the developmental stage of insects. Although handling advanced developmental stages of insects is more efficient, silencing effects are more prominent in earlier stages. For instance, in second instar larvae of *Rhodnius prolixus*, the gene *nitropin 2* was knocked down for 42%, but no silencing was observed in the fourth instar individuals, even though both larval stages were treated with the same concentration of dsRNA [59]. In *Spodoptera frugiperda*, fifth instar larvae presented higher gene silencing as compared to adult moths [60].

Another consideration that can affect the RNAi silencing efficiency is the presence of insect nucleases and gut pH. For instance, feeding assays with *Lygus lineolaris* showed no mortality effects because the saliva of *L. lineolaris* contains dsRNA-degrading activity. Thus, dsRNA ingested did not result in siRNA fragments, rather were completely digested to monomers [61]. Also, dsRNA degradation is reduced during molting period and further reduced by starvation in some insects [62]. The stability of the dsRNA in the midgut could be affected not only by enzymatic but also by chemical hydrolysis [63]. In both cases, gut pH is an important factor; particularly, it is quite variable among insect orders, with variation even among gut regions [64]. For example, in general, lepidopterans exhibit a strong alkaline gut (up to pH 10.5 in some species), which provides a highly hostile environment for dsRNA [64]; therefore, this order is particularly recalcitrant to gene silencing by RNAi.

4. Overview on the use of RNAi to control insects by transgenic plants

Most of the current transgenic crops with specific control against insect pests are based on *Bacillus thuringiensis* (Bt) toxins, which act in gut epithelial cell membrane in susceptible insects. Bt toxins are highly specific against certain orders of insects, where the most successful use was achieved against Lepidoptera and Coleoptera. However, continuous exposure of those insects to Bt crops evolved field-resistance, affecting the efficiency in controlling those pests [4, 65]. Also, there are limitations of Bt transgenics to manage some other important agricultural pests, such as the sap-sucking insects (Hemiptera). This encouraged the development of new strategies to help in controlling agricultural pests.

In 2007, two studies demonstrated the concept of plants expressing dsRNAs derived from hairpin vectors that directed dsRNAs to target gene regions of economically important agricultural pests: the cotton bollworm (*Helicoverpa armigera*; Lepidoptera; [44]) and the Western corn rootworm (WCR; *Diabrotica virgifera virgifera*; Coleoptera; [43]). After the demonstration of plants resistant to insects, the application of RNAi by transgenic plants became a potential new approach to control important agricultural pests, which led to the flourishing of a new field of research. So far, searches on the publication database "Web of Science" from Thomson Reuters (August 2015), identified 543 published studies based on the combination of the topics "RNAi," "plant" and "insect," and only one quarter was published before 2008.

To implement RNAi in agricultural pest control, the target insect should uptake the dsRNA autonomously, e.g., from transgenic plants expressing dsRNA. This feeding should be continuous, since insects lack an amplification mechanism based on RdRP, such as *C. elegans*. Many of the main agricultural pest species have already been targeted by RNAi technology using various genes and delivery methods [66]. However, three orders have been the major focus of the development of transgenic plants expressing target gene regions for RNAi: Coleoptera, Hemiptera, and Lepidoptera. Here, we list some of the most recently published RNAi transgenic plants studies performed against those insect orders (Table 1).

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Specie	Order	Crop	Target Gene	Remarks	Reference
Diabrotica v. virgifera	Coleoptera	*Zea mays*	*vATPase*	Mortality	[67]
Leptinotarsa decemlineata	Coleoptera	*Solanum tuberosum*	*β-actin, Shrub*	Mortality	[68]
Helicoverpa armigera Spodoptera exigua	Lepidoptera	*Nicotiana tabacum*	Nuclear receptor complex of 20-hydroxyecdysone (*HaEcR*)	Molting defect and larval lethality	[45]
Helicoverpa armigera	Lepidoptera	*Nicotiana tabacum*	Molt-regulating transcription factor gene (*HR3*)	Developmental deformities and larval lethality	[70]
Helicoverpa armigera	Lepidoptera	*Arabidopsis thaliana*	*HaAK*	Developmental Deformities and larval lethality	[71]
Myzus persicae	Hemiptera	*Arabidopsis thaliana and Nicotiana benthamiana*	*MpC001, Rack1*	Progeny reduced	[72]
Myzus persicae	Hemiptera	*Arabidopsis thaliana*	*serine protease*	Progeny reduced	[73]
Myzus persicae	Hemiptera	*Nicotiana tabacum*	*hunchback(hb)*	Inhibited reproduction	[74]
Bemisia tabaci	Hemiptera	*Nicotiana rustica*	*vATPase*	Mortality	[75]

Table 1. Overview of the recently published studies on the use of plant-RNAi against different insect pests

4.1. Coleoptera

The coleopterans are likely to be the first target to be controlled by the new generation of transgenics, the "RNAi-plants." *Diabrotica virgifera virgifera (*Western corn rootworm, WCR) is one of the most important agricultural pests, and this species, along with other coleopterans, requires little effort to have genes silenced by RNAi, independent of the delivery method and gene target. The significant breakthrough was demonstrated when WCR presented significant larval stunting and mortality, causing less injury to maize roots that express a hairpin version of *V-ATPase A* [43]. Since then, many studies have been conducted using various target genes, while not focusing only on pest control, but also to characterize the mechanism of action of the RNAi [46]. A recent study with WCR demonstrated that long dsRNAs of *Dv V-ATPase C* expressed in maize provides highly efficient root protection, but the siRNA population generated in the transgenic plant does not lead to lethal RNAi responses when consumed by the insect [67].

Studies have also been performed in other species, such as the important potato pest *Leptinotarsa decemlineata* (Colorado potato beetle, CPB). So far, this is the first study to compare efficacy at controlling pest insects by dsRNAs expressed either in the chloroplast or in the cytoplasm. Transgenic potato plants expressing hairpin versions of *β-actin* and *Shrub* genes (both insectidal dsRNAs) in the chloroplasts (transplastomic plants) conferred the most potent insecticidal activity (insects died after 5 days), while the conventional expression from nuclear transgenics did not affect the beetles [68].

An explanation for this result is that since choroplasts do not have cellular RNAi machinery [69], the dsRNAs produced inside these organelles are not cleaved by a plant Dicer and the beetles ingest almost entirely long dsRNA. In contrast, beetles fed on nuclear-transformed plants consumed mostly siRNAs; previous dsRNA-feeding studies already indicated that ingested long dsRNAs were much more effective than ingested siRNAs. It should be highlighted that all the other studies have been based on an efficient cytoplasm-derived dsRNA in various crops, indicating that possibly potato plants process long dsRNA more effective than the plants from those other studies (Table 1 [69]).

4.2. Lepidoptera

Plants producing *Bt* proteins were the first generation of transgenic plants to control insects, and most of the lepidopterans were successfully managed by Bt crops for years. However, the durability of Bt technology appears to be unsure. The number of pest species that evolved Bt resistance in the field, reducing transgenic efficacy, increased from one in 2005 to five in 2010 [4]. Among those five species, four are lepidopterans [4]. The lepidopterans could be the first and main targets for RNAi crops if they were not as recalcitrant to gene silencing, without any definite explanation for the limited and unstable RNAi responses [28, 29]. For instance, the concentration of dsRNA necessary to knock down a specific gene by feeding *Diatraea saccharalis* (Lepidoptera) neonate larvae is much higher than the one required for the same larval stage of *D. v. virgifera* (Coleoptera).

The first successful RNAi plant protected against a lepidopteran (*Helicoverpa armigera*) was demonstrated by silencing the *CYP6AE14* gene, necessary for detoxifying gossypol from cotton (*Gossypum hirsutum*) [44]. Studies have been conducted to explore alternative target genes to control and understand the RNAi mechanism in lepiopterans. Larval lethality and molting defects were detected in *H. armigera* fed with transgenic tobacco plants expressing dsRNA targeted to the nuclear ecdysone receptor complex (*HaEcR*), absolutely required for insect development [45]. The transgenic tobacco expressing dsRNA of *HaEcR* had an improved resistance to another lepidopteran pest, *Spodoptera exigua*. This cross-species effect might indicate a risk to affect nontarget insects, highlighting the importance of biosafety studies that should be carefully conducted [45].

The expression of dsRNA in both *Escherichia coli* and transgenic tobacco plants to silence a molt-regulating transcription factor gene (*HR3*) of *H. armigera* resulted in developmental deformity and larval lethality [70]. Transgenic *Arabidopsis thaliana* plants expressing dsRNA targeted to *arginine kinase* (*AK*) of *H. armigera* (*HaAK*) led to a 55% mortality rate in first instar larvae, while retarding growth in surviving larvae [71]. However, no lethal phenotypes were

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observed for the third instar larvae, although transcript levels of *HaAK* were distinctly suppressed [71].

4.3. Hemiptera

Hemipterans are characterized as piercing/sucking insects, representing major agricultural pests that inflict direct damages by sucking sap, or indirectly by acting as a vector of several viruses and bacterial infections. Since hemipterans feed through sucking the phloem, only systemic chemical insecticides are effective against these insects, resulting in high residual toxicity. The problem is further aggravated as no Bt toxin has been identified as exhibiting adequate insecticidal effects against hemipterans. Transgenic crops based on RNAi offer a large potential to control hemipteran, requiring expression of target gene dsRNAs on the phloem. One first report was published in 2011 about developing transgenic *Nicotiana benthamiana* and *A. thaliana* expressing dsRNA targeting genes expressed in *Myzus persicae* gut (*RACK1*) and salivary glands (*MpC002*) [72]. A reduction of the expression level of the target genes and a decrease in *M. persicae* fecundity were observed, but no lethal effects [72].

The same phenotype was observed when *M. persicae* fed on *A. thaliana* expressing dsRNA of a serine protease gene (*MySP*), with no lethal effects, but reduced fecundity [73]. Once again, reduced reproduction, but no lethal phenotype was observed when *M. persicae* was fed on tobacco plants expressing dsRNA targeting the *hunchback* gene [74]. A possible explanation for not achieving the expected phenotypes (mortality) after target gene depletion is that the dsRNA level produced by RNAi transgenic plants could not be sufficient for an efficient uptake of dsRNA or siRNA by sap-sucking insects [74]. However, more recently, mortality rates were observed in *Bemisia tabaci* fed in tobacco plants expressing dsRNA of *v-ATPaseA* [75]. This result provides a proof-of-concept that plants expressing dsRNA, at an efficient level and targeting crucial genes, could resist the attack of Hemipteran pests [75].

5. Conclusion and future perspectives

Since the concept of a transgenic plant expressing dsRNA targeted to a specific essential gene in the insect that affects its viability was first demonstrated in 2007, the technology has been extended to a large number of insect species from various orders. Elucidating the various mechanisms and components of the RNA interference pathway has progressed, but many aspects remain to be clarified. Many differences in components and mechanisms among insect orders and between insects and other organisms still need to be worked out. Some of these differences (e.g., genes involved, gene number, and level of expression) may explain variation in recalcitrance among insect species and need to be further investigated. Of particular interest are the mechanisms of dsRNA uptake, signal amplification, and systemic spread in the major pest species. Additional insect- or order-specific characteristics, such as gut pH, presence of dsRNA-degrading activity in digestive system, among others that could be associated with differences in recalcitrance to RNAi need to be dissected and clarified.

Due to the variety of RNAi response to RNAi in insects, no single protocol is suitable for all species. Issues related to the choice of effective target genes, including determining the size of optimal dsRNA length and ideal gene region. Assuming that the method of choice to deliver dsRNA is transgenic plants, a major question still to be addressed is the impact of plant dsRNA processing in the effective RNAi-induced silencing. There is still a need for investigation in this area. The choice of a suitable inducible promoter for expressing the dsRNA construct is another point barely explored.

Based on the recent publications reviewed in this chapter, the progress in developing "RNAi-plants" to control important insect pests widely demonstrated the potential of this technology to complement or replace Bt crops, providing resistance against a broad variety of insect pests. However, to be applied on a commercial level, several issues related to the RNAi mechanism and biosafety still need to be addressed. As a new technology, risk assessments and government regulations still have to be developed. However, RNAi transgenic crops are expected to have wider acceptance and reduced biosafety requirements for RNAi traits, in comparison to a protein incorporated into a plant, such as a Bt transgenic [39]. Thus, RNAi-mediated pest control will open a new paradigm in insect pest management.

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