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In Search of New Methodologies for Efficient Insect Pest Control: "The RNAi "Movement"

Anna Kourti, Luc Swevers and

Dimitrios Kontogiannatos

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Abstract

The development of insecticide formulations with new mechanisms of action (modes of action, MOAs) is a huge priority for pesticide industry. This priority has become apparent during the last few years after (a) the observed increase in insect resistance for the most widely used active substances and (b) the harmful effects of the excessive use of pesticides on human health, environment, beneficial insects and fish. Silencing of genes by RNAi (RNA interference) technology provides an alternative, selective to species level, environmentally friendly strategy to combat insect pests. Double-stranded RNA molecules (double-stranded RNAs, dsRNAs) targeting important developmental genes are taken up by the digestive tract of the targeted insect speciesand induce RNAi, which results in inhibition of growth, development and reproduction of the targeted insect species. After the rapid development of RNAi technology in the past 10 years, biotech industry is seeking for new applications aimed at producing environmentally friendly genetic insecticides or genetically modified plants (GMPs) that induce environmental RNAi in the targeted insect species. These technologies are expected on the market at the end of this decade. In this chapter, we exploit established methods involving recent initiatives of RNAi technology with respect to the development of new bio-insecticidal formulations.

Keywords: modes of action, RNA interference, double-stranded RNAs, genetically modified plants, agricultural biotechnology

1. Introduction

Eukaryotic cells are equipped with a conserved mechanism by which exogenously added or endogenously expressed RNA duplexes (double-stranded RNA or dsRNA) are directly

degradedin to their complementary endogenously encoded messenger RNA (mRNA), resulting in targeted gene silencing. This phenomenon is generally referred to as RNA interference (RNAi) [1, 2]. In plant and animal organisms, RNAi provides a line of defense against viruses and parasitic genetic elements (transposons) while a similar, but mechanistically separated, mechanism regulates tissue-specific gene expression at translational level [3]. Induction of RNAi begins when high-molecular-weight dsRNAs are selectively degraded by type III ribonucleases encoded by RNAse III-related genes known as Dicer or Dicer-like genes. The Dicer enzymes appear to specifically act on the dsRNAs leading to their degradation (digestion) by their 5'–3' ends resulting in the production of small 21–24 base (ds) ribonucleotides known as small interfering RNAs (siRNAs) [4]. The double-stranded siRNAs are incorporated into a multiprotein complex known as the RNA-induced-silencing complex (RISC) that is actually induced by the presence of the RNAs. In this complex, the "passenger" (sense) strand of siRNAs is degraded and the antisense or guide strand is directed to the mRNA target. The guide sequence enables Watson-Crick complementarity to the mRNA target which is enzymatically degraded by a family of proteins called "Argonauts" (Argonaute proteins), thereby preventing translation of the mRNA. Arguably, the mechanism of gene silencing is specialized and targeted at the nucleotide sequence level. The possibility of specific gene downregulation through RNAi has proven mainly in insects which lack methods for functional genomic analysis [5]. The extremely interesting observation that dsRNA molecules taken up through the digestive tract of the nematode *Caenorhabditis elegans* induce gene-specific downregulation [6, 7] created hopes that oral dsRNA uptake could regulate gene expression in other invertebrates as well, including insects. This technology could be used for the reasonable purpose of developing environmentally friendly pesticides to combat agricultural insect species.

The first-historical publications describing the use of RNAi technology in crop insect control were those of Baum et al. [8] and Mao et al. [9]. These researchers showed that genetically modified plants expressing dsRNAs of entomological origin could influence the development and growth of phytophagous insect species which were feeding on these plants. Corn plants expressing hairpin dsRNAs, which target the A subunit of the ATPase gene in *Diabrotica virgifera virgifera* (western corn rootworm, WCR), showed significant protection from the damage caused by this insect species [8]. Following previous discoveries, *Arabidopsis* plants expressing double-stranded RNA hairpins targeting the cytochrome P450 monooxygenase gene in the corn pest *Helicoverpa armigera* showed decreased resistance to the sesquiterpene gossypol [9]. The above developments in gene-targeting research gave birth to a new term known as environmental RNAi [10]. Environmental RNAi involves the phenomenon in which RNAi is induced after environmental exposure to insect dsRNAs by oral or topical administration. Successful environmental RNAi has been reported in a wide range of insect families belonging to the classes of Coleoptera, Diptera, Dictyoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera and Orthoptera [11]. The technology of environmental RNAi is the best candidate for replacing conventional insecticides and avoiding collateral damages to the ecosystem, beneficial insects and human health.

2. dsRNA delivery systems

2.1. dsRNA injection

The most widely used methodology for administrating dsRNA molecules in insect cells is by injecting volumes of *in vitro* synthesized dsRNAs into their hemocoel. This methodology was mostly used for functional analysis of genes in model and non-model insects from the early 2000s till date. DsRNAs could be *in vitro* synthesized using different approaches (there are many alternatives but we describe the one which was extensively used in our laboratory). In this approach, the gene of interest should be cloned in both sense and antisense directions in plasmids flanked with at least one T7 promoter. Then, the plasmids are linearized with restriction enzymes and transcribed in two different reactions (using T7 RNA polymerase) to synthesize sense and antisense ssRNAs. The complementary RNA molecules are then mixed and annealed. For the annealing step, the two transcripts should be mixed in precisely equimolar amounts (**Figure 1**). We have been using this methodology for synthesizing a wide range of targets for functional analysis of genes of the lepidopteron *Sesamia nonagrioides*. For the juvenile hormone esterase-related gene of *Sesamia*, we have targeted different parts of the full gene transcript by synthesizing *in vitro* three different dsRNAs, corresponding to a 472-bp part of its 5'-translated region, a 1276-bp part of its central translated, 3'-translated and part of its 3'-untranslated region and a 1725-bp part encompassing both of the above regions, which spanned 94% of the total cDNA. All transcripts resulted in gene-specific knockdown of *SnJHER* but only the 1725 bp dsRNA was able to cause a significant phenotype [12]. In order to silence the 1276-bp part, we ligated a SnJHER 1276-bp fragment into the MCS of the RNAi L4440 vector (**Figure 2A**). The L4440/ SnJHER1276 plasmid was then linearized with either XhoI or NcoI (to create sense and antisense plasmids) and used as template for RNA synthesis. After DNase treatment (to destroy the DNA templates), sense and antisense ssRNAs were annealed to form dsRNA and analyzed in agarose gels before injection to insects (**Figure 2B**).

In other insect species, a wide range of successful experiments of intra-hemolymph RNAi have already been published and some of them will be described below:

- In adult fruit flies, RNAi could be induced by injecting dsRNAs into the abdomen of anesthetized individuals targeting genes which were expressed in their central nervous system (CNS) [13].

- In *Bombyx mori*, injection of double-stranded RNA corresponding to the silkworm white gene (Bmwh3) into preblastoderm eggs of wild-type silkworm induced phenotypes similar to those observed with mutants of the white egg 3 locus. The induced phenotypes were characterized by the presence of white eggs and translucent larval skin [14].

- Additionally, parental silencing of the hemolin gene from the Giant silkmoth, *Hyalophora cecropia*, demonstrated that hemolin is crucial for the normal development of embryos. When RNAi females were mated, no larvae emerged from their eggs and when dissected, the eggs revealed malformed embryos [15].
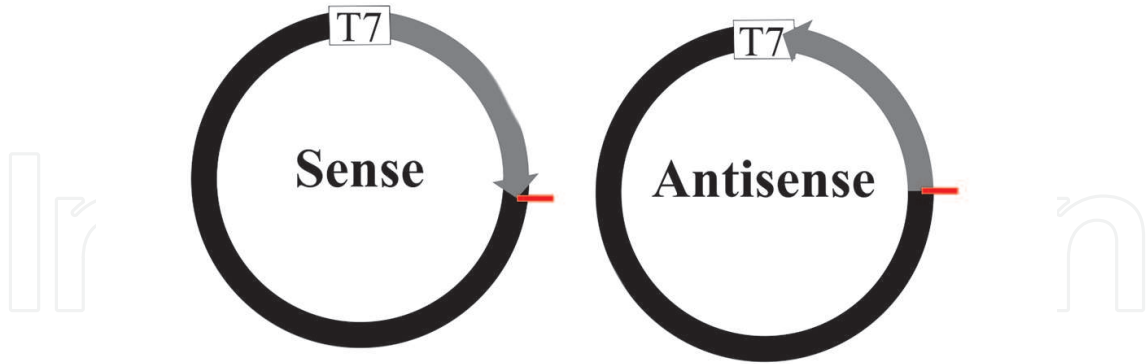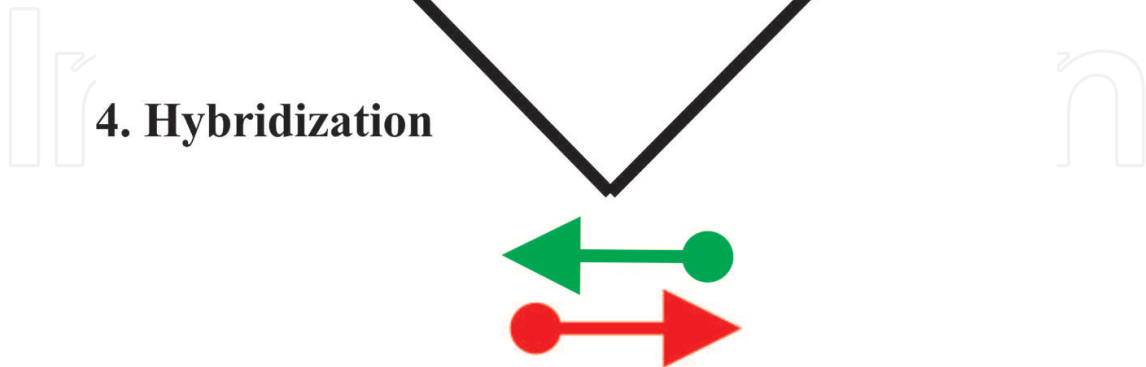
Figure 1. *In vitro* dsRNA synthesis strategy. Plasmid sequences are indicated in black while inserts for RNA productions downstream of the T7 promoter (cloned in two different orientations) are displayed in gray. Sense and antisense RNAs are indicated in red and green, respectively.

• In *Manducta sexta* injection of double-stranded integrin-beta1 RNA into larvae resulted in decreased integrin beta1 expression in plasmatocytes and significantly suppressed encapsulation [16].
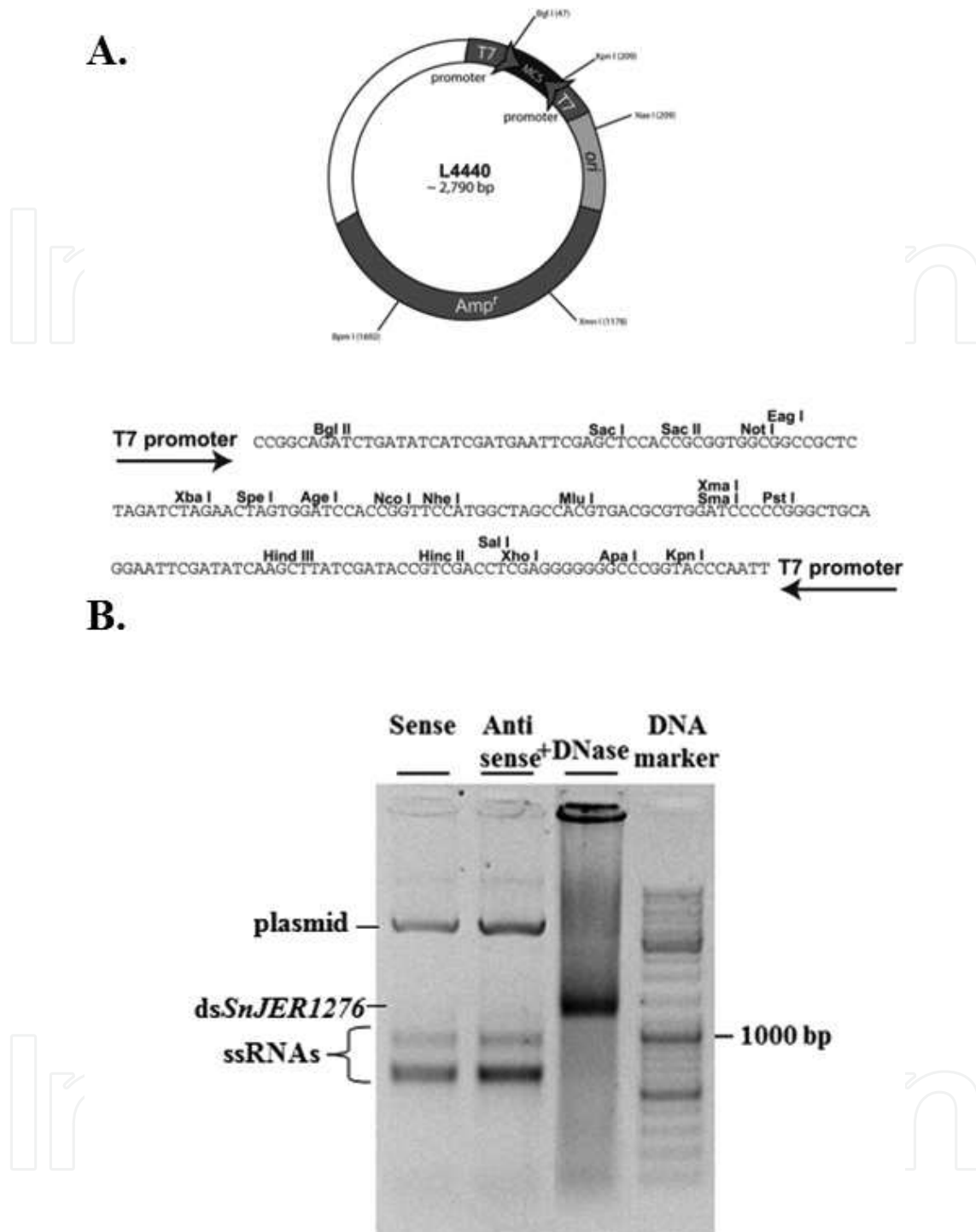
Figure 2. Production of dsRNA corresponding to the gene *SnJHER*. **A**. Schematic representation of L4440 plasmid and its polylinker for cloning gene fragments for dsRNA production (https://www.addgene.org/1654/). The polylinker is flanked on either side by a T7 RNA polymerase promoter for RNA synthesis. **B**. Synthesis of ds*SnJHER*1276. Sense and antisense ssRNAs running in 1% w/v agarose gel (first and second lanes counting from the left). The two ssRNAs were annealed and subjected to DNase treatment (third lane). DNA marker is being shown on the fourth lane.

- While in *Aedes aegypti* injection of double-stranded RNA corresponding to the broad gene (BR) isoform Z2 led to a significant decrease in the expression of the *Vitellogenin* (Vg) gene at 8 and 24 h post blood meal. Knockdown of Z1 or Z4 resulted in enhanced Vg expression beyond its normal expression time [17].

- Injection of both dsRNA/siRNA could induce the silence of chitin synthase gene A (CHSA), which is an important gene for the growth and development of cuticles and trachea in beet armyworm, *Spodoptera exigua*. The cuticle of CHSA-silenced insects was disordered and the epithelial walls of larval trachea did not expand. Moreover, injections significantly increased abnormalities relative to control larvae [18].

- In *Tribolium castaneum*, injection of dsRNA prepared using the common or isoform-specific regions of ecdysone receptor (EcR) and ultraspiracle (USP) genes as templates caused derailment of development [19].

- In *S. frugiperda*, silencing of the allatostatin AS-C-type (Spofr/Manse-AS) or the allatotropin AT 2 (Spofr-AT 2) genes after intra-hemolymph administration resulted in reduction of their transcript levels in brain and gut of last instar larvae and adults. This suppression led to an increased JH titer in larvae [20].

- In *Gryllus bimaculatus*, injection of the circadian clock gene period (*G. bimaculatus* period, Gb'per) dsRNA into the abdomen of third instar nymphs knocked down the mRNA levels to 25% of that in control animals. Most Gb'per dsRNA-injected nymphs lost their circadian locomotor activity rhythm, while those injected with DsRed2 dsRNA as a negative control clearly maintained the rhythm [21].

- The membrane-bound trehalase genes of *S. exigua SeTre-1 and SeTre-2* were analyzed by dsRNA injections. The RNA interference (RNAi) of either SeTre-1 or SeTre-2 was gene-specific and effective, with efficiency rates up to 83% at 72 h post injection. After RNAi of SeTre-1 and SeTre-2, significant higher mortality rates were observed during the larval-pupal and pupal-adult stages [18].

- In *D. virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), injection of laccase 2 DvvLac2-specific double-stranded RNA resulted in the prevention of post-molt cuticular tanning, while injection of chitin synthase 2 DvvCHS2-specific dsRNA reduced chitin levels in midguts. Silencing of both DvvLac2 and DvvCHS2 was confirmed by real-time polymerase chain reaction (RT-PCR) and quantitative RT-PCR [22].

- DsRNA-based gene silencing resulted in a dramatic reduction in the levels of the corresponding mRNA in the *Locusta migratoria manilensis* (Meyen) nymphs injected with dsRNA of chitin synthase 1 LmCHS1, or either of its two variants, LmCHS1A and LmCHS1B. mortalities of 95, 88 and 51% were observed in the locusts injected with the LmCHS1, LmCHS1A and LmCHS1B dsRNA, respectively [23].

- In *Leptinotarsa decemlineata*, specific interference of Ldace1, an ortholog of *Anopheles gambiae Agace1* by means of dsRNA injection, resulted in a reduction of AChE activity to an approximate 50% compared to control, while interference of Ldace2 reduced AChE activity to an approximate 85%. Interference of Ldace1 in CPB adults caused a significant increase in mortality (43%) as early as 3 days post injection (p.i.). Interference of Ldace2 also caused a significant increase in mortality (29%) compared to control, although at seven days p.i. [24].

- In *G. bimaculatus* and the firebrat *Thermobia domestica*, a dose-dependent effect of dsRNA was observed to achieve knockdown of clock genes. However, this effect was affected by the particular gene that was silenced and the insect species (*Gryllus* versus *Thermobia*) [25].

- Injection of CHS dsRNA interfered with egg development in the ovary and the eggs that were laid were dark of color and not viable. Fluorescence microscopy demonstrated reduced deposition of chitin in previtellogenic and vitellogenic oocytes in the ovaries [26].

- In a later work, HSP70/HSC70 knockdown of *Rhodnius prolixus* insects showed lower resistance to prolonged starvation in comparison to appropriate controls, dying between 32 and 40 days after dsRNA injection. After blood feeding, the physiological effects of HSP70/HSC70 knockdown were more prominent and the insects died even earlier, within 14–20 days after feeding (21–27 days after dsRNA injection). These bugs showed impaired blood processing and digestion; reduced energetic metabolism and the midgut immune responses were compromised [27].

2.2. Oral delivery of dsRNAs

Oral delivery of dsRNAs aims to silence the selected gene after gut-mediated uptake and transport to the insect cells. If oral delivery is efficient, then much higher possibilities exist to formulate a dsRNA-based insecticide. For orally delivering dsRNAs, dsRNAs should be *in vitro* synthesized as described previously. Then, the dsRNAs are incorporated to the artificial diets of the insects or even sprayed in the plants which are used to be fed on. Important examples (highlights) from the literature are given below:

- Walshe et al. [28] first demonstrated specific gene knockdown by feeding in dipteran species. This was a first example of RNAi in a blood-sucking insect by including dsRNA in its blood meal. Delivery of dsRNA through the blood meal of *Glossina morsitans* was as effective as dsRNA injection with respect to the silencing of the midgut-expressed gene TsetseEP. By contrast, the gene 2A192 that is expressed in the fat body was only knocked down after dsRNA injection. Feeding of dsRNA reduced significantly the mortality rates of the flies compared with the injection treatment.

- Bautista et al. [29] silenced a P450 CYP6BG1 gene by dsRNA droplet feeding in *Plutella xylostella* in order to show its involvement in permethrin resistance. Quantitative real-time PCR showed efficient reduction of expression of CYP6BG1 transcripts in midgut and carcass after oral delivery, which was reflected in reduced total P450 activities of microsomal preparations and which resulted in significant reduction in resistance to the insecticide permethrin. The experiments indicate the participation of overexpressed CYP6BG1 in the resistance mechanism against permethrin [29].

- RNAi has been used successfully to silence endogenous honey bee genes by feeding [30]. This was the first successful large-scale real-world use of RNAi for insect-disease control. RNAi was shown to prevent bees from succumbing to infection from Israeli Acute Paralysis Virus, IAPV, under laboratory conditions. In this study, IAPV-specific homologous dsRNAs were used in the field, under natural beekeeping conditions in order to prevent mortality and improve the overall health of bees infected with IAPV [30].

- In the whitefly, *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae), Lü and Wan [31] explored the role of heat-shock protein (Hsp) genes in both male and female sexes by dsRNA feeding.

- Chen et al. [32] examined the effects of using a feeding-based RNAi technique to target the gene trehalose phosphate synthase (TPS) in the brown planthopper, *Nilaparvata lugens*. DsRNA feeding resulted in rapid and significant reduction in expression levels of TPS mRNA and enzymatic activity. Developmental abnormalities were observed in dsRNA-fed *N. lugens* larvae which resulted in lethal effects.

- Hunt et al. [33] developed an RNAi-mediated bioassay to explore proposed connections between expression of hexameric storage proteins and worker versus gyne (potential future foundress) castes in naturally founded colonies of the wasp genus *Polistes* (*P. metricus*). They targeted the hexamerin 2 gene in fifth (last) instar larvae by feeding with double-stranded hexamerin 2 RNA directly to larvae in naturally founded colonies in the field. Their results pave the way for functional genomic research that can contribute significantly to learning the interactions between environment and development and its significance for paper wasp evolution and behavioral ecology [33].

- Luan et al. [34] developed a new and high throughput methodology to silence whitefly genes using a leaf-mediated dsRNA-feeding method. A leaf-mediated dsRNA-feeding method was developed to test silencing of whitefly genes in high-throughput format. While silencing of ecdysone biosynthetic and regulatory genes had little effect on survival and fecundity of adult whiteflies, reduced survival and delayed development were observed during the treatment of the nymphal stages.

- Moreover, Mao and Zeng [35] performed RNAi at the second instar stage to knock down *hunchback* (*hb*) expression in the pea aphid, *Acyrthosiphon pisum*. Continuous feeding of *Aphb* dsRNA mixed in their artificial diet led to reduction of *Aphb* transcripts and rise of insect lethality. Their results indicated that the gene *hunchback* was an efficient RNAi target for managing populations of aphids.

- Li et al. [36] introduced dsRNAs of P450 CYP6CM1 genes corresponding to the B and Q biotypes into the insect body of *B. tabaci* adults through membrane feeding. RNAi of the P450 CYP6CM1 gene reduced gene expression, increased mortality and inhibited the ability to detoxify a pesticide or a plant secondary metabolite in both biotypes of *B. tabaci*, with better efficacy in B biotype than in Q biotype.

- Finally, Abd El Halim et al. [37] evaluated oral delivery of dsRNAs targeting sodium ion channel paralytic A *(TcNav)* gene in *T. castaneum* as a viable means of controlling this insect pest. Oral delivery of dsRNA caused dose-dependent mortalities between 19 and 51.34% accompanied by a significant knockdown in gene expression following 3 days of continuous feeding. The majority of larvae injected with, or fed, dsRNA died during the final larval stage prior to pupation. This work provided evidence of a viable RNAi-based strategy for insect control.

2.3. Bacterial-mediated RNAi

Bacterial dsRNA administration is based on the observations of Timmons and Fire [6] which showed that ingestion of bacterially expressed dsRNAs could produce specific and potent genetic interference in *C. elegans*. This approach uses an RNase III-deficient *Escherichia coli*

strain known as HT115 (DE3) [F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen: lavUV5 promoter -T7 polymerase]. In this methodology, the gene of interest is being cloned between two T7 promoters on a special RNAi plasmid known as L4440 (T7p, T7p, lacZN, OriF1) (**Figure 2A**). The plasmid is being transformed in HT115 cells and dsRNA production is achieved after induction with IPTG. The induced cells are then introduced in the worm's growth media and RNAi is achieved after a short period of incubation.

Similarly in insects the IPTG-induced bacteria are incorporated in their artificial diets or they are even sprayed in plant organs that insects are feeding on and RNAi is induced after a period of continuous feeding (**Figure 3**).

The successful application of bacterial-mediated RNAi has been reported in several cases:

- Tian et al. [38] showed that the growth and development of *S. exigua* larvae fed with HT115 cells expressing dsRNAs of the chitin synthase gene A (SeCHSA) was disturbed, resulting in lethality. The survival rates of fifth instar larvae, prepupal and pupal stages were significantly lower than those of controls.

- Similarly, Li et al. [39] used genetically engineered HT115 *E. coli* cells. Engineered bacteria were generated that produce specific dsRNAs targeting several essential genes in *Bactrocera dorsalis*, such as the ribosomal protein Rpl 19, the type V ATPase D subunit, the fatty acid elongase Noa and the small GTPase Rab11. Quantitative real-time PCR indicated
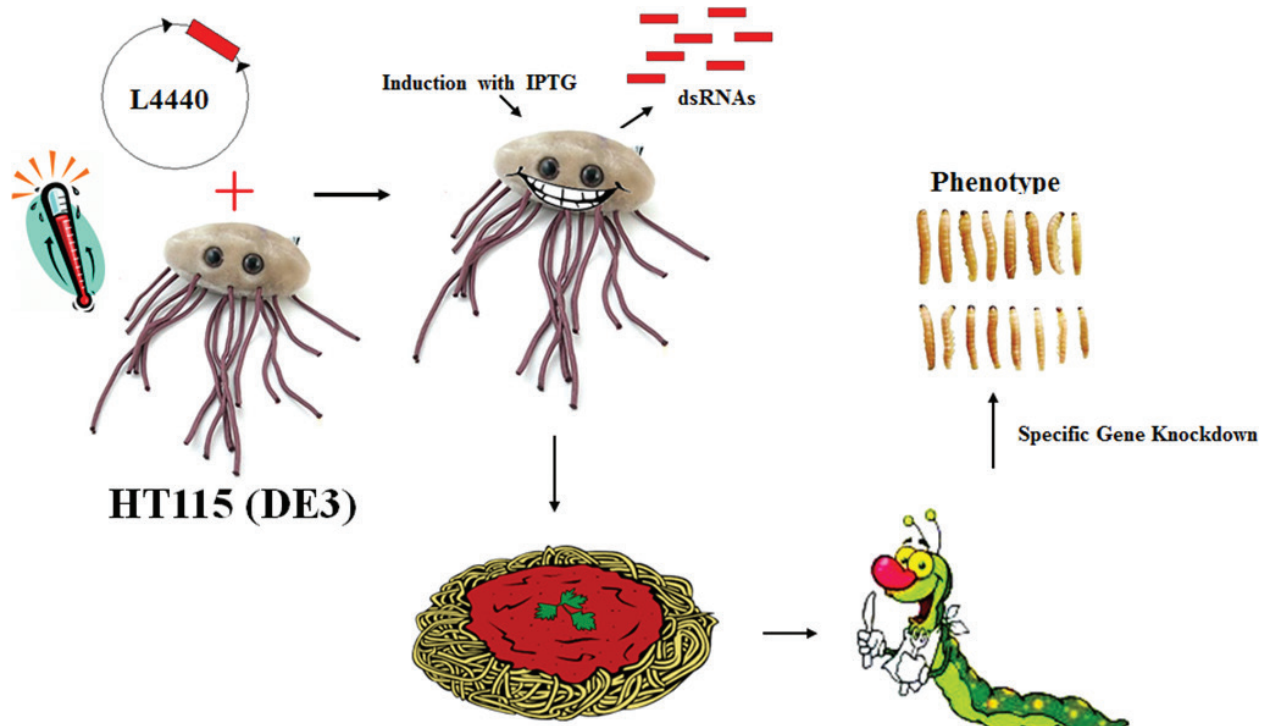

Figure 3. Strategy behind bacterial-mediated RNAi. The gene of interest is being cloned in the RNAi L4440 plasmid and transformed to competent HT115 cells. The transformed cells are then induced with IPTG and incorporated in insect's diet in order to induce RNAi.

that feeding of both engineered bacteria and isolated dsRNAs proved effective at silencing the four targeted genes when compared with nonspecific (EGFP) dsRNA.

- In the Colorado potato beetle, *L. decemlineata* (Say) (CPB), Zhu et al. [40] successfully triggered silencing of five target genes by dsRNA feeding resulting in significant mortality and reduced body weight gain in the treated beetles. These results suggested that the efficient induction of RNAi using bacteria to deliver dsRNA is a possible method for the management of CPB.

- Moreover, Zhang et al. [41] demonstrated the efficacy of RNAi in the cotton bollworm, *H. armigera*, using bacterial-mediated dsRNA expression of CYP6B6 gene. Gene and protein expression levels of CYP6B6 were reduced in *H. armigera* larvae fed with HT115 bacteria expressing CYP6B6 dsRNAs.

- Taracena et al. [42] silenced *R. prolixus* heme-binding protein (RHBP) and catalase (CAT) genes after feeding nymphs and adult triatomine insects with dsRNA expressing HT115 bacteria. The RNA interference effect was systemic and temporal. RHBP expression in the fat body was reduced by 99% three days after feeding and CAT expression was reduced by 99 and 96% in the ovary and the posterior midgut, respectively, 5 days after ingestion.

Essential for successful RNAi-feeding experiments is correct dsRNA induction before the oral administration step. A general protocol for bacterial-mediated dsRNA expression is given as follows. In this experiment, we cloned the 1276 bp part of *S. nonagrioides* juvenile hormone esterase-related gene in the L4440 plasmid and then we used it to transform HT115 (DE3)-competent cells. We used empty L4440-transformed HT115 cells as control. Single colonies of HT115/L4440-SnJHER1276 and HT115/L4440 cells were cultured in LB at 37°C with shaking at 220 rpm overnight. The cultures were diluted 50-fold in 100 ml LB supplemented with 100 μg/ml ampicillin plus 15 μg/ml tetracycline (Sigma) and cultured at 37°C to OD600 = 0.5. After induction of T7 RNA polymerase expression with 400 μM IPTG for 4 h with continuous shaking at 37°C, the bacteria were centrifuged (5000 g, 10 min) and the pellet was re-suspended with 0.5 ml of water. Total RNAs were extracted from bacterial cells using Trizol (Sigma). To remove excess ssRNA, RNA samples were treated with 1 μg of RNase-A (Sigma) in the presence of 0.3 M NaCl, which protects against digestion of dsRNA. The reaction occurred for 10 minutes at 37°C. The length and the quality of the produced dsRNAs were confirmed by electrophoresis on 1% agarose gel (**Figure 4**). As shown in **Figure 4** in the no RNase-A lane, no band was observed for the control L4440-transformed bacteria while a band running to ~1200 bp was observed in the L4440-SnJHER1276-transformed bacteria. After adding 1 μg of RNase-A, all RNAs were degraded leaving unaffected the DNA band. In the third lane after co-incubating dsRNAs with 1 μg of RNase-A in salinity of 0.3 M NaCl, only ssRNAs are degraded while dsRNAs remain unaffected. It was reported that RNase-A in high salinity buffers selectively digests ssRNAs leaving undigested the dsRNAs [43]. An alternative of the previous experiment was published in our laboratory by Kontogiannatos et al. [12]. In this experiment, we used a JHER hairpin expressed in pGEM T-easy plasmid which was used to transform HT115 cells and induce dsRNA expression.

Figure 4. Bacterial-mediated production of SnJHER1276 in HT115 cells. Total RNA isolated from L4440 or L4440-SnJHER1276-transformed HT115 cells was analyzed in 1% w/v agarose gel. RNA extracts were treated with RNase A or RNase A+0.3 M NaCl. M: Molecular marker. Production of ds*SHER*1276 is indicated.

There is an alternative procedure for bacterial-mediated RNAi in which dsRNAs are first isolated from bacteria before injection into insects. In the following experiment, we silenced *S. nonagrioides ultraspiracle* gene after injecting bacterially expressed dsRNAs into its hemo-lymph. For doing that, we cloned a 689-bp fragment of *SnUSP* partial cDNA sequence (GenBank: JN704569) to the L4440 plasmid which was then used to transform competent HT115 cells. The protocol described previously was used to induce dsRNA expression. HT115 ds*SnUSP* expressing bacterial cells were Trizol treated and total RNAs were extracted and analyzed in 1% w/v agarose gels. In this case, we did not perform RNase-protection

assay since dsRNAs were clearly visualized (**Figure 5B**). Note that in **Figures 4** and **5B**, a 1000-bp band exists in the empty L4440-transformed bacteria which disappear when the gene of interest is cloned. Total RNA extracts were purified and injected in fifth instar larvae at day 3 (L5d3) and specific phenotypes were observed (**Figure 5C**). A proportion of ds*SnUSP689*-injected animals died presenting a large range of developmental abnormalities. Gene-specific downregulation was observed after RT-PCR analysis (data not shown).

2.4. Plant-mediated RNAi

The observation that genetically modified plants expressing dsRNAs targeting specific insect genes could induce RNAi in the insect pests was first reported in independent publications of Baum et al. [8] and Mao et al. [9]. Baum et al. showed that corn plants expressing hairpin dsRNAs that target the A subunit of ATPase gene in the western corn rootworm were sig-
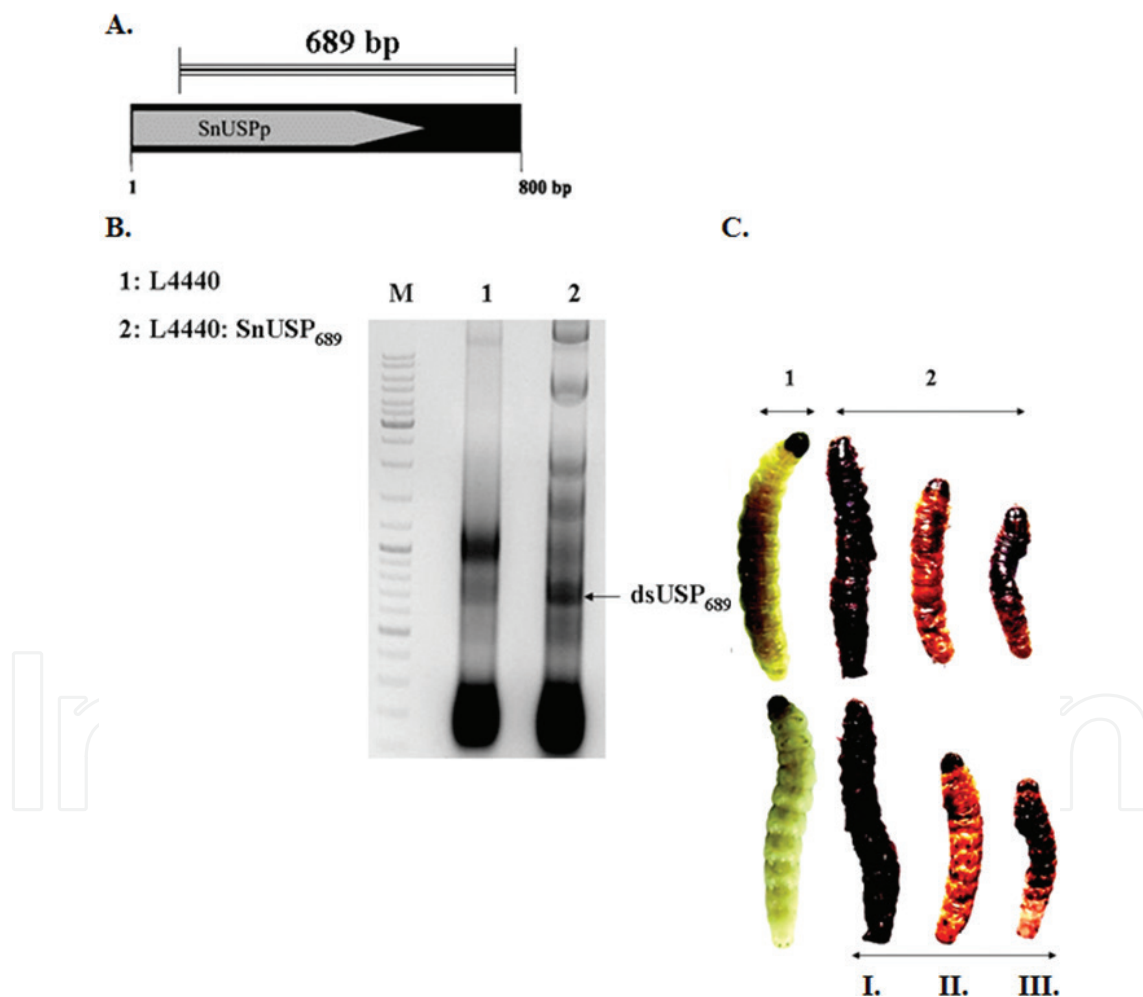

Figure 5. Phenotypes observed after knockdown of *SnUSP* by dsRNA produced in bacteria. **A**. Schematic representation of the partial cDNA sequence of *Sesamia nonagrioides ultraspiracle* gene (GenBank: JN704569). The bar above the sequence indicates the 689 bp fragment used for RNAi experiments. **B**. Confirmation of dsUSP689 synthesis in HT115 IPTG-induced bacteria. Total RNAs isolated from IPTG-induced HT115/L4440 (1) and HT115/L4440:SnUSP689 (2) bacteria were analyzed in 1% w/v agarose gels. **C**. Lethal phenotypes of L5d3-injected *S. nonagrioides* larvae injected with total RNAs isolated from the HT115/L4440:SnUSP689 (2) bacteria. Control HT115/L4440-injected animals are also indicated (1).

nificantly protected by the damage caused by this pest [8]. Furthermore, *Arabidopsis* plants expressing dsRNA hairpins targeting the cytochrome P450 monooxygenase gene in the corn pest *H. armigera* led to decreased resistance to the sesquiterpene gossypol to the feeding insects [9]. Following these publications of proof-of-principle, several other reports have documented the successful application of plant-mediated RNAi:

- In *H. armigera*, a molt-regulating transcription factor gene was selected to be used in plant-mediated RNAi experiments. Four different fragments covering the coding sequence of *HaHR3* were initially tested by bacterial-mediated RNAi. The most effective fragment (in terms of RNAi efficiency) was used for *Nicotiana tabacum* agrobacterium-mediated transformation. When *H. armigera* larvae were fed the *E. coli* or transgenic plants, the HaHR3 mRNA and protein levels dramatically decreased, resulting in developmental deformities and larval lethality [44].

- Similarly, dsRNAs of the gap gene hunchback (hb) of *Myzus persicae* were overexpressed in transgenic tobacco. Continuous feeding of neonate aphids on transgenic diet reduced *Mphb* mRNA level in the fed aphids and inhibited insect reproduction [35].

- In the brown planthopper *N. lugens*, a common 360-bp fragment between ecdysone receptor (EcR) NlEcR-A and NlEcR-B genes was used to construct a transgenic RNAi rice line. After newly hatched nymphs of *N. lugens* fed on the transgenic rice lines, effective RNAi was observed. The NlEcR expression levels were decreased in all lines compared with the controls. In all lines, survival rates of nymphal stages were nearly 90%, but the average number of offspring per pair in the treated groups was significantly less than that observed in the control, with a decrease of 44.18–66.27% [45].

- Efficient RNAi after plant-mediated dsRNA delivery was reported in the *Triticum-Sitobion* system. *S. avenae* fed on transgenic *T. aestivum* lines expressing dsRNAs of its carboxylesterase (CbE E4) gene presented reduced CbE E4 gene expression. The number of aphids grown on transgenic *T. aestivum* lines was lower than the number raised on non-transgenic plants. CbE E4 enzyme isolated from *S. avenae* fed on dsCbE plants hydrolyzed only up to 20–30% Phoxim solution within 40 min whereas a solution of the enzyme from CbE E4 fed on control plants hydrolyzed 60% of Phoxim solution within 40 min [46].

- Efficient plant-mediated RNAi was also reported in *H. armigera* [47]. Researchers used this technology to silence the *arginine kinase* (*AK*) gene of *H. armigera* (*HaAK*), encoding a phosphotransferase that plays a critical role in cellular energy metabolism in invertebrate species. Transgenic *Arabidopsis* plants producing *HaAK* dsRNAs were generated by *Agrobacterium*-mediated transformation. The feeding bioassays clearly showed that resistance of transgenic *Arabidopsis* plants to *H. armigera* was improved and levels of *HaAK* transcripts were drastically suppressed.

- Parental RNAi after plant-mediated dsRNA delivery was observed in *M. persicae*. DsRNA producing *A. thaliana* lines were constructed to target genes with different functions in the aphid. RNAi-mediated knockdown in aphids was achieved independently of gene identity and function and could reduce original expression levels by 70% between 4 and 8 days after feeding on dsRNA-producing transgenic *A. thaliana*. Target genes were also

downregulated in nymphs born from mothers exposed to dsRNA-producing transgenic plants and the RNAi effect lasted twice as long (12–14 days) in these nymphs [48].

2.5. Virus-mediated RNAi

The use of viruses is a less common methodology to transfer dsRNAs into the insect tissues. Virus-mediated-RNAi involves the expression of a dsRNA transgene into a virus which is then used to infect the insect cell or a tissue in order to express dsRNAs intracellularly. This methodology has not been used extensively because of the general viral interference with normal cell physiology; for instance, baculoviruses cause high lethality and potential phenotypes could not be distinguished between dsRNA-producing and control viruses. In addition, viruses can produce inhibitors of RNAi, thereby lowering silencing efficiency [49].

In order to successfully distinguish effects of virus-mediated RNAi, wild-type viruses should be somehow inactivated or at least should not cause highly toxic effects in the insect host. The first report of successful viral dsRNA delivery was made by Hajos et al. [50]. In this paper, the researchers used a recombinant baculovirus, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), to express *Heliothis virescens* juvenile hormone esterase (JHE) gene in antisense orientation, driven by the viral p10 promoter. Infection with this recombinant virus greatly reduced the hemolymph JHE levels and resulted in aberrant morphogenesis of final-instar *H. virescens* larvae. This was the first time that baculovirus-mediated gene silencing could be accomplished and utilized to dissect insect development and to design a new class of baculovirus-based insecticides.

One of the most interesting virus-mediated RNAi reports is by Uhlirova et al. [51]. In this paper, researchers used a recombinant Sindbis virus as a tool to silence the gene encoding for the transcription factor Broad-Complex (BR-C) in *B. mori*. Sindbis virus with a BR-C antisense expression cassette reduced BR-C mRNA expression levels in infected tissues via an RNAi mechanism. BR-C silencing resulted in developmental arrest at the larval-pupal transition or in defective differentiation of adult compound eyes, legs and wings. Also the programmed cell death of the larval silk glands was prevented after RNAi of BR-C. *B. mori* nucleopolyhedrovirus (BmNPV)-mediated RNAi was demonstrated by our laboratory in a work by Kontogiannatos et al. [12]. Even if BmNPV's spectrum is extremely narrow, infection of the non-target species *S. nonagrioides* is feasible. In order to assess which kind of baculovirus is suitable for infection of *S. nonagrioides* larvae, we performed bioassays with genetically modified AcMNPV and BmNPV viruses expressing a GFP cassette under the control of *B. mori* actin promoter. Infected insects with 10^7 pfu/ml of the AcMNPV-BmA::GFP virus had significant higher mortality rates in almost 90% of the total injected animals. These animals presented all typical symptoms of polyhedrosis. In addition, the survived animals presented several developmental abnormalities, failing to complete normally their developmental cycle. In contrast to AcMNPV, infected insects with 10^7 pfu/ml of the BmNPV-BmA::GFP virus were able to proceed through their developmental stages and none of them presented signs of polyhedrosis. Both viruses were located mostly in the fat body tissues, in hemolymph (**Figure 6**), in epidermal cells and in tracheoles of the infected animals [12].
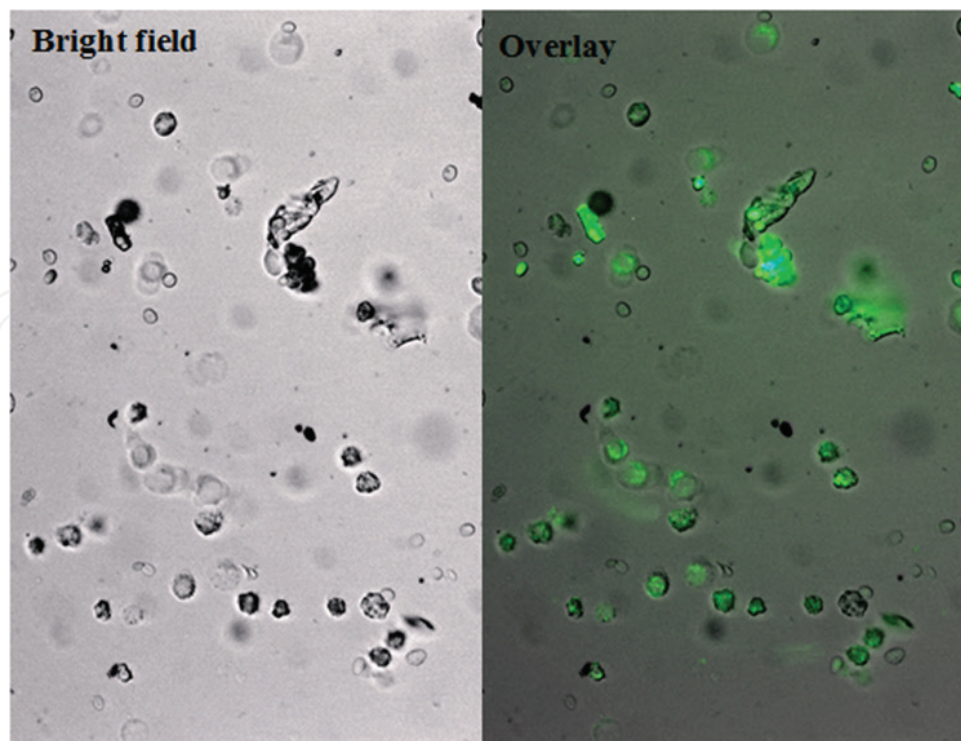
Figure 6. Hemolymph cells (20× magnification) of *S. nonagrioides* fifth instar larvae infected with the BmNPV-BmA::GFP virus, 7 days PI. Left. Bright field imaging of hemolymph cells infected with BmNPV-BmA::GFP. Right. Overlay of bright and fluorescence field images of infected *S. nonagrioides'* hemolymph cells.

The previous experiments showed that BmNPV should be the appropriate vector to transfer dsRNAs in *S. nonagrioides* cells. But this methodology should not be used for functional analysis of genes implicated in larval-pupal transformation since we observed that when insects were infected with either the AcMNPV-BmA::GFP or the BmNPV-BmA::GFP virus in the prepupal stage, larval-pupal transition was blocked while singular adults emerging from surviving pupae were also abnormal. Taking the above observations into consideration, it was nevertheless clear that the infection of *S. nonagrioides* larvae with a BmNPV-BmA::GFP-JHER472 hairpin-expressing virus resulted in specific gene downregulation with similar phenotypes than those after intra-hemolymph dsRNA administration [12].

Therefore, this methodology should be improved in order to create genetically modified baculoviruses that will cause even less physiological impact to the infected cells and to allow more clearly the distinction between infection-related effects and those caused by RNAi.

3. Two bottlenecks for efficient RNAi in insects: dsRNA stability and dsRNA uptake

Recently, several studies were published that focused directly on the causes for the variability of RNAi efficiency among different insect groups [52–54]. Beetles (Coleoptera) and cockroaches (Blattaria) are very sensitive to RNAi that is administered by injection or feeding;

locusts (Orthoptera) are sensitive to RNAi by injection but are refractory to RNAi by feeding, while caterpillars (Lepidoptera) are refractory to RNAi by both injection and feeding. A series of experiments with insects as well as derived cell lines investigated differences in cellular uptake of dsRNA as well as degradation of dsRNA among the different groups. One important finding was that dsRNA degradation correlated negatively with RNAi efficiency, that is, insects with low efficiency in RNAi (e.g. lepidopterans) degrade dsRNA faster in hemolymph and midgut than insects with high efficiency in RNAi (e.g. coleopterans) [52, 54]. The second important finding relates to the cellular uptake of dsRNA: while both lepidopteran and coleopteran cells can take up efficiently labeled dsRNA from the extracellular medium by endocytosis, this results in the production of siRNAs and silencing of target genes only in the coleopteran cells [52, 53]. The use of pH-sensitive dyes coupled to dsRNA molecules suggests that in lepidopteran cells endocytosis of dsRNA is followed by fusion of endosomes with lysosomes and subsequent degradation while in coleopteran cells presumably endosomal escape can occur and subsequent interaction of dsRNA with the RNAi machinery [53]. In lepidopteran cells, the core RNAi machinery works very efficiently [55] and the obstacle seems to be mainly the efficient arrival of the dsRNA trigger at the intracellular RNAi machinery.

These investigations indicate that the use of "naked" dsRNA to trigger RNAi by feeding will only work efficiently in beetles and cockroaches and that for other insects special delivery systems need to be developed. In the first instance, those delivery systems need to protect the dsRNA trigger from degradation in the midgut, an effort which is especially difficult to achieve for lepidopterans in which the alkaline gut content is prone to destabilize dsRNA even in the absence of nucleases [56]. Second, methods need to be developed for more efficient "functional" uptake of dsRNA in locusts and caterpillars in the sense that the internalized dsRNA is not degraded but efficiently presented to the RNAi machinery. Besides naked dsRNA, other delivery methods using bacteria, algae, plants, symbionts and viruses have been tested with variable efficiency [e.g. 36, 55, 57–59] but mechanistic details of how dsRNA escapes from the different vehicles to enter the cellular cytoplasm are lacking and should become an active area of research in the future.

Also synthetic nanoparticles are being tested for delivery of dsRNA in insects [23, 60]. Of note, chitosan dsRNA/siRNA nanoparticles have been reported to efficiently trigger RNAi in mosquito larvae [61, 62] and detailed protocols were published to achieve efficient gene silencing by this method [62]. In relevance to the discussion above, nanoparticles can be engineered to stimulate endosomal escape [63] and cell-penetrating peptides can be harnessed to deliver nucleic acid cargo directly to the cytoplasm [64, 65]. Moreover, fusions with viral capsid proteins have been used to deliver protein toxins to the hemocoel of insect pests [66]. Thus, research into the mode by which insect viruses can penetrate the midgut epithelium can lead to new biotechnological applications for efficient delivery of dsRNA/siRNA cargo to specific insect pests [67].

4. Commercialization of RNAi for crop protection

Because of the increasing burden of chemical pesticides (residual toxicity in the environment, pesticide resistance), interest for developing biological pesticides has expanded significantly during recent years. Biological pesticides are pesticides derived from natural materials such

as animals, plants, bacteria and certain minerals [68, 69]. Biological pesticides include microbial pesticides (e.g. parasitoid wasps, predatory bugs), microbial pesticides (e.g. *Bacillus thuringiensis* (Bt) spores), semiochemicals (e.g. pheromones as attractants), natural products (e.g. fermentation products from bacteria such as *Streptomyces* and *Saccharopolyspora*) and plant-incorporated protectants (e.g. transgenic plants that produce Bt toxins). Advantages of biological pesticides are their reduced toxicity and reduced risk of persistence in the environment, increased specificity toward targeted pests and decreased risk for development of resistance. Biological pesticides often also represent a new mode of action and can thus be employed in combination with conventional pesticides to reduce their dose and environmental impact. The field of biological pesticides is now considered as a big opportunity for expansion since farming is becoming more and more environmentally responsible in the world. In the European Union, for instance, new guidelines are being developed for the promotion of the use of safer pesticides and biological pesticides are considered a major part for this solution [70].

RNAi technology is one of the most recent trends in the field of crop protection and conceptually approaches the "ideal" of the perfect pesticide: it targets only the intended pest and is predicted to have minimal impact on non-target organisms (pollinators, parasitoids, predators and vertebrates). Furthermore, it is biodegradable and therefore with minimal risk for human health and the environment. Because dsRNA is either produced enzymatically in vitro or in genetically transformed bacteria, it can be considered as a "natural product" (biological pesticide). However, RNAi technology, as with most biological pesticides, may be less effective than conventional chemical pesticides (slower killing may necessitate multiple applications) and associated with higher cost of production. Because of the "biological" nature, shelf life may be lower. Because of the novelty of the technology, delays can occur during registration for commercialization.

A major issue with RNAi technology so far concerns its efficiency. The success of RNAi to control pests seems to be mainly determined by the efficiency of delivery of dsRNA, the trigger of RNAi. Major obstacles in the success of RNAi are the uptake of dsRNA/siRNA by the cells and its stability (resistance to degradation) in the gut and the tissues [71–73]. Because of these issues, RNAi as an economically viable approach to control insect pests so far is limited to two coleopteran species, the western corn rootworm (*D. virgifera*) and the Colorado potato beetle (*L. decemlineata*), that are extremely sensitive to environmental RNAi [8, 74]. For other species, most notably non-coleopterans, it is perceived that the development of specialized "formulations" is necessary to deliver dsRNA with sufficient efficiency to cause an impact on larval growth and crop damage [75]. Specialized formulations could be tailored to the ecological and physiological characteristics of the targeted pests to stimulate oral uptake (e.g. food attractants), stability in the gut (e.g. chitosan nanoparticles) and interaction with gut epithelium (e.g. specific interaction with membrane proteins).

For the control of the two coleopteran pests mentioned above, two different strategies are used. A spray of dsRNA can be used to control infestation of potato plants by *Leptinotarsa*; this approach requires techniques for the (cheap) production of large amounts of dsRNA [74, 76]. For the control of *Diabrotica*, on the other hand, transgenic corn can be used that produces RNA hairpins targeting the genes of the pest [8]. Both approaches seem to be close

for commercialization. While RNAi technology could be used as a method to directly affect insect growth and mortality, its use as a "synergist" to enhance the effects of other pesticides also can have important applications. For instance, RNAi is proposed as a "pyramided" insect protection trait in genetically modified crops [49]. In "pyramided" protection traits, different insecticidal compounds are employed that act with an independent mode of action in insect pests. RNAi technology and Bt toxins provide such an example because their mode of action is entirely different and also the dynamics of their toxicity is complementary: while Bt toxins act fast but are less persistent, dsRNA achieves its strongest effects after extended periods. Because of the decreased risk of development of pesticide resistance, the combination of two independent protection traits in a crop can be considered as an alternative to the high-dose approach of a single protection trait and will reduce the refugee requirements for genetically modified crops.

RNAi technology can also be used to increase the efficiency of conventional pesticides [77]. RNAi can be used to decrease the expression of both the targets (for instance, acetylcholinesterase in case of organophosphates and carbamates) and the detoxifying enzymes (for instance, cytochrome P450 enzymes, carboxylesterases and glutathione-S-transferases) of chemical insecticides to increase their effectivity. In combination with RNAi, chemical pesticides might be employed effectively at lower doses, thereby increasing the safety of their applications. Thus, the employment of RNAi technology in pesticide formulations is an important area for further applied research with potential for commercialization.

5. Conclusion

RNAi has been successfully applied in entomological research to analyze gene function in homeostasis, development, immunity and reproduction of insects. Furthermore, the potential of RNAi to control agricultural insect pests and vectors of human disease was revealed. In this review, an overview was presented of the success of the main methods of RNAi delivery (injection and feeding) and the use of different delivery vehicles was discussed (naked dsRNA versus bacterial-, plant- and virus-mediated RNAi) together with highlights of our own experiences with the lepidopteran pest *S. nonagrioides*. Our experiences and a survey of the literature indicate the usefulness to try different approaches for the delivery of dsRNA to achieve successful gene silencing. Regarding injections of dsRNA, we observed much more efficient gene silencing and phenotypic effects in the prepupal stage than in the larval stage and this effect was dependent on the length of the injected dsRNA fragment [12]. Thus, the success of the method of dsRNA injection may be dependent on the size of the dsRNA and the developmental stage of the insect. For feeding of bacteria expressing dsRNA, silencing of targeted genes was observed but phenotypic effects were limited, indicating insufficient delivery of the dsRNA trigger in our experimental system. Finally, in our work [12], we have pioneered the method of virus-mediated RNAi. Baculoviruses that express an RNA hairpin directed against the *JHER* gene could induce specific developmental phenotypes during metamorphosis in *Sesamia*. This approach, however, needed large groups of animals for evaluation since common effects on metamorphosis were observed after infection with

baculoviruses that express specific (targeting *JHER*) and non-specific (targeting *luciferase*) hairpins. Engineered baculoviruses that are deficient in genes targeting the insect hormonal system (e.g. ecdysteroid glucosyltransferase) could be tested in future experiments to allow easier evaluation of phenotypes.

While clear successes in RNAi-mediated gene silencing were achieved and commercial application is expected for pest control in a few instances before the end of the decade, it is equally clear that much needs to be learned about the RNAi process in insects. Further research should focus on the deeper understanding of the process of RNAi in insects, especially with respect to the uptake of dsRNA from the environment. Recent research indeed indicates that efficient delivery of dsRNA is essential for robust RNAi-mediated-silencing effects. Improvements in RNAi should focus on increased stability of dsRNA in the environment, gut content and insect tissues and the development of efficient vehicles for effective release of dsRNA in the cytoplasm (as opposed to endocytotic vesicles) of the targeted cells. Only detailed understanding of the process of RNAi in insects, taking into account their different physiology and ecology, will allow us to develop the tools for robust triggering of gene silencing and to realize its full potential for insect pest control.

Author details

Anna Kourti[1]*, Luc Swevers[2] and Dimitrios Kontogiannatos[1]

*Address all correspondence to: akourti@aua.gr

1 Department of Biotechnology, School of Food, Biotechnology and Development, Agricultural University of Athens, Athens, Greece

2 Insect Molecular Genetics and Biotechnology Group, Institute of Biosciences & Applications, National Centre for Scientific Research "Demokritos", Athens, Greece

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