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FORUM

Insecticidal Gene Silencing by RNAi in the Neotropical Region

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Abstract
Insecticidal gene silencing by RNA interference (RNAi) involves a post-transcriptional mechanism with great potential for insect control. Here, we aim to summarize the progress on RNAi research toward control of insect pests in the Neotropical region and discuss factors determining its efficacy and prospects for pest management. We include an overview of the available RNAi information for Neotropical pests in the Lepidoptera, Coleoptera, Diptera, and Hemiptera orders. Emphasis is put on significant findings in the use of RNAi against relevant Neotropical pests, including diamondback moth (*Plutella xylostella* L.), Asian citrus psyllid (*Diaphorina citri* Kuwayama), and the cotton boll weevil (*Anthonomus grandis* Boheman). We also examine the main factors involved in insecticidal RNAi efficiency and major advances to improve screening of lethal genes, formulation, and delivery. Few studies detail resistance mechanisms to RNAi, demonstrating a need for more research. Advances in formulation, delivery, and resistance management tools for insecticidal RNAi in the Neotropics can provide a basis for efficient field application.

Introduction

In agriculture, chemical pesticides are still the major approach for controlling insect pests, yet their use is associated with significant hazards to the environment and human health. Safer and more environment-friendly alternatives include the use of microbial biopesticides or commercial biotechnology based on the expression of insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) in transgenic crops (Koch *et al* 2015). However, sustainability of these technologies is threatened by the development of field resistance in selected pests of relevance to the Neotropical region, including *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae), *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae), *Helicoverpa zea* (Boddie), and *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Tabashnik & Carriere 2017). Consequently, new biotechnological approaches to pest control are urgently needed.

Insecticidal gene silencing by RNA interference (RNAi) is one of the biotechnological advancements expected to revolutionize pest control (Huvenne & Smagghe 2010). As a tool for functional genomics, RNAi has greatly facilitated studies linking phenotype and gene function (Scott *et al* 2013, Bellés 2010), yet the rapidly expanding literature on potential applications of insecticidal RNAi highlights possibilities for agricultural pest management (Palli 2012, Katoch *et al* 2013, Christiaens *et al* 2018b, Zotti *et al* 2018, Vogel *et al* 2019). Further evidence for the increased relevance of this technology for agriculture is the recent approval for commercialization of a new genetically modified (GM) crop event (MON87411) combining RNAi with production of insecticidal Bt proteins to control the western corn rootworm (*Diabrotica virgifera virgifera*, LeConte) (Coleoptera:

Chrysomelidae) (EPA 2017). Interestingly, recent reports also suggest a potential interaction between RNAi and bacterial infections in insects (Baradaran *et al* 2019), which needs to be further explored.

The mechanism of insecticidal RNAi has been recently reviewed elsewhere (Cooper *et al* 2019) and is activated by the uptake of long double-stranded RNA (dsRNA) molecules in cells. Delivery of dsRNA to insects has been documented by different procedures, including injection, feeding on dsRNA synthesized in vitro or produced by bacteria or transgenic plants, soaking, or produced in viral and bacterial vectors (Christiaens *et al* 2018b). The efficiency of RNAi varies depending mostly on the insect, delivery method, target gene, target tissue, and refractoriness to dsRNA, among other factors (Cooper *et al* 2019). Consequently, no single protocol can be applied to every gene in every insect and target tissue (Scott *et al* 2013). Thus, adaptability of RNAi methodology to control a particular pest must be carefully evaluated prior to deployment, maximizing efficacy and sustainability through appropriate management (Davis-Vogel *et al* 2018a). In this manuscript, we summarize the progress on the use of insecticidal RNAi against pests with occurrence in the Neotropical region and discuss factors determining its efficacy considering the agricultural systems in the Neotropics (Fig 1).

Research on Insecticidal RNAi Targeting Pests from the Neotropical Region

In this section, we provide an overview of the available studies with insect pests that occur in the Neotropical region, focusing on the target-genes, observed phenotype effects, and the main implications of these studies toward pest control.

Lepidoptera

The first report of successful RNAi with a lepidopteran pest of relevance in the Neotropical region was the knockdown of a *tryptophan oxygenase* gene through dsRNA injection in *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) embryos (Fabrick *et al* 2004). Later, RNAi-mediated gene silencing was used as a tool to test functional linkage of three *aminopeptidase N* (*APN*) genes with susceptibility to the Cry1Ab toxin from Bt in *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) (Yang *et al* 2010). Similarly, RNAi was used to test the role of a trypsin protein (SfT6) in susceptibility to the Cry1Ca1 toxin from Bt in *S. frugiperda* (Rodríguez-Cabrera *et al* 2010). Additional studies in lepidopteran species of relevance to the Neotropical region have used RNAi as a tool to establish the functional relevance of *cytochrome P450* and *esterase* genes to susceptibility against pesticides (Tang *et al* 2012, Hu *et al* 2014) and *APN*, *cadherin*, and *ABC transporter* genes in susceptibility to Bt proteins (Porta *et al* 2011, Guo *et al* 2015, Wang *et al* 2017). Additional RNAi experiments using diverse lepidopteran pests found in the Neotropical region are available, including *Helicoverpa armigera* Hübner (Asokan *et al* 2013, 2014), *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Camargo *et al* 2016), *H. zea* (Wang *et al* 2018a), and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Christiaens *et al* 2018a). Common findings in these studies revealed that lepidopterans are generally refractory to effective gene silencing by RNAi, especially when using oral delivery of dsRNA. The two most likely causes for this refractoriness are the rapid degradation of ingested dsRNA in the digestive fluids of Lepidoptera and uptake of dsRNA into degradative cellular organelles (Yoon *et al* 2017). Recent reports suggest strategies to improve RNAi efficiency in Lepidoptera, such as the use of modified or concatemerized dsRNAs (Sharath *et al* 2018; Christiaens *et al* 2018a). Limitations to efficient RNAi in Lepidoptera due to degradation of dsRNA in digestive fluids will be discussed in the "dsRNA persistence in the insect body" section.

The diamondback moth (*P. xylostella*) has been one of the target lepidopteran Neotropical pests tested for susceptibility to RNAi (Bautista *et al* 2009, Gong *et al* 2013). This species is a relevant pest of brassicaceous crops mainly because of its extreme ability to evolve resistance to many classes of insecticides (Talekar & Shelton 1993). A study involving *serpin* genes in the innate immune response revealed highly efficient RNAi-mediated knockdown in *P. xylostella* (Han *et al* 2014). Almost complete knockdown of three individual *serpin* genes after injection of respective dsRNAs only resulted in marginal larval mortality, yet simultaneous injection of the three dsRNAs doubled the levels of mortality. Addition to the dsRNAs of a fungal peptide (*destruxin A*) specifically suppressing the humoral immune response (Pal *et al* 2007) resulted in higher levels of toxicity and larval melanization due to lack of serpin regulation (Han *et al* 2014). Knockdown of a new *ABC transporter* gene in *P. xylostella* (*PxABCH1*) resulted in lethal phenotypes in larvae and pupae, suggesting that this gene may be a good target gene for insecticidal RNAi in this pest (Guo *et al* 2015). In contrast, the knockdown of genes involved in juvenile hormone and ecdysteroid hormonal signaling regulation resulted in more marginal levels of mortality (Chaitanya *et al* 2017). Importantly, improved uptake of dsRNA and RNAi efficiency in *P. xylostella* have been recently reported by using concatemerized dsRNAs targeting the *acetylcholinesterase* (*AChE*) gene (Sharath *et al* 2018). The use of these concatemerized dsRNAs led to the relative formation of a greater number of specific pools of small interfering RNAs (siRNAs) from the dsRNA, enhancing the RNAi efficiency and resulting in almost a 2-fold increase in larval mortality when compared to nonconcatemerized dsRNA formulations.

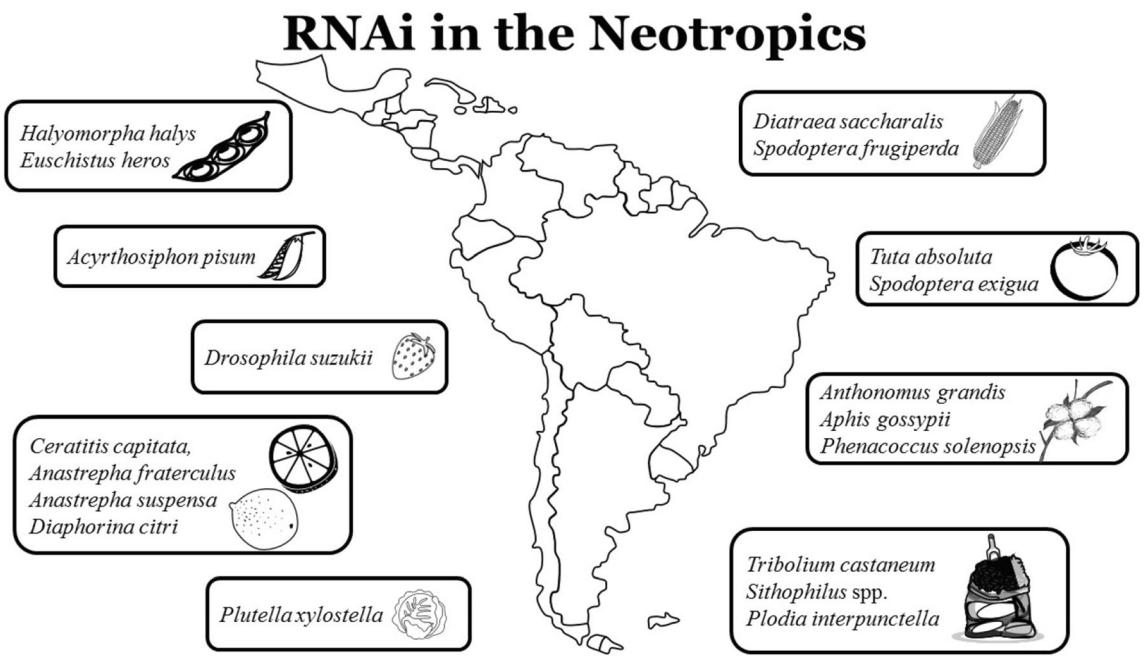

Fig. 1 Insect pests studied in the Neotropical region using the RNAi technique.

Coleoptera

A coleopteran model for RNAi studies has been the red flour beetle, *Tribolium castaneum* (H.) (Coleoptera: Tenebrionidae), given its relevance as major pest of stored grains and cereal products worldwide, and being a well-characterized insect genetic model (Tomoyasu *et al* 2008, Cao 2018, Knorr *et al* 2018). The availability of genetic tools facilitated large-scale RNAi screens for identification and selection of target genes for effective control (Ulrich *et al* 2015).

Other important pests of stored cereals such as *Sithophilus* spp. (Coleoptera: Curculionidae) were studied for exploring gene-specific functions using the RNAi technique (Vallier *et al* 2009). The genus *Sitophilus* includes three species in the Neotropics: the cosmopolitans *Sitophilus oryzae* and *Sitophilus zeamais* and *Sitophilus granarius* present in Argentina, Chile, and Mexico. These species have endosymbionts (γ-Proteobacteria) that balance the insect diet and improve energetic metabolism, impacting insect fitness, flight ability, and invasive power (Anselme *et al* 2008, Heddi *et al* 1993). Vallier *et al* (2009) showed that the injection of dsRNA in *S. zeamais* larvae led to a knockdown of 98% in a gene specifically expressed in the symbiotic organ (*wpgrp1*). Considering the relevance of the endosymbionts to fitness, these results suggest a potential target for the application of RNAi as a control strategy for this pest of stored products. Delivery of dsRNA to control pests of store products may be improved by using lure and kill methods, which involve attractants to draw insects to a location where they are exposed to the dsRNA molecules. These dsRNA molecules can also be applied to surfaces or cracks and crevices in empty bins, equipment, or structures where food material accumulates, and it is conceivable that dsRNA could thus be used for control of adults (Perkin *et al* 2016).

In Brazil, studies with the cotton boll weevil, *A. grandis* Boheman (Coleoptera: Curculionidae), support that knockdown of the *chitin synthase II* (*ChSII*) gene in larvae through microinjection significantly affected development, resulting in 100% adult mortality (Macedo *et al* 2017). Importance of dsRNA stability for gene silencing in *A. grandis* is exemplified by significantly reduced degradation and increased susceptibility to dsRNA targeting the *chitin synthase II* (*ChSII*) gene by previous knockdown of three nuclease genes by microinjection (Garcia *et al* 2017). Current efforts are focused on methods to increase the RNAi efficiency, mostly through the protection of the dsRNA molecule with chitosan nanoparticles or fusion proteins. In an alternative strategy, the formation of a ribonucleoprotein particle (RNP) from a chimeric protein combined with dsRNA provided a 2-fold increase in the gene silencing efficiency in *A. grandis* upon oral delivery (Gillet *et al* 2017).

Diptera

Few studies have reported positive use of insecticidal RNAi to control dipteran pests of interest in the Neotropical region. The use of RNAi to knockdown expression of genes involved in sex determination was suggested as a potential strategy for production of insects for use in the sterile insect technique (SIT) (Schetelig *et al* 2012, Gabrieli *et al* 2016) in the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera:

Tephritidae). In *C. capitata*, the knockdown of the *transformer-2* (*tra-2*) gene in embryos led to 95% of adults phenotypically male (Salvemini *et al* 2009), while silencing of *innexin-5* (*inx5*) produced sterile males (Gabrieli *et al* 2016). Importantly for SIT, these RNAi-generated sterile males remained sexually competitive with wild-type rivals and were able to induce similar post-mating responses.

Recently, the first evidence of functional RNAi in the South American fruit fly, *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae), was reported in Brazil (Dias *et al* 2019). Larvae soaked in dsRNA solution targeting the V-ATPase gene showed 100% of knockdown after 48 h and up to 40% larval mortality. The silencing effect persisted up to 72 h. Also, the expression of *Dcr2* and *Ago2* increased upon exposure to dsRNA, confirming the robust response of this species to RNAi.

Another dipteran pest of economic interest in the Neotropical region is the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). Oral delivery of dsRNA did not result in significant silencing or mortality in *D. suzukii* adults, yet combining dsRNAs targeting essential genes (including *alpha-coatomer protein* isoform A, *ribosomal protein S13*, and the *V-ATPase E subunit*) with a transfection reagent (Lipofectamine®2000) resulted in low, albeit significant, levels of larval mortality (Taning *et al* 2016b). Among these target genes, the highest mortality was observed for the *V-ATPase E subunit* (42%). Engineering of yeast (*Saccharomyces cerevisiae*) to express dsRNAs targeting *γ-tubulin-23C* and *V-ATPase* 16 kDa *subunit-1* genes was proposed as a delivery method that capitalizes on the symbiotic interactions between *Drosophila* and yeast that are naturally found growing on the surface of fruit crops (Murphy *et al* 2016). This strategy resulted in reduced locomotor activities and reproductive fitness in adult *D. suzukii*, while in larvae, it significantly reduced survival (23% compared to controls) to pupation. These results support the need for optimization of delivery and formulation for oral applications for effective insect control using an RNAi-based approach, as discussed above for lepidopteran pests.

Hemiptera

Efforts on the use of RNAi against hemipteran pests of economic importance in the Neotropical region are recent. The number of successful insecticidal RNAi descriptions in the literature increased, especially after 2017. The main target pest has been the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Hunter *et al* 2012, Taning *et al* 2016a, Andrade & Hunter 2017, Galdeano *et al* 2017, Yu & Killiny 2018), vector of the phloem-limited bacteria *Candidatus Liberibacter americanus* and *Candidatus Liberibacter asiaticus*, the causal agents of the devastating citrus greening (Huanglongbing) (Galdeano *et al* 2017). Since the report of the effective RNAi pathway in *D. citri* (Taning *et al* 2016a), potential target genes have been identified for control. These genes include *carboxylesterases* (Kishk *et al* 2017), *cathepsin D*, *chitin synthase* and *inhibitor of apoptosis* (Galdeano *et al* 2017), *arginine kinase* (Andrade & Hunter 2017), *muscle protein-20* (Yu *et al* 2017), *juvenile hormone acid O-methyltransferase* and *vitellogenin* (Ghosh *et al* 2018), and the fertility gene *boule* (Yu & Killiny 2018). Strategies for delivery of dsRNA against *D. citri* have included feeding of plant leaflets, soaking, root drench, and injections in trees. Andrade & Hunter (2016) described an RNAi feeding bioassay, called in plant system (iPS), in which they used vegetative new growth citrus flush to deliver dsRNA to *D. citri* during natural feeding. This system delivered dsRNA to *D. citri* adults 72 h after feeding. Recent studies support that the RNAi effects on *D. citri* female adults could be transferred in a trans-generational manner to their eggs and progeny, commonly referred to as parental RNAi (Yu & Killiny 2018).

In the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), the knockdown of *carboxylesterase* reduced the resistance to omethoate insecticide (Gong *et al* 2014). The results of this study suggest that this gene would be a propitious target against resistant aphid population control. Similar results were obtained with knockdown of *inositol 1,4,5-trisphosphate* receptor in *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) resistant to cyantraniliprole (Guo *et al* 2017). Screenings identified additional effective RNAi target genes in *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), *Phenacoccus solenopsis* (Hemiptera: Pseudococcuidae), and stink bugs *Halyomorpha halys* (Stål) and *Euschistus heros* (F.) (Hemiptera: Pentatomidae) (Peiffer & Felton 2014, Jaubert-Possamai *et al* 2017; Cao 2018, Castellanos *et al* 2018, Mogilicherla *et al* 2018). In the study with the Neotropical brown stink bug (*E. heros*), seven target genes that resulted in 95% of nymphal mortality were identified (Castellanos *et al* 2018). The class of target genes selected in this study represented varied functions, such as signaling pathways, intracellular transport, degradation of proteins, cell energization, pH homeostasis, transcriptional regulation, protein synthesis, and muscle movement. The Southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae), was confirmed to contain RNAi machinery components (Davis-Vogel *et al* 2018b), although the efficacy of RNAi in this pest needs to be further assessed.

Factors Involved in the RNAi Efficiency in Insects

Several factors affect the efficiency of insecticidal RNAi, including the RNAi machinery in the target species, dose and concentration of the dsRNA, insect life stage and tissue used, dsRNA molecule delivered (type and length), duration of exposure, and measurement points. Many of these factors

were reviewed by Christiaens *et al* (2018b) in a study to support the environmental risk assessment of RNAi-based genetically modified plants. These factors should be particularly defined according to the objectives of each RNAi bioassay and experimental setup and are crucial for appropriate insect RNAi research. In the following sections, we will discuss the recent advances and limitations related to these factors.

RNAi machinery genes

The presence of the so-called core RNAi machinery genes (*Dcr2*, *Ago2*, and *R2D2*) in the genome is necessary for efficient and robust gene silencing response. Although RNAi is a conserved mechanism throughout Eukaryota, considerable differences in pathways and functioning proteins in these pathways exist among insect groups (Christiaens *et al* 2018b). Yoon *et al* (2016) screened 50 genes for potential functions in RNAi by exposing cells to dsRNA target genes followed by incubation with dsRNA targeting the *inhibitor of apoptosis-1* gene and using apoptosis from silencing of this gene as reporter. Among the tested genes, 29 showed a significant effect on RNAi, and silencing of the *Ago1*, *Ago2* (isoforms a and b), *Aubergine*, and *V-ATPase 1* blocked RNAi, suggesting that these genes are essential for the functioning of RNAi. In contrast, the knockdown of both Dicer proteins Dcr2a and Dcr2b in these cells only partially blocked RNAi.

Studies show that duplication and loss of core RNAi pathway genes have occurred multiple times and that variations of the RNAi efficiency among insects could be explained by a diversity of RNAi pathway genes and their expression levels (Dowling *et al* 2016). Differences in the number of isoforms and expression levels of dsRNA-binding and argonaute proteins among *D. v. virgifera*, *S. frugiperda*, and *N. viridula* were proposed to contribute to distinct susceptibility to insecticidal RNAi in these species (Davis-Vogel *et al* 2018b). More recently, transcriptome analysis in *A. fraterculus* showed three copies for the *Dcr2* gene and two for *Ago2*, while *Drosophila* has only one copy for both genes; this factor may be associated with the strong knockdown results found in *A. fraterculus* larvae (Dias *et al* 2019). The duplications or loss of core RNAi-related genes may lead to subfunctionalization or neofunctionalization in RNAi pathways and could explain observed differences in the efficiency of RNAi across different insect groups (Dowling *et al* 2016).

dsRNA uptake and systemic RNAi

The uptake of dsRNA and RNAi systemic amplification differ among organisms. In the nematode *Caenorhabditis elegans*, the gene responsible for the uptake and systemic RNAi is *sid-1*, a transmembrane dsRNA-gated channel (Winston *et al* 2002, Tijsterman *et al* 2004). In *Drosophila* cells, intake of dsRNA leads to localized gene silencing without systemic distribution of the RNAi signal (Roignant *et al* 2003). In a recent study, putative orthologs of *sid-1* were identified in the transcriptomes of 68 species representing almost all major insect lineages, except species belonging to Antliophora (i.e., Diptera, Mecoptera, and Siphonaptera) (Dowling *et al* 2016). This study also identified multiple copies of *sid-1* in some insect pests, such as *Frankliniella cephalica* (Crawford DL) (Thysanoptera: Thripidae) and *T. castaneum*. Phylogenetic analyses in this study supported that *sid-1* was present in the last common ancestor of insects and that it was lost in Diptera.

Delivery methods

Methods of dsRNA delivery can significantly vary among the different insect species and strongly influence the efficiency of gene silencing and its potential for insect pest control, especially considering that gene silencing is limited to cells that uptake the dsRNA (Yang *et al* 2011, Terenius *et al* 2011). Delivery of dsRNA by microinjection, a commonly used technique in laboratory approaches and basic functional studies, has the advantage of direct delivery to the target tissue or into the hemolymph, and therefore, it is widely used (Yu *et al* 2013). However, microinjection can be accompanied by high mortality due to tissue damage (Jaubert-Possamai *et al* 2007) and is not a logistically valid option for field application.

A delivery method more adaptable to insecticidal field applications is spray-induced gene silencing (SIGS), based on oral delivery or soaking of dsRNA (Wang & Jin 2017). This method has the advantage over transgenic crops producing dsRNAs of a reduced regulatory burden (Zotti & Smagghe 2015). However, the main drawback of SIGS is the need for multiple applications to protect new plant growth, implying higher costs to farmers (Cagliari *et al* 2018). Nonetheless, dsRNA sprays on leaves but also injection in trunks or soil applications can be optimized for the development of pest control strategies (Hunter *et al* 2012, Andrade & Hunter 2016).

Engineered microorganisms are powerful tools for basic research on RNAi and potentially for field application. Horizontal transfer of symbiont-engineered microorganisms may potentialize the spread of RNAi signal within a population, even with minimal initial inoculum (Whitten *et al* 2016). Engineered virus may also have a similar effect, as the target sequence is inserted in the viral genome. Upon infection, the host RNAi immune response is triggered, inducing the target gene suppression (Kolliopoulou *et al* 2017). In plants, this technique is largely used for functional genomic studies; however, currently, only three silencing vectors were developed based on insect-specific virus species (Kontogiannatos *et al* 2013; Gu *et al* 2011, Taning *et al* 2018). Moreover, the

use of insect-specific virus and symbiotic microorganisms adds another layer of selectivity to RNAi-based pest control methods, depending on the host specificity of the selected vector. Also, the use of RNAi through endosymbionts is a friendly control alternative for stored product pests, such as *P. interpunctella*, *T. castaneum*, and *Sitophillus* spp., compared to the main control tactic available in the Neotropical region which is the pesticide fumigant phosphine, highly toxic to animals (Perkin *et al* 2016).

RNAi effect variation related to the target gene

Target gene selection for RNAi silencing can greatly affect the silencing outcome. Differences in development and metabolism among insect groups explain why an effective RNAi target in one species may not have the same effect in another species (Katoch *et al* 2013). Additional explanations for this variation include degradation in a sequence-specific manner of the dsRNAs or resulting siRNAs and efficient feedback mechanisms for certain genes that may be able to prevent the depletion of mRNA by increasing their rate of transcription (Terenius *et al* 2011). Genes with lower transcript abundance require less dsRNA for silencing; thus, the functional characterization of the candidate genes should always be considered in initial RNAi studies.

dsRNA persistence in the insect body

The major factors contributing to the variability in RNAi efficiency in insects is the persistence of dsRNA in the insect body. The dsRNA molecules can be rapidly degraded by nucleases present in the saliva, intestinal fluids, or hemolymph (Christiaens *et al* 2018b). Given these factors, the delivery of dsRNA as a topical application in crops will highly depend on formulations which bypass degradation of dsRNA, facilitating its delivery into insect cells (Lim *et al* 2016). Potential delivery auxiliary agents include liposomes, polymers, and nanoparticles (Gillet *et al* 2017).

Soaking of neonate *Drosophila* larvae in dsRNA encapsulated with liposomes (Lipofectamine®2000) for 1 h resulted in a 10-fold reduction in *β-glucuronidase* transcript levels compared to soaking in naked dsRNA (Whyard *et al* 2009). Similar results were observed for *D. suzukii*, using the same transfection reagent (Taning *et al* 2016b). However, for an important fruit fly pest, *A. fraterculus*, delivery of naked dsRNA into larvae by soaking showed high silencing efficiency (Dias *et al* 2019).

Lepidopterans have alkaline gut environment (pH > 9.0) and a strong intestinal nucleolytic activity, making the use of products to protect the dsRNA against nucleolytic degradation necessary. In an RNAi study targeting the knockdown of the essential *chitin synthase B* gene in *S. exigua*, polymers with high guanidine content provided good protection against nucleolytic degradation at pH 11, protecting the dsRNA for up to 30 h (Christiaens *et al* 2018a). The larval mortality in the polymer-protected dsRNA treatment increased to 53% compared to only 16% with the naked dsRNA, highlighting the importance of these formulation strategies in preventing dsRNA degradation in the alkaline pH and strong nucleolytic gut of lepidopteran insects.

Degradation of dsRNA by saliva can be of critical importance for RNAi in some insects, especially hemipteran species relying on extra-oral digestion of the plant tissue before ingestion (Peiffer & Felton 2014). For instance, a strong dsRNA-degrading capacity was detected in the saliva from the hemipteran *E. heros* (Castellanos *et al* 2018). Protection of the dsRNA by liposomes or EDTA increased nymph mortality compared to naked dsRNA, again supporting the use of these protective methods to enhance insecticidal gene silencing by RNAi.

Resistance to dsRNA

As with any other insecticidal technology, it is expected that insects may acquire resistance to dsRNA. Proactive identification of possible resistance mechanisms will enhance our ability to use the technology in a sustainable manner, yet currently, information on resistance mechanisms to RNAi is scarce.

Several possible mechanisms of resistance to RNAi in insects can be proposed. Stability of dsRNA in the insect gut is an important factor for a successful RNAi response (Garbutt *et al* 2013, Garcia *et al* 2017, Spit *et al* 2017), and increased nuclease expression can lead to degradation of dsRNA and subsequent RNAi failure. Artificial reduction of *Dcr2* and *Ago2* expression has been reported to confer complete protection to *D. virgifera virgifera* adults against an insecticidal dsRNA, with no phenotypic effects in adults or larvae (Vélez *et al* 2016). These observations suggest that mutations in the RNAi machinery genes are also a potential resistance mechanism. However, it is important to consider that these RNAi machinery genes encode proteins essential for normal processing of endogenous siRNAs and microRNAs (Carthew & Sontheimer 2009), which increases fitness costs and thus reduces the likelihood of resistance (Wu *et al* 2017). Another plausible mechanism of resistance is mutations in the target gene sequence such that siRNAs cannot identify the target mRNA. However, each insecticidal dsRNA molecule can produce numerous siRNAs with different sequences, and a single 21-bp match to the target sequence is efficacious (Bolognesi *et al* 2012); this possibility appears more difficult.

Since most of the possible resistance mechanisms act independently of the dsRNA sequence, it is important to assume the same initial mode of action for all naked dsRNA molecules. Thus, diverse dsRNAs should have similar cellular uptake mechanisms and stability in the gut lumen

environment of the insect, even if the dsRNA molecules target completely different genes. This is a valid concern and will have to be carefully considered when developing novel pest control strategies based on the RNAi technology and when estimating the associated resistance risks (Spit *et al* 2017).

The sole experimental evidence on resistance to RNAi in an insect comes from a recent study by Khajuria *et al* (2018). In this study, a population of field-collected *D. v. virgifera* was used for screening and selection for resistance against maize expressing dsRNA targeting the *DvSnf7* gene. Resistant *D. v. virgifera* showed significantly lower mortality compared to susceptible insects while feeding on maize expressing dsRNA. These insects were also cross-resistant to other insecticidal dsRNAs, suggesting that the resistance mechanism is not sequence-specific. Importantly, no cross-resistance to the Cry3Bb1 toxin was observed, thus supporting the combined use of the two technologies to delay the onset of resistance (Moar *et al* 2017). Similar stability of dsRNA incubated in vitro with digestive fluids from resistant and susceptible *D. v. virgifera* indicated that nucleases were not associated with resistance. In contrast, reduced uptake of fluorescently labeled dsRNA in midgut cells from resistant insects compared to susceptible rootworms and the lack of siRNAs corresponding to the *DvSnf7* dsRNA in resistant insects supported impaired uptake of dsRNA as a candidate resistance mechanism (Khajuria *et al* 2018). These results have high significance as they provide the first insight into resistance mechanisms against insecticidal dsRNAs.

Selection experiments with dsRNA targeting the *inhibitor of apoptosis 1* gene in a cultured cell line of coleopteran led to resistance (Yoon *et al* 2018). In these resistant cells, the levels of expression of *StaufenC*, a dsRNA-binding protein required for processing of dsRNA, was reduced when compared with susceptible cells. The same study showed that coleopteran-specific *StaufenC* is required for RNAi in *T. castaneum*, supporting that its reduced expression may result in RNAi resistance. The diversity reported so far on mechanisms of resistance to dsRNA highlights the need for characterization of resistance in multiple target pests and involving distinct delivery methods, to ensure the maintenance of this technology in the field for more time.

Differences among insect populations/lineages

The implications of RNAi efficiency on distinct populations are seldom discussed in the literature. Results from bioassays comparing *T. castaneum* field populations from diverse locations support low variation in susceptibility to dsRNA (Wang *et al* 2018b). The authors reported limited variations in the LD50 values for the *VATPase-E* gene, which ranged from 0.10 to 0.29 ng/larva among the laboratory strain and the seven field populations. A study in *D. v. virgifera* targeting a highly expressed *cysteine protease* gene showed variable degrees across three different populations of the western corn rootworm (Chu *et al* 2014). These results suggest that the genes potentially subjected to differential selections in the field should be avoided as target genes for RNAi-based insect pest management. Also, the target gene selection should consider the function related to the gene, once isolation mechanisms among cryptic species show differences in insect biology and behavior (Cladera *et al* 2014).

Concluding Remarks

Research on the use of insecticidal RNAi against insect pests of economic importance to the Neotropical region has increased in the last decade. Major advances have been made, including increasing efficiency of dsRNA delivery methods, screening of target genes, improved dsRNA formulations, and insights of possible mechanisms of resistance to RNAi.

Understanding differences in insect response to RNAi is central to the development and proper implementation of RNAi-based crop protection. The process of selecting a target gene for knockdown is of paramount importance, particularly in cases where the end goal involves pest control. Additional relevant aspects to be considered include the presence of RNAi machinery genes, RNAi uptake in the cells, the delivery method selected, dsRNA persistence in the insect body, and insect population target.

The main current limitation for the use of RNAi-based products via spray application is the low persistence of the dsRNA molecules in the field conditions. This concern follows the same problem for applying bioinsecticides (especially based on fungi and viruses). The protection of molecules from degradation by high temperature and UV radiation deserves more attention from researchers and companies. Once this barrier for field application is solved, the RNAi technology can be compatible with different agricultural systems (tropical and temperate). In countries such as Brazil, registration of the RNAi-based products can follow the same process as for biological products, which are registered for a specific target pest regardless of crop. Thus, the same RNAi product can be registered in different cultivated crops to control polyphagous pests. This would increase the probability of farmers adopting this technology.

The development of strategies to avoid off-target effects is also a crucial factor, although reduced risks are expected considering that RNAi is probably the most specific pesticide known to date due to its dependence on nucleotide sequence complementarity. Besides, RNAi technology can increase the efficiency of conventional pesticides by decreasing the expression of detoxifying enzymes (for instance, cytochrome P450 enzymes, carboxylesterases, and glutathione *S*-transferases). In combination with RNAi, chemical

pesticides might be employed effectively at lower doses, thereby increasing their safety (Kourti *et al* 2017).

As with every novel pest control strategy, RNAi is not exempt from the concern of resistance development in insect pests. In addition to pyramiding technologies with different modes of action, implementation of integrated pest management (IPM) and effective insect resistance management (IRM) strategies would ensure durability and optimization of the technology. However, comprehensive information on resistance mechanisms obtained from studies of different insects and delivery approaches are needed to assist in technical recommendations to improve insecticidal RNAi and delay the evolution of resistance to this control technique in the future.

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