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RNAi in Agriculturally-Important Arthropods

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1. Introduction

This chapter was inspired by rapid developments in the field of RNA interference (RNAi), an evolutionarily-conserved cellular mechanism that directs protection against nucleic-acid invaders (e.g., viruses and repetitive DNA sequences including transposable elements) in plants, animals, protozoans and fungi. It is now evident that prokaryotes also possess an RNA-based defense system, though completely distinct from that in eukaryotes. The recent discovery of post-transcriptional gene silencing (PTGS) has generated tremendous interest in basic and applied research, including the development of *in vitro* and *in vivo* therapeutic approaches to reduce expression of disease-associated genes. This cutting-edge technology has already been successfully used in genomic manipulations of insect disease vectors, such as development of Dengue-resistant mosquitoes (Blair et al., 2006; Franz et al., 2006 ; Mathur et al., 2010).

Our aim in this chapter is to provide an overview of the profound knowledge accumulated in recent years from invertebrate RNAi studies, but with a focus on agriculturally important arthropods. We start with a brief discussion of the RNAi mechanism to introduce readers to key concepts that underlie the practical application of RNAi discussed in the remainder of the chapter. Our discussion will include a number of important issues that should be carefully considered when working with multi-cellular organisms, including bidirectional transport of silencing signals, processing of dsRNA, homology-driven mRNA degradation and subsequent gene silencing.

We will look at examples of gene silencing in different arthropod systems to illustrate commonalities and differences found in their silencing machinery. Arthropods are a diverse group of organisms, including ticks, mites, spiders, crabs, and insects, many of which are economically significant pests of agricultural crops, parasites of commercially managed pollinators and vectors of livestock diseases.

Considering the huge economic impact of honey bees on crop production, and particularly on pollination of specialty crops, a substantial part of this chapter will be dedicated to honey bee research. Recently, catastrophic losses of honey bee colonies drew national media attention and ignited a renewed interest in basic and applied bee research (Aronstein et al., 2006; Campbell et al., 2010; Dearden et al., 2009; Maori et al., 2009a, 2009b; Nelson et al., 2007). The latest achievements in basic honey bee research provide a better understanding of bee physiology and behavior. Analysis of gene function by selective gene silencing has been

a powerful tool to dissect the complex mechanisms regulating biological processes involved in bee development, immunity, olfaction, learning and memory. Applied studies have focused on practical implementation of RNAi for control of honey bee diseases and parasites that cannot be achieved using conventional management techniques (Liu et al., 2010; Maori et al., 2009a, 2009b; Paldi et al., 2010). Examples will include: 1) development of RNAi-based control for *Nosema ceranae,* an intracellular parasite infecting adult bees, and 2) development of an antiviral treatment to protect bees against Israeli Acute Paralysis Virus (IAPV) and Chinese Sacbrood Virus (CSBV).

The success of RNAi technology as a control method for agricultural pests depends heavily on target specificity, gene-silencing efficiency and systemic spread of silencing. Originally, systemic RNAi was thought to be unique to plants and nematodes, however recent research revealed that systemic transfer of a silencing signal occurs in many arthropods. The first commercial use of RNAi in agriculture was demonstrated via oral RNAi in transgenic plants (*in planta* RNAi) toward several economically-important coleopteran pests, including the Western corn rootworm, *Diabrotica virgifera virgifera* and Colorado potato beetle, *Leptinotarsa decemlineata (*Baum et al., 2007). Oral RNAi has also been reported in Diptera, Hemiptera, Hymenoptera and Lepidoptera (Araujo et al., 2006; Aronstein et al., 2006; Lehane et al., 2008; Turner et al., 2006; Walshe et al., 2009). Our discussion will focus on the successful use of this strategy, as well as the challenges encountered by researchers seeking to use this sensitive, targeted approach to pest control.

We include an overview of the development of RNAi in the coleopteran model and stored product pest, the red flour beetle, *Tribolium castaneum*. The fact that the *T. castaneum* genome has been sequenced, coupled with the beetle's ability to mount a robust, systemic RNAi response to injected dsRNAs, makes it an excellent model for RNAi. Of particular interest is the discovery of new pest control targets through a candidate gene approach to RNAi. While much can be learned from small-scale RNAi studies, we will also discuss a large-scale RNAi-based screen in this beetle.

The first high-throughput RNAi screens in insects were performed in *D. melanogaster* cell lines (Bellés, 2010; Boutros & Ahringer, 2008; D'Ambrosio & Vale, 2010). Since then, genome-wide screens have revealed the function of genes involved in phenotype (physiology/morphology), neurobiology, signal transduction, ion transport, pathogen response, as well as metabolic and gene-processing pathways, among others (Mummery-Widmer et al., 2009). Currently efforts are underway to apply whole-genome RNAi screens in economically important arthropods, such as the cattle tick, *Rhipicephalus microplus* (Kurscheid et al., 2009) and *T. castaneum* (Angelini et al., 2009; Lynch et al., 2009). These and other RNAi-based studies will enable functional analysis of homologous genes in different arthropod species, as well as provide insight into the function of vertebrate homologs that could possibly aid in the identification and validation of drug targets.

2. RNAi mechanisms in arthropods

Like other organisms, arthropods, have evolved efficient homology-driven gene silencing mechanisms for protection against nucleic acid invaders (Deddouche et al., 2008; Gaines et al., 1996; Lu et al., 2004; Olson et al., 1996). Since its discovery, significant efforts have been made to unravel the molecular mechanisms of RNAi. While the core components of RNAi appear to be well conserved across phyla, molecular mechanisms underlying signal amplification and systemic spread of silencing are highly diverged. Improvements in

dsRNA design algorithms and delivery methods triggered an avalanche of new research projects in over 30 insect species (Orthoptera, Dictyoptera, Isoptera, Hemiptera, Coleoptera, Neuroptera, Hymenoptera, Lepidoptera, and Diptera). The most extensive work has been facilitated by sequenced genomes and performed in model insects, including *D. melanogaster*, *T. castaneum* and the silkworm, *Bombyx mori.* The use of RNAi in honey bee research has attracted enormous interest, driven by their economic importance and the sharp increase in colony losses in recent years. Below, we'll discuss some of these issues in attempt to decipher the ambiguities in research findings that, in some cases, generate more questions than answers. Understanding the critical steps in the RNAi process will facilitate the transfer of this technology to additional arthropod species.

2.1 Core RNAi components

The post-transcriptional silencing of gene function is a very rapid process where double-stranded RNA (dsRNA) directs sequence-specific degradation of mRNA. In general, this complicated chain of reactions can be viewed as a two-step process. First, a long dsRNA is cleaved into small interfering RNAs (siRNA), and second, siRNAs are incorporated into silencing complexes (RISC). Following RISC assembly, siRNAs guide degradation of homologous mRNAs (Hammond et al., 2000).

The core components of the RNAi machinery have been thoroughly studied using a combination of biochemical, genetic, and bioinformatic approaches (Hamilton et al., 2002; Hammond et al., 2000; Rolff & Reynolds, 2009; Tomoyasu et al., 2008; Zamore et al., 2000). When dsRNA is introduced into insect cells, it is recognized by a dsRNA-specific RNase-III type ribonuclease called Dicer and cleaved into siRNAs that are 21–23 nt in length. Unlike most animals, insect genomes encode two Dicer-like proteins. One of them, Dcr-2 is involved in RNA interference in *Drosophila*, whereas Dcr-1 recognizes precursors of miRNAs. With the assistance of dsRNA-binding motif proteins (dsRBM), the next phase in the RNAi pathway involves the loading of siRNAs into RISCs. Most evidence indicates that RISCs contain only one siRNA strand, specifically the "guide ssRNA". Therefore, careful selection of siRNA sequences that favor incorporation of the antisense strand into the RISC may improve efficacy and specificity of RNAi. Another essential member of the RISC complex is the RNase H enzyme Argonaute (AGO) that mediates recognition of the target mRNA. Using the siRNAs as a guide, AGO finds complimentary sequences and cleaves homologous mRNA, consequently leading to its degradation.

While most core RNAi components were characterized in *D. melanogaster*, many have been identified in other insects, nematodes and chelicerates. Recently analyzed genomes of the honey bee, red flour beetle, and silkworm (Consortium, 2008; Consortium, 2006; Richards & Consortium, 2008) indicate they each encode the core components of the RNAi machinery, including Dicer enzymes, Ago1 and 2, dsRBMs and other members of the cell-autonomous RNAi machinery.

2.2 Mechanism of dsRNA up-take

In the model nematode, *Caenorhabditis elegans*, systemic RNAi can be induced by dsRNA injection, ingestion or immersion. However, microinjection of dsRNA in *Drosophila* failed to induce systemic RNAi, resulting in the erroneous conclusion that RNAi is a cell-autonomous process in insects (Boutla et al., 2001; Kennerdell & Carthew, 1998, 2000; Roignant et al., 2003). Since then systemic gene silencing has been demonstrated in many

insect species (Amdam et al., 2003; Aronstein et al., 2006; Bucher et al., 2002; Hughes & Kaufman, 2000; Miller et al., 2008; Posnien et al., 2009).

Injection, and in some cases ingestion of long dsRNA, produces very robust silencing effects both locally and in tissues distant from the site of introduction in arthropods, suggesting that the systemic nature of RNAi is conserved among the Arthropoda. This discovery promoted the use of RNAi technology in chelicerates and in a wider range of insect species, especially those for which transgenic protocols have not been developed. However, questions remain about the longevity of this effect in different arthropod species. Most studies report a time or stage when gene silencing was confirmed, but the effect was short-lived and did not necessarily coincide with the observed phenotypic change. In general, RNAi in arthropods is transient. However, in some cases the trans-developmental effects can persist long enough to be observed over several developmental stages (e.g., embryonic, larval and pupal) (Grossmann et al., 2009; Liu & Kaufman, 2004; Ronco et al., 2008; Tomoyasu & Denell, 2004).

In the honey bee, injections of *vitellogenin* (*Vg*) dsRNA into newly emerged workers knocked-down *Vg* expression and dramatically affected the behavior of aged bees, causing a premature shift from nesting tasks to those of foraging (Amdam et al., 2003; Nelson et al., 2007). Injection or oral administration of *Am18w* dsRNA into 5-day-old bee larvae resulted in silencing of this Toll-like receptor transcript and produced significant morphological defects (Fig. 1) in both pupae and adults (Aronstein et al., 2006; Aronstein & Saldivar, 2005).

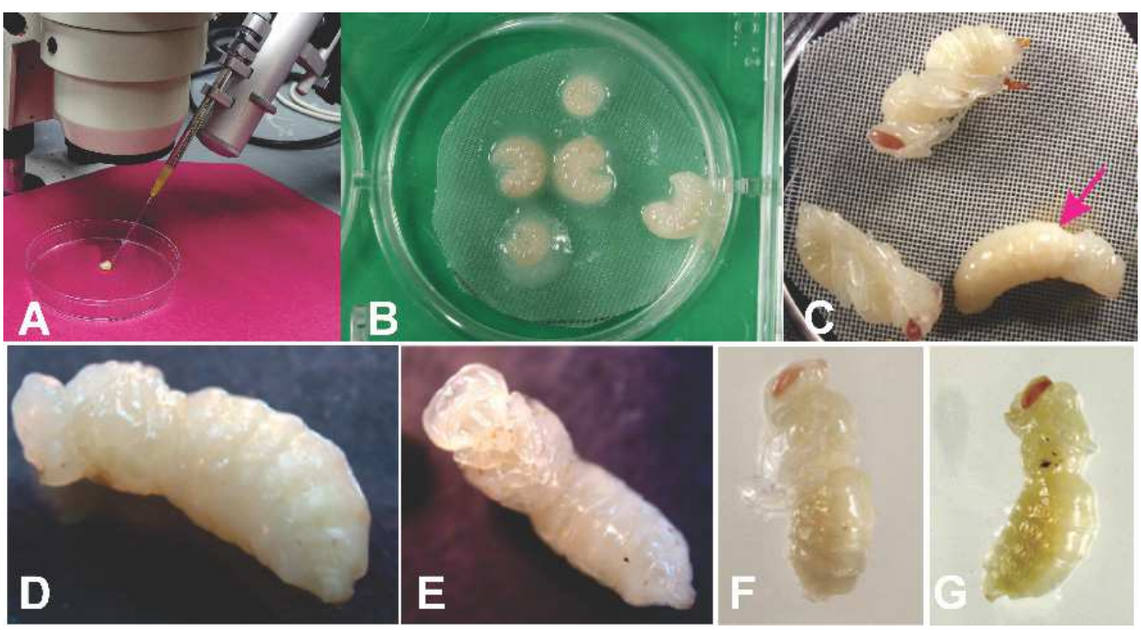

Fig. 1. RNAi-mediated silencing of the honey bee (*Apis mellifera*) *Am18w* encoding Toll-like receptor: 5-day-old-larvae were injected A) or fed/soaked B) with *Am18w* dsRNA as described by Aronstein and Saldivar (2005) and Aronstein et al. (2006). Silencing of *Am18w* resulted in severe morphological defects observed in pupae (C - E) and adult bees (F - G). The abnormalities were most evident in the thorax and head of pupae (C, red arrow), such as complete absence of or shortened appendages, as well as fused body segments.

In the Indian mealmoth, *Plodia interpunctella*, and *T. castaneum*, silencing of a gene encoding tryptophan oxygenase in embryos resulted in loss of eye-color pigmentation in 1st instar

larvae (Fabrick et al., 2004; Lorenzen et al., 2002). Interestingly, the loss-of-function phenotype persisted until pupation in the beetle, approximately four weeks, while the phenotypic changes were not tracked past the initial larval stage in the mealmoth.

In addition to trans-developmental effects, trans-generational RNAi has also been observed in insects. For example, zygotic expression of multiple target genes has been silenced by injecting dsRNA into female pupae or adults (i.e., parental RNAi) in *T. castaneum*, a parasitic wasp, *Nasonia vitripennis,* crickets, *Gryllus bimaculatus* or milkweed bugs, *Oncopeltus fasciatus* (Bucher et al., 2002; Lynch & Desplan, 2006; Mito et al., 2011; Ronco et al., 2008; Tomoyasu et al., 2008; Werren & Loehlin, 2009). Recent *Drosophila* studies demonstrated that flies too have the ability to activate systemic RNAi, albeit in response to viral infection (Saleh et al., 2009).

The ability of RNAi signals to move cell-to-cell within the organism suggests the existence of molecular mechanisms for transporting signals bidirectionally across cell membranes, and from the site of introduction to distant tissues. Moreover, to support the long lasting effect of silencing, there may be an additional mechanism for RNAi amplification. It appears that arthropods can efficiently import and export silencing signals, although molecular mechanisms underlying these processes are still debated. Below we discuss recently proposed models explaining mechanisms of import and export of silencing signals in different arthropod systems.

2.2.1 Sid-dependent up-take

Organisms exhibit substantial differences in their ability to take up and distribute dsRNA. Some are capable of both up-take and systemic spread (Araujo et al., 2006; Dong & Friedrich, 2005; Saleh et al., 2006; Soares et al., 2005; Walshe et al., 2009), while others apparently readily take up dsRNA, but have significant difficulties with systemic distribution (Dietz et al., 2007; Roignant et al., 2003; Van Roessel et al., 2002). However, the inability of an organism to foster systemic effects of dsRNA could be due to shortcomings in methodology which may be mitigated by improved applications, such as extended hairpin RNA-based transgenic RNAi (Artymovich, 2009; Carthew, 2003; Kennerdell et al., 2002), as well as other improvements in RNAi protocols discussed in Section 4.

In nematodes, a two-step model has been proposed to explain the complex mechanism of large (~500 bp) dsRNA transport (Winston et al., 2007). According to this model, ingested dsRNA does not require Sid-1 (a systemic-interference-defective) to pass through the gut lumen in *C. elegans*. Instead, endocytosis of dsRNA by Sid-2, an intestinal luminal transmembrane protein, is proposed for the import of dsRNA across the midgut lining. Vesicles containing dsRNA bud off from the gut lumen and release their content into the body cavity, where they are taken up by cells in different tissues via Sid-1-assisted passive diffusion in a concentration dependent manner (Feinberg & Hunter, 2003; Jose & Hunter, 2007). Several other genes (*Rsd-2*, *Rsd-3* and *Rsd-6*) are involved in the systemic spread of dsRNA in *C. elegans*, which was previously attributed to endocytosis, suggesting that transport of dsRNA in *C. elegans* can also use a Sid-1-independent mechanism.

While some core processes remain common among different arthropod phyla, it appears that mechanisms of dsRNA up-take and transport are largely diverged. A single *sid-1* gene has been identified in most sequenced insect genomes, with the exception of three *sid-1* homologs found in *T. castaneum* and *B. mori* genomes (Aronstein et al., 2006; Aronstein & Saldivar, 2005; Huvenne & Smagghe, 2010; Tomoyasu et al., 2008). However, the correlation

between the presence of *sid-1,* or *sid-1-like* (*sil*), in insect genomes and systemic RNAi is uncertain (Tomoyasu et al., 2008). One reason for this uncertainty is the lack of one-to-one orthology between the *sil* genes and *sid-1*. It has been proposed that the three *sil* genes in *T. castaneum* are orthologus to *C. elegans tag-130,* rather than *sid-1* (Tomoyasu et al., 2008). While *tag-130* is not required for systemic RNAi in nematodes, it is possible that the *sil* genes in *T. castaneum* are orthologous to *tag-130*, but still function in systemic RNAi. On the other hand, it is also possible that the *sil* genes are orthologous to *sid-1,* but play no role in systemic RNAi. However, the most satisfying explanation to date is that the *sil* genes are functional *sid-1* orthologs. This observation correlated with the fact that flour beetles and honey bees are proficient in systemic RNAi, and dipterans (which lack *sil* genes) are extremely poor. The existence of a Sid-2-mediated mechanism is also questionable, as *sid-2* orthologs have not been found in any animal genomes other than *C. elegans*, but this may be due to the rapid evolution of *sid-2* homologs.

2.2.2 Sid-independent transport

While substantial progress has been made towards understanding dsRNA up-take in nematodes, the molecular mechanisms for the transport of silencing signals in other arthropods are still mostly unknown. A new model explaining dsRNA entry and initiation of RNAi silencing in arthropods has been proposed (Saleh et al., 2006; Ulvila et al., 2006). According to this model, dsRNA up-take relies on receptor-mediated endocytosis followed by an active spread of the silencing signal by vesicle-mediated intracellular trafficking (Tomoyasu et al., 2008; Ulvila et al., 2006). The role of SR-CI and Eater in the up-take of dsRNA has been recently tested in *Drosophila* S2 cells, demonstrating that more than 90% of dsRNA up-take depends on these two receptors (Ulvila et al., 2006). However, silencing of 8 Toll and 19 scavenger receptors did not significantly affect the inhibition of RNAi, indicating that other members of pattern recognition receptors must be tested to evaluate the receptor-mediated endocytosis model (Saleh et al., 2006). Furthermore, systemic transport of endogenous silencing signals may be stimulated by specific physiological conditions, such as exposure to viruses. Saleh et al. (2009) demonstrated that receptor-mediated endocytosis is involved in an antiviral response in *Drosophila*. Nevertheless, specific mechanisms associated with dsRNA up-take and transport throughout the body in other insects continues to be the subject of ongoing research.

2.2.3 Mechanism of signal amplification

To sustain silencing, some organisms may employ a strategy for signal amplification. In *C. elegans,* primary siRNAs are amplified through an RNA-directed RNA polymerase (RdRP)-dependent mechanism leading to generation of secondary siRNAs and amplification of silencing (Hamilton et al., 2002). While RdRP activity has not been demonstrated in other organisms, molecular components of this amplification mechanism have recently been identified in the cattle tick genome (Kurscheid et al., 2009). Although insect genomes do not encode a canonical invertebrate RdRP homologue, there is a possibility that RdRP-like activity may occur via other enzymes (Lipardi & Paterson, 2009). The existence of the amplification mechanism in nematodes and possibly in the chelicerate arthropods provides an interesting perspective on the existence of alternative RNAi mechanisms in evolutionarily-diverged groups of animals.

3. Applied research projects using RNAi

Understanding the mechanisms involved in the different phases of gene silencing is absolutely essential for the development of effective RNAi-based applications to control pests of agricultural crops, vectors of livestock disease, and predators and parasites of beneficial insects. RNAi also holds considerable potential as a therapeutic approach to silence disease-causing genes in beneficial insects, particularly important in arthropods deficient in protein-based adaptive immune responses. The identification and utilization of these new approaches in different insect systems may provide more effective control applications. For example, receptor-mediated endocytotic machinery could offer a starting point for novel dsRNA delivery strategies. Below, we describe some of the most recent research findings that harness RNAi technology and hold promise for the development of a new class of therapeutic drugs and pest-control applications.

3.1 RNAi in beneficial arthropods and other non-pests

As honey bees are primary pollinators for most agricultural crops, substantial resources have been devoted to solving recent problems with honey bee health. Therefore, we begin our discussion of RNAi as it relates to agriculture with an overview of the success of the technology related to applications in honey bee research.

3.1.1 Disease control in Honey Bee colonies

The unprecedented loss of honey bee colonies over the past several years has endangered not only the honey bee industry, but also threatens to wipe out agricultural production of crops dependent on pollination. Since 2006, significant colony losses have been reported in many countries around the world, challenging the beekeeping industry to meet pollination demands. The worldwide economic value of the pollination service mainly provided by bees is estimated at $217 billion USD, particularly in added value of specialty crops such as nuts, berries, fruits, and vegetables (Gallai et al., 2009). Among numerous threats, honey bees face diverse parasites and pathogens, some of which are implicated in the massive colony losses, termed Colony Collapse Disorder (CCD) (Genersch, 2010).

Microbial pathogens and parasites in the honey bee colonies are traditionally controlled by antimicrobial drugs and pesticides. Although necessary, these activities, often result in the over exposure of bees to synthetic chemicals that ultimately affect the bee's health and behavior. Misuse of chemicals has also been suspected in some colony losses reported by beekeepers. Therefore, development and implementation of RNAi technology holds great potential for new non-toxic applications for disease control in bee hives. This approach has rapidly emerged as a genetic tool for combating microsporidial and viral diseases in bees. Some bee pathogens, such as *Nosema ceranae* (Higes et al., 2008; Higes et al., 2009) and Israeli Acute Paralysis Virus (IAPV) or a combination of the two (Bromenshenk et al., 2010) , were recently implicated in world-wide losses of bee colonies. Analysis of the *N. ceranae* genome demonstrated the presence of RNA silencing machinery in this species, suggesting that RNAi can be exploited for control of infection within the host (Cornman et al., 2009). Indeed, *in vivo* experiments targeting expression of *N. ceranae* ADP/ATP transporter genes demonstrated inhibiting effects on *Nosema* development, as well as the level of pathogen in the host when fed dsRNA (Paldi et al., 2010). However, activity against *Nosema* in these experimental treatments declined sharply within two to three weeks post treatment.

RNAi has also emerged as an important antiviral defense in insects. Since most honey bee viruses are positive-stranded RNA viruses that generate dsRNA in the process of viral replication, they are particularly vulnerable to the insect's silencing machinery. RNAi applications based on silencing the internal ribosome entry site (IRES) of IAPV were recently tested and showed great potential for developing a novel antiviral drug for use in bee colonies (Maori et al., 2009a, 2009b). One dsRNA product, "Remebee," is currently being tested in a large–scale field trial (Hunter et al., 2010). This product is delivered to bees during routine feeding and is recommended for multiple applications in bee colonies, subject to FDA approval. To control another bee virus, Chinese Sacbrood Virus (CSBV), second instar *Apis. cerana* larvae were fed dsRNA targeting VP1 structural protein (Liu et al., 2010). Silencing of the target gene (*VP1)* was observed 12 h post feeding, but long-term effects of this treatment on the level of CSBV in *A. cerana* have not been evaluated.

We are currently assessing an RNAi–based approach for the control of sexual reproduction in the most prevalent bee fungal pathogen, *Ascosphaera apis* (Aronstein, unpublished). In this study, dsRNAs target newly identified *A. apis* mating type transcription factors (MAT-1 and MAT-2) (Aronstein et al., 2007). If successful, RNAi-based control methods could potentially fill the current void (i.e. no chemical treatments are available for the prevention and/or control of chalkbrood disease in bee colonies).

3.1.2 Silkworm and other beneficials

In *B. mori*, RNAi has been used primarily as a tool to investigate gene function at different developmental stages (Goldsmith et al., 2005). Specifically, RNAi has been used to delineate a number of physiological processes in silkworm, including cocoon pigmentation (Tabunoki et al., 2004), the sex pheromone biosynthetic pathway (Ohnishi et al., 2006), segmentation and appendage formation (Masumoto et al., 2009), and programmed cell death (Lee et al., 2009). Transgenic *B. mori* have been developed with a heat shock inducible and inheritable RNAi system to further probe gene function (Dai et al., 2007). RNAi is also being used to address problems in silkworm culture. For example, transgenic *B. mori* larvae expressing dsRNA targeting a baculovirus gene, *baculoviral immediate early-1* (*ie-1*), induced strong protection against infection by the *B. mori* nucleopolyhedrovirus (Kanginakudru et al., 2007).

Another beneficial, *Nasonia* wasps, primarily parasitize large pest flies, making these four closely related parasitoid species a useful tool for biocontrol. After the honey bee genome, *N. vitripennis* is only the second species of Hymenoptera with a sequenced genome (Rütten et al., 2004; Werren et al., 2010), and is rapidly emerging as a powerful model organism for functional studies. Rapid advances in *Nasonia* genomics have already helped to delineate basic mechanisms of embryonic development (Lynch & Desplan, 2010) and sex determination (Verhulst et al., 2010) in this species, and will help to further improve our understanding of the systemic nature (Werren & Loehlin, 2009) and trans-generational effects of gene silencing (Lynch & Desplan, 2006).

3.2 Using RNAi for pest control

RNAi applications have already demonstrated great success in silencing essential biological functions of many arthropod-pests (Price & Gatehouse, 2008). However, current *in vivo* applications will require substantial improvements in silencing efficiency, stabilization of dsRNAs and improved formulations to be implemented in the field.

3.2.1 Red flour beetle

The red flour beetle has been associated with human agriculture for at least four thousand years. It is a major pest of stored grains worldwide, causing millions of dollars in damage annually. However, *Tribolium* also has become one of the best understood model organisms in biology in the past few decades, and is the first beetle to have a sequenced genome (Richards & Consortium, 2008). As previously mentioned, injection of dsRNA elicits a robust systemic RNAi response in *T. castaneum*, and can be performed during any life stage (Fig. 2).

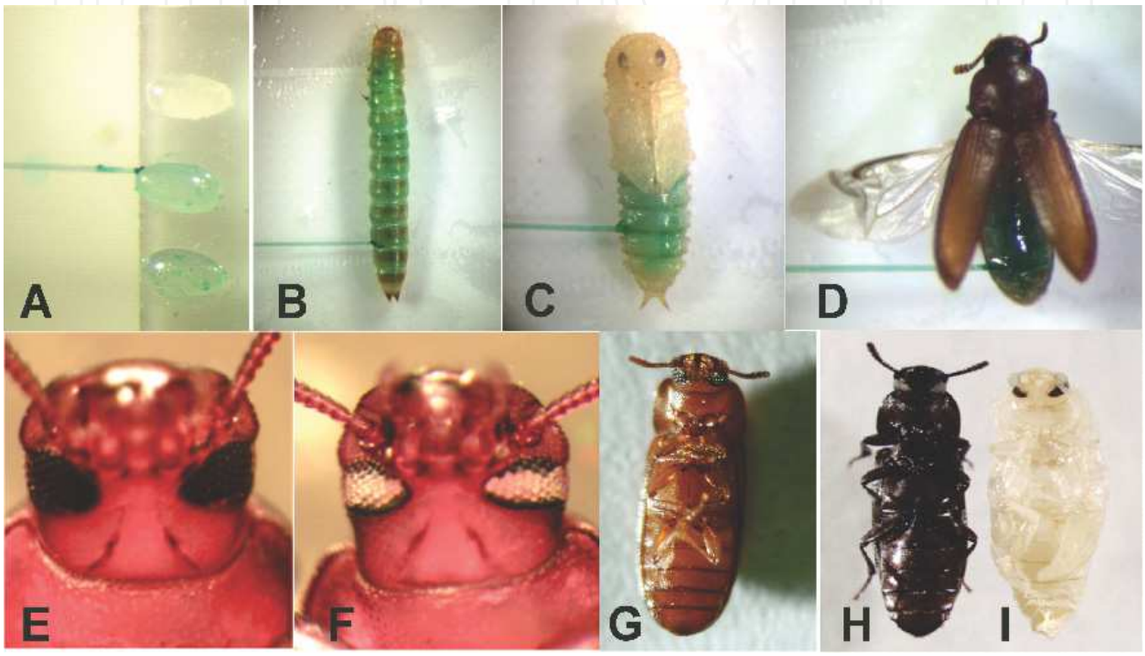

Fig. 2. RNAi-mediated gene silencing in the red flour beetle, *Tribolium castaneum*. RNAi can be performed at any life stage in flour beetles by injecting dsRNAs into A) embryos, B) larvae, C) pupae or D) adults. Injected dsRNAs are generally dyed to provide confirmation of injection (in this case a green dye was used). Effect of *T. castaneum scarlet* (*Tcst*) dsRNA on eye pigmentation. Last-instar larvae were injected with *Tcst* dsRNA and observed as adults; E) uninjected individual with wild-type eye color, F) wild-type individual that was injected with *Tcst* dsRNA during last larval stage. Note absence of eye pigmentation in the ommatidia (black "mascara" around eye is from a different pigment pathway). Effect of *T. castaneum laccase-2* (*TcLac2*) dsRNA on body color. G) wild-type body color, H) "black" body color mutant, I) "black" mutant that was injected with *TcLac2* during last larval stage.

From its initial use in 1999 to phenocopy null mutations of the *T. castaneum Deformed* gene (Brown et al., 1999), to its wide use in functional genomic screens, RNAi has become an extremely valuable tool both to elucidate gene function and to identify potential pest control targets. Here we will discuss key *Tribolium*-based RNAi studies with significance to pest control.

Arthropods are known for their hard chitinous exoskeleton. The chitin/cuticle pathway that gives rise to this arthropod-specific structure has been the object of much interest due to its relevance as a pest control target. While researchers have studied this pathway for many

years, RNAi has revolutionized studies in this field. For example, a group in Manhattan, Kansas (USDA-ARS and Kansas State University) has been using RNAi to identify *T. castaneum* genes that encode proteins responsible for exoskeleton biosynthesis and degradation. Candidate genes from other insects have been selected and orthologs identified via blast analysis of the *T. castaneum* genome database, BeetleBase (http://beetlebase.org/). Prior to RNAi analysis, the spatiotemporal pattern of gene expression is determined via RT-PCR to ensure RNAi is performed at appropriate time points. Chitin/cuticle pathway genes, including those required for chitin synthesis (Arakane et al., 2005b; Arakane et al., 2008), molting, survival and fecundity (Arakane et al., 2010b; Arakane et al., 2009; Arakane et al., 2008; Broehan et al., 2010; Hogenkamp et al., 2007; Zhu et al., 2008) and tanning of the epidermal cuticle (Arakane et al., 2010a; Arakane et al., 2009; Arakane et al., 2005a) have been functionally characterized, revealing a wealth of potential biotargets for arthropod-specific pest control.

While the candidate gene approach has been of enormous value in *T. castaneum*, it imposes limitations due to reliance on sequence conservation. Therefore a genome-wide RNAi screen is underway (Lynch et al., 2009). This project, known as iBeetle, targets all genes (identification based on gene predictions, as well as expression data) at two life stages. Specifically, dsRNAs are injected into 5th-instar larvae (analysis of injected animal), as well as into female pupae (analysis of offspring). Alterations in cuticular phenotypes, fluorescently marked muscles, melanotic stink glands, metamorphosis control and fertility are being scored (G. Bucher, personal communication). Approximately 5,500 genes will be screened by fall 2011, with completion of the project scheduled for fall 2014. The genome-wide collection of PCR templates for dsRNA synthesis in the iBeetle-Library will be made available to the community (http://ibeetle.uni-goettingen.de/resources.html). This invaluable resource will facilitate subsequent RNAi screens for additional processes.

3.2.2 Plant-mediated RNAi in crop pests

Although a comprehensive review of RNAi in plants is beyond the scope of this chapter, it is important to highlight transgenic approaches for generating RNAi-based insect-resistant plants (Artymovich, 2009; Baum et al., 2007; Mao et al., 2007). RNAi applications have been effective in silencing target genes in some insects upon oral administration of dsRNA. Our examples detail how this approach has enhanced plant resistance to economically important agricultural pests, such as the cotton bollworm, *Helicoverpa armigera*, and Western corn rootworm (WCR), *D. v. virgifera*, in commercially produced crops (e.g., corn, cotton, and tobacco).

The key to successful *in planta* RNAi depends not only on the identification of suitable gene targets, but also on the expression and delivery of sufficient amounts of intact dsRNA for up-take by the insects. Examples of successful gene silencing by oral delivery have been described in insect species from different orders (Table 1). With few exceptions, most oral RNAi assays in insects have targeted mRNAs in the gut. The first report of oral RNAi in an insect was in a lepidopteran, the light brown apple moth, *Epiphyas postvittana*, with temporary knockdown of a gut target, carboxylesterase 1, and an adult antennae target, pheromone binding protein 1 (Turner et al., 2006). Since then, additional reports of ingested dsRNA leading to knockdowns in Lepidoptera (Bautista et al., 2009; Mao et al., 2007; Whyard et al., 2009), Coleoptera (Baum et al., 2007; Whyard et al., 2009) , Diptera (Walshe et al., 2009; Whyard et al., 2009), Hemiptera (Price & Gatehouse, 2008), and Isoptera (Zhou et al., 2008) , suggest that knockdown of specific targets may be feasible in most insects.

Order	Insect	Gene Target	LC_{50}	Percent Mortality[1]	Reference
Coleoptera	*Diabrotica virgifera virgifera*	subunits of vacuolar ATPase and others	2-5 ng/cm^2	80-95	Baum et al., 2007
Coleoptera	*Diabrotica undecimpunctata howardii*	subunits of vacuolar ATPase	780 ng/cm^2	45	Baum et al., 2007
Coleoptera	*Leptinotarsa decemlineata*	subunits of vacuolar ATPase	52 ng/cm^2	90	Baum et al., 2007
Coleoptera	*Tribolium castaneum*	vacuolar ATPase E	0.003 mg/g diet	70	Whyard et al., 2009
Diptera	*Drosophila* spp.	vacuolar ATPase E and tubulin γ	0.2-0.6 mg/ml	40-70	Whyard et al., 2009
Diptera	*Glossina morsitans morsitans*	tsetse-EP, a major midgut protein	435 ng/µl	40	Walshe et al., 2009
Hemiptera	*Acyrthosiphon Pisum*	vacuolar ATPase E	0.003 mg/g diet	60	Whyard et al., 2009
Hemiptera	*Rhodnius prolixus*	nitroporin 2	1 µg/µl	not reported	Price & Gatehouse, 2008
Isoptera	*Reticulitermes flavipes*	cellulase hexamerin[2]	15.3 µg/cm^2 6.6 µg/cm^2	75 45	Zhou et al., 2008
Lepidoptera	*Epiphyas postvittana*	carboxylesterase 1 pheromone binding protein 1	1 µg	not reported	Turner et al., 2006
Lepidoptera	*Helicoverpa armigera*	cytochrome P450 (CYP6AE14)	3 mg/g diet	not reported	Mao et al., 2007
Lepidoptera	*Manduca sexta*	vacuolar ATPase E	0.01 mg/g diet	50	Whyard et al., 2009
Lepidoptera	*Plutella xylostella*	cytochrome P450 (CYP6BG1)	662-824 µg/µl	90[3]	Bautista et al., 2009

[1]Values are the maximum observed mortality and are approximate (exact values were not given).
[2]Administered in combination with juvenile hormone.
[3]Decrease in resistance to permethrin was 1-2.6-fold in dsRNA fed larvae compared to control.

Table 1. Reports of oral delivery of dsRNA that reduce the levels of target RNA in insects.

RNAi directed against a number of gene targets (e.g., *b-tubulin*, *v-ATPase A* and *v-ATPase E*) was effective in several economically important coleopteran pests (Baum et al., 2007). Oral RNAi targeting *v-ATPase* subunits and others resulted in significant larval mortality in perhaps the most economically important pest in the U.S., *D. v. virgifera*. It was further demonstrated that *D. v. virgifera* is highly sensitive to knockdown of a gene encoding the delta subunit of the coatomer complex (COPI) that is involved in intracellular protein trafficking. Similar results were obtained with orthologs for *v-ATPase* in Southern corn rootworm, *D. undecimpunctata howardii*, and Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the cotton boll weevil, *Anthonomus grandis*. However, increased sensitivity to the cotton metabolite gossypol was reported in a lepidopteran, the cotton bollworm, *Helicoverpa armigera*, with a silenced cytochrome P450 gene, CYP6AE14 (Mao et al., 2007).
Accordingly, transgenic plants are being engineered to express a variety of dsRNAs to silence important biological functions in insects (Artymovich, 2009). Transgenic corn

expressing a *v-ATPase* subunit A hairpin RNA demonstrated significant protection to damage by WCR larvae (Baum et al., 2007), while cotton engineered to express dsRNA targeting cytochrome P450 *CYP6AE14* caused knockdown of the transcript in the *H. armigera* gut and reduced larval growth (Mao et al., 2007). Thus far, initial laboratory tests of transgenic RNAi approaches to plant protection appear to be as successful as those based on the expression of insecticidal toxins against coleopteran and lepidopteran herbivores. However, further development and refinement of this technology, as well as large-scale field tests will likely be required to know the true potential of *in planta* RNAi.

3.2.3 Chelicerates (ticks, mites, spiders)

Double-stranded RNA has been successfully delivered to ticks via injection (various developmental stages and tissues), infection (viral vector), ingestion (oral), and incubation (whole body soaking) (de la Fuente et al., 2007). Oral RNAi was used to knockdown an anticomplement gene (*isac*) in the blacklegged tick, *Ixodes scapularis*. Affected nymphs weighed less than those fed a control dsRNA (*lacZ*) and had lower spirochete loads when infected with *Borrelia burgdorferi* (Soares et al., 2005). Another medically relevant study demonstrated that dsRNA can be transmitted through the blood-brain barrier in *I. scapularis* (Karim et al., 2008). Cy3-labeled dsRNA specific for either *β-actin* or *Na+-K+-ATPase* was injected into the abdomen of unfed adult females, and β-actin protein or Na+-K+-ATPase activity was measured after supplying a partial blood meal. Labeled dsRNAs were detected in the synganglia (CNS), and effective knockdown was confirmed via tissue-specific RT-PCR.

R. microplus is an economically significant tick ectoparasite that transmits a variety of pathogens, such as *Anaplasma marginale*, thereby increasing cattle exposure to vector-borne infectious diseases. Silencing of a defensin gene, *varisin*, by injection of dsRNA into male ticks reduced their ability to infect calves with *A. marginale* (Kocan et al., 2008). Additional RNAi targets, such as those affecting tick mortality and fecundity, have been identified through RNAi screens in *R. microplus* (Kurscheid et al., 2009).

Parental RNAi was used to knock-down expression of a homeobox gene, *Distal-less* (*Dll*), in the two-spotted spider mite, *Tetranychus urticae* (Khila & Grbic, 2007). Injection of either *Tu-Dll*-specific dsRNA or siRNA into adult female mites resulted in offspring with truncated and fused leg segments. This experiment demonstrated the conserved nature of Dll function and illustrates the power of RNAi in mites.

Efforts to use RNAi to control a major mite parasite of the honey bee are underway. The gene encoding glutathione S-transferase, involved in detoxification of pesticides used to control mites in the hive, was targeted successfully in *Varroa destructor* with dsRNA (Campbell et al., 2010). Two different dsRNA delivery methods were evaluated, microinjection and soaking mites in dsRNA solution. Although injection of dsRNA produced up to 96% silencing of the target (*VdGST-mu1)* gene for over 72 h, there were problems with high mortality in controls. However, encouraging results were obtained by soaking mites in a solution of dsRNA in 0.9% NaCl. The method enables high-throughput screening to identify the best targets for control of this devastating pest of honey bees, even though the exact mechanism of dsRNA up-take is unknown.

3.2.4 Improvements to other insect control methods

Current insect control methods benefit from RNAi studies. For example, the symbionts *Heterorhabditis bacteriophora* (a nematode) and, *Photorhabdus luminescens* (a bacterium) are a

lethal combination in some insects. Using genes described in *C. elegans* as a model, various phenotypes were disrupted in *H. bacteriophora* soaked in dsRNA (Ciche & Sternberg, 2007). These studies facilitate the discovery of genes involved in symbiosis and/or insect pathogenesis.

RNAi has also been used to explore host-pathogen interactions in mosquitoes. One study explored the role of an antibacterial peptide encoded by a defensin family member (DEF) in *Anopheles gambiae* (Blandin et al., 2002). Targeting this *A. gambiae* defensin with gene-specific dsRNA resulted in *DEF* knock down (up to 12 days) and increased the mosquito's vulnerability to Gram-positive bacteria (Blandin et al., 2002). However, *DEF* knock down had no effect on the malaria parasite, *Plasmodium berghei*. To identify genes involved in mosquito immunity to *P. berghei*, researchers (Michel et al., 2005; Osta et al., 2004) targeted a number of candidate genes in *A. gambiae*. They discovered that reduction of transcripts encoding a type-C lectin, a leucine-rich protein or a serpin (SRPN2) were sufficient to make the mosquito refractory to infection by *P. berghei* in the midgut. Such studies may inspire the development of new methods to control protozoan infections in humans (Lehane et al., 2008; Solis et al., 2009; Walshe et al., 2009).

Researchers are also using RNAi to create mosquitoes that are resistant to dengue fever. Franz and colleagues made transgenic *A. aegypti* that express an inverted-repeat specific for the dengue type 2 (DEN-2) virus (Franz et al., 2006). Careful promoter selection resulted in transgenic *A. aegypti* that mount an RNAi response to DEN-2 virus in midgut epithelial cells immediately after a bloodmeal. Moreover, the authors demonstrated that after viral infection, transgenic mosquitoes expressing the hairpin RNA had reduced viral loads and DEN-2 virus-derived siRNAs in the midgut compared to control insects.

In agriculture, RNAi applications are being developed as control strategies for the *Asian citrus psyllid* which vectors Citrus Greening disease (Hunter et al., 2008), the Colorado potato beetle (Zhu et al., 2011), and *Varroa* mites as described above in section 3.2.3. Results such as these bode well for the future of RNAi in pest control strategies.

4. Future studies for practical implementation of RNAi technology

The success of RNAi depends both on the biology of the organism, and the method of dsRNA delivery. Intrinsic factors, such as the efficiency of dsRNA up-take, relative turnover of target mRNA/protein, signal amplification and systemic spread are important for robust RNAi. Extrinsic factors amenable to manipulation include dsRNA concentration, target gene selection, transcript localization, dsRNA synthesis and route of introduction. One of the most important considerations is determining if the observed phenotype is gene-specific or an "off target" effect. Another critical requirement is the stability and intracellular availability of the RNAi signal. Often such requirements can be met by improved delivery systems, such as stable, transgenic expression of hairpin RNAs (Kennerdell & Carthew, 2000; Tavernarakis et al., 2000), or alternatively, by expression of dsRNA in non-pathogenic bacteria that can be fed directly to the target organism, as demonstrated in nematodes (Timmons & Fire, 1998) and arthropods (Tian et al., 2009; Zhu et al., 2011).

Injection of dsRNA can elicit a stress response in some insects and potentially compromise the study. Moreover, mechanical damage to the cuticle and underlying tissue can stimulate innate immunity (Aronstein et al., 2006; Aronstein & Saldivar, 2005; Brey et al., 1993; Han et al., 1999), complicating interpretation of gene expression data. While appropriate controls

(buffer and/or "control" dsRNA) can address some of these issues, alternative approaches for non-viral and virus-mediated dsRNA delivery have demonstrated great potential (Leng et al., 2009; Yuan et al., 2011). Direct delivery of dsRNA (soaking and/or feeding) is particularly popular in arthropod studies and appears to be feasible in at least some hemipteran, coleopteran, lepidopteran, and hymenopteran insects (Araujo et al., 2006; Aronstein et al., 2006; Baum et al., 2007; Eaton et al., 2002; Mao et al., 2007; Turner et al., 2006). However, most currently used methods reveal significant impediments preventing their application in large-scale *in vivo* trials.

To increase the efficiency of RNAi, current and future studies are directed at improving existing methodologies and adapting innovative technologies. A new bacterially-expressed dsRNA delivery technology, TransKingdom RNAi (*tk*RNAi) (Keates et al., 2008; Tian et al., 2009; Xiang et al., 2006; Xiang et al., 2009), recently emerged as a powerful tool for the control of parasites and disease agents in mammalian systems. This method utilizes attenuated, non-pathogenic bacteria that are safe, effective, and inexpensive vectors for delivering RNAi to target cells. Similar methods have been used successfully in nematodes (Newmark et al., 2003; Timmons & Fire, 1998**)**, trophozoites (*Entamoeba histolytica*) (Solis et al., 2009) and other organisms (Keates et al., 2008; Nguyen & Fruehauf, 2008). Bacteria-based RNAi is technically suitable for production of large quantities of dsRNA, and therefore opens interesting perspectives for mass screening of novel gene targets and development of environmentally-safe pest control applications.

The application of RNAi to pest control, while still at a formative stage, is already taking different forms; 1) *in planta* dsRNA expression for the direct protection of crops; 2) bacteria-based dsRNA expression for the indirect control of parasites and pathogens of beneficial organisms; and 3) *in vivo* dsRNA expression for generating disease refractoriness in arthropod vectors of disease. Unlike *in planta* and bacteria-based RNAi, the use of RNAi for the spread of disease refractoriness requires genetic drive to push inheritance of the effecter gene (i.e. hairpin RNA) through target populations.

Among the most promising gene-drive candidates is a synthetic "*Medea*" element (Chen et al., 2007) which is based on the genetic principles observed in the Maternal-Effect Dominant Embryonic Arrest (*Medea*) factors found in *Tribolium* (Beeman et al., 1992; Lorenzen et al., 2008). Heterozygous (*M*/+) females transmit dominant-lethal activity to hatchlings by maternal action, but the lethal effect is manifested only in those progeny that fail to inherit an *M* allele from either parent. Thus, each *M* allele is bifunctional, encoding both a maternally loaded "poison" and a zygoticly expressed "antidote". Current and future efforts need to focus on both the development of improved RNAi effecter genes, as well as on improved methods for driving population replacement if the goal of controlling arthropod vectors of disease by *in vivo* dsRNA expression is to be realized.

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6. Disclaimer

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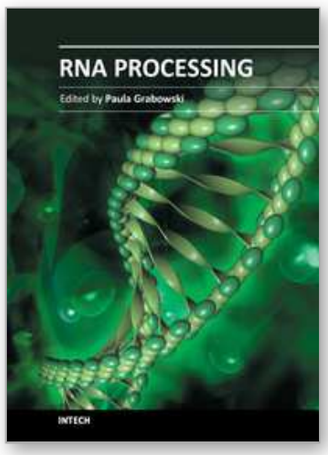

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RNA functions broadly as informational molecule, genome, enzyme and machinery for RNA processing. While these functions reflect ancient activities, they also remain vital components of contemporary biochemical pathways. In eukaryotic cells RNA processing impacts the biogenesis of RNA molecules of essentially every shape and function. The collection of articles in this volume describes the current state of understanding of the broad array of RNA processing events in animal and plant cells, key unanswered questions, and cutting edge approaches available to address these questions. Some questions discussed in this volume include, how viruses subvert the RNA processing machinery of the host cell, how the coordination of co-transcriptional RNA processing is regulated at the level of chromatin, the status of RNA processing in plant organelles, and how micro RNA machinery is biosynthesized and regulated.

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