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Review Article

BIOLOGICAL CONTROL OF GYPSY MOTH (*LYMANTRIA DISPAR*): AN RNAI-BASED APPROACH AND A CASE FOR DNA INSECTICIDES

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Abstract: The discovery of the post-transcriptional gene silencing (PTGS) mechanism, widely known as RNAi (RNA interference), has contributed towards the elucidation of the cellular machinery involved in the response against viral infections based on gene silencing, and in developmental regulation of translational suppression. The application of RNAi in insect pest management (IPM), and gene functional analysis, has been of enormous importance. Unfortunately, as RNAi has many times proven to be difficult to examine in Lepidoptera, focus has shifted to other potential post-genomic options in IPM. Special attention has afforded to novel DNA insecticides based on preparations of short single-stranded fragments of baculovirus anti-apoptosis genes, which represent a safe and relatively rapid alternative approach for IPM. This paper focuses on the drawbacks and advantages of DNA insecticides used in gypsy moth control and based on RNAi.

Key words: RNAi-based insect pest control; DNA insecticides; gypsy moth control

INTRODUCTION

In forest and agricultural systems, insect pests cause a wide range of damage to trees and crops, mainly through leaf damage and the subsequent decrease in the physiological functions of affected plants, which by extension disrupts the food web leading to the agro-ecological imbalance of an area with consequent loss in profits for farmers, gardeners, investors and the economy. Worldwide pest control costs billions of dollars each year. Chemical pesticides (pyrethroids, neonicotinoids, carbamates, etc.) are still the major approach for controlling insect pests, even though they are nonselective and are associated with significant hazards to the environment and human health. Biological preparations for gypsy moth control are an environmentally safer alternative to chemical insecticides.

In practice, the most commonly used biological preparations for gypsy moth control are based on the bacterium *Bacillus thuringiensis*. The endospores of the bacterium as well as its diamond-shaped crystal endotoxins (cry proteins) are used against gypsy moth. The use of *B. thuringiensis* is threatened by the development of resistance in some species, such as *Ostrinia nubilalis* (Lepidoptera, Crambidae) and *Heliothis virescens* (Lepidoptera, Noctuidae) [1].

Biological control of gypsy moth based on *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) is also used on a large scale. LdMNPV preparations are made on the basis of polyhedral occlusion bodies containing an occlusion-derived virus that starts an infection process in the epithelial cells in the midgut of the insect. Besides the slow action, the major drawback in the use of LdMNPV and *B. thuringiensis* in microbial insecticides is that microbial pathogens must be produced in living cells. Production is labor-intensive, costly and thus, availability

is low. As a result, there is an urgent need to develop economically and ecologically sound alternatives for pest control.

Most eukaryotic organisms, including insects, possess a common machinery for sequence-specific post-transcriptional gene silencing that is triggered by the presence of double-stranded RNA (dsRNA), resulting in the degradation of the target mRNA [2]. In the past decade, RNAi demonstrated potential in the control of insect pests. Unfortunately, to date the RNAi approach has some drawbacks in creating commercially available RNAi products against lepidopteran insects for agriculture and forestry. For example, it is always a concern that based on the mechanism of gene silencing, RNAi treatments may in some cases induce off-target effects in non-target organisms [3]. Another interesting post-genomic approach to insect pest control is based on the topical use of short single-stranded fragments of baculovirus anti-apoptosis genes. This novel idea and its application have recently shown reliable results in gypsy moth control [4-6].

RNAi

RNAi is an RNA-dependent gene silencing process that is managed by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in cytoplasm, where they interact with Argonaute (Ago) proteins, essential catalytic components of the RISC. This gradual development of a conserved post-transcriptional gene silencing mechanism is hypothesized to represent an active organismal response to infections of a viral nature [7], and when the dsRNA is exogenous (coming from infection by a virus with an RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved into short fragments by the Dicer enzyme. The initiating dsRNA can also be endogenous (originating in the cell), as in pre-microRNAs expressed from RNA-coding genes in the genome. The pathway for RNAi in the cell is initiated by a ribonuclease Dicer enzyme known as the RNase III enzyme that processes dsRNAs into short (21-25 nucleotides long) small interfering RNAs. Basically, the processes are divided into three steps [8]. A long endogenous or exogenous dsRNA molecule expressed in or introduced into the cell is processed into small RNA duplexes by Dicer, a ribonuclease III (RNase III). However, depending on the organism, there may be one or more than one Dicer enzyme, each responsible for a different type of short dsRNA product [9]. Dicer 1 is mainly used to produce microRNAs, while Dicer 2 is responsible for the processing of long dsRNA into siRNA products [10]. At the second level of the RNAi mechanism, the formation of siRNAs from the Dicer activity allows for complex formation with the RISC. The siRNA is unwound and the sense strands are degraded while the antisense strands remain fused with the RISC. In the third level, the RISC finds target messenger RNAs (mRNA) with complete or partial sequence corresponding to the dsRNA and subsequently silencing the expression of a target gene or genes. The discovery that cells respond to dsRNA by silencing the target genes has changed the view of gene regulation and this in itself is proposed to be very relevant in practical application for different purposes, including insect pest control.

RNAi as a method for IPM

Gene knockdown by RNAi has now become a valuable tool in the study of the function of gene(s) in different organisms. This technique has also led to the development of new methods for the insect-pest control of agriculturally important crops by genetically engineering plants to express dsRNA favorable to the specific conditions. The proposal to using RNAi to protect plants against insect pests by regulating essential gene functions in insects has been well conceived [11]. The selectivity of RNAi is conferred by the nucleotide sequence identity of the dsRNA to its target gene sequence. For example, the feeding of each unique dsRNA to target insects resulted in the selective killing of species whose dsRNA sequence was matched to its target [12-15].

In insect pest management, further progress in RNAi use has become possible as more insect genomes and their parts become available from day

to day. However, this approach has yet to deliver its practicality in the field of plant defense against invading insect pests. For effective insect pest management by RNAi, there should be regular and autonomous uptake of the dsRNA by the insect, which can be facilitated by feeding and digestion in the insect gut. Out of the three insect gut regions, the midgut is the best target for dsRNA uptake because it is the main site of absorption in insects [16]. Research in recent years has afforded new insights into the dsRNA uptake mechanisms in insects, with emphasis on uptake through the midgut and body tissues, e.g., the transmembrane channel-mediated uptake and the "alternative" endocytosis-mediated uptake. RNAi is now being considered as a potential future approach for the control of insect pests, as the effects of RNAi in insects have been documented and reviewed [3,17]. In the order of Lepidoptera, the application of dsRNA in an artificial diet resulted in the knockdown of targeted genes in *Epiphyas postvittana* [18] and *Plutella xylostella* [19]. However, in recent years, insects were observed to act differently to the uptake and action of dsRNA, even though some insects, such as coleopterans, showed high susceptibility, whereas hemipterans and lepidopterans were found to possess resistance to RNAi [4]. In Coleoptera [20], the success of RNAi in *Diabrotica virgifera virgifera* has been shown by feeding plant material expressing hairpin dsRNA. Baum et al. [21], who first used feeding assays, identified 14 genes whose knockdown by low levels of dsRNA killed Western corn rootworm larvae. A striking RNAi effect was also demonstrated in *Phyllotreta striolata*, where silencing of the invertebrate-specific phosphotransferase arginine kinase was done via feeding. The development of the beetle was severely impaired due to the disruption of cellular energy homeostasis [22]. Interestingly, conclusive proof for the involvement of the RNAi pathway, such as detection of specific siRNAs derived from administered dsRNAs [3], has not been reported.

An RNAi approach to insect pest management may include, but not be limited to partial reduction of gene activity. Hypothetically, it is also possible that the reduced levels of gene expression could be enough to fulfill some or all of the endogenous cellular roles of the gene. Not all of them bing about death of target insects. However, with prolonged research, the control of insect pests using RNAi looks promising. Of note, currently there is no research showing the insecticidal potential of an RNAi approach for gypsy moth control.

Beyond the fact that RNAi treatments may in some cases induce off-target effects in non-target organisms, the RNAi approach for insect pest management includes two main drawbacks. Firstly, the chemical synthesis of large amounts of relatively long dsRNA is currently too costly to be compared with chemical insecticides for effective pest control under field conditions, and thus more research into cost-effective methods for dsRNA production should be encouraged. Secondly, the question of delivery of RNAi to target insects has not been solved because the use of microinjection is unrealistic for pest control in the field, and delivery through feeding requires high concentrations of dsRNA. RNAi-based crops are expensive to produce and have a high risk of resistance breakdown: topical application is underway as a non-transformative approach that might enable RNAi-based insect management in species with low responses to environmental RNAi [11,23]. Another avenue in the post-genomic approach to insect pest management includes preparations of DNA insecticides based on short single-stranded DNA fragments of baculovirus anti-apoptosis (IAP) genes [4-6].

DNA Insecticides

DNA insecticides are a novel approach for insect pest management and attention is given to gypsy moth (*Lymantria dispar*), an insect pest having devastating effects on forests in North America and Eurasia. Gypsy moth control with DNA insecticides is based on the topical application of solutions with two single-stranded DNA (ssDNA) fragments from BIR (baculoviral IAP repeat; sense chain; 5'-GCC GGC GGA ACT GGC CCA-3') and RING (really interesting new gene; antisense chain; 5'-CGA CGT GGT GGC ACG GCG-3') domains of the LdMNPV IAP-3 (inhibitor of apoptosis) gene, which induces a significantly

higher mortality of LdMNPV-free I-III instar gypsy moth caterpillars on the 3rd-12th day in comparison with the application of control solutions [4-6].

Mode of action of DNA insecticides

In our opinion, the mode of action of DNA insecticides is consistent with the mechanisms of action similar to RNAi [24], DNA interference [25] and antisense oligonucleotides [26]. Two classes of baculoviruses anti-apoptosis genes, p35 and IAP, inhibit apoptosis in host cells [27-29]. Phylogenetic analysis of baculoviral IAP genes has indicated their host origin; the capture of these genes from the insect genome likely occurred at least twice in the course of evolution [30,31]. Our research shows that non-infected gypsy moth caterpillars contain mRNA that has a very high similarity output (> 90%) to a part of the LdMNPV IAP-3 gene cloned with BIR- and RING-domain fragments as primers, which supports our previous ideas that these fragments could affect the function of homologous anti-apoptosis genes of the host [5,6]. In our opinion, RING-domain fragments interact complementarily with the mRNA of a host IAP gene, blocking its expression and helping to initiate apoptosis in gypsy moth cells that eventually leads to insect death.

Moreover, our studies on LdMNPV-infected insects show that the antisense RING-domain fragment in the same concentration also has a faster significant insecticidal effect on I-III instar gypsy moth caterpillars in comparison with a control composed of LdMNPV-infected insects. The possibility of the joint use of very short (18-20 bases long) antisense fragments of the LdMNPV anti-apoptosis genes and LdMNPV preparations (one after another) may catalyze a more efficient use of the latter.

Obviously, the outcome of the application of DNA insecticides on the basis of the LdMNPV IAP-3 gene, particularly the antisense RING-domain fragment, depends on the dynamics of mRNA synthesis and the breakdown of the target anti-apoptosis gene. In our opinion, in the case of non-infected gypsy moth caterpillars, a host anti-apoptosis mRNA serves as a target mRNA, while in the case of LdMNPV-infected caterpillars, the mRNA of the LdMNPV IAP-3 gene is a complementary sequence which initiates the post-transcriptional silencing of the target IAP gene and subsequently leads to apoptosis of insect cells and insect death. In the case of LdMNPV-infected insects, we have assumed a stronger effect of DNA insecticides, since an optimal dose of LdMNPV provides a sufficient amount of target viral mRNA. Consequently, healthy and LdMNPV-free caterpillars are not likely to have a sufficient concentration of target host IAP mRNA to provide a robust initiation of post-transcriptional silencing of the target IAP gene. In our experiments, DNA insecticides work better on LdMNPV-infected gypsy moth caterpillars.

Selectivity of DNA insecticides

DNA insecticides designed for gypsy moth caterpillars can be selective, and thus unharmful to non-target insects, such as black cutworm and tobacco hornworm [5,32]. It is worth noting that 1st and 2nd instar gypsy moth caterpillars were significantly affected during the same period of time (7-14 days) by the smaller dose of DNA insecticides based on the same fragments of BIR and RING domains of the LdMNPV IAP3 gene. Gypsy moth caterpillars have a similar or comparatively greater average body mass than tobacco hornworm (8 to 6 mg, respectively), and an approximately 4-fold bigger body mass in comparison with black cutworm (8 to 2 mg, respectively) at this stage of development. Thus, a higher dose of DNA insecticides per 1 mg of insect tissue was used for non-target insects than for gypsy moth, but they were not significantly affected. This implies that DNA insecticides based on the fragments of BIR and RING domains of the LdMNPV IAP3 gene have a reliable margin of safety in action and harmlessness for non-target insects [5,6,32]. By evaluating alkaline phosphatase activity, glucose concentration and biomass accumulation, we also determined that DNA insecticides do not have a long-term negative effect on plants such as wheat (*Triticum aestivum*) [33]. This paves the way to the creation of selective insecticides that are well-tailored to target insect pests.

Perspective use of DNA insecticides

We believe that the practical advantage of the use of short (18-nucleotide-long) insect-specific DNA insecticides is that it could be easily synthesized without active human participation and that it will be cost-efficient. In comparison, manufacturing baculovirus preparations remains unattractive to many companies in North America and Europe that are unfamiliar with mass insect culture as a mainstream production technique, and while *in vivo* production remains capable of meeting the current market needs, the ability to produce the amounts of baculovirus needed for large-scale field-crop protection is far from certain [34,35].

High amounts of dsRNA have been used in many studies, raising the question of the specificity of effects. In most studies, "standard" amounts of double-stranded RNA is injected to achieve RNAi in lepidopterans, varying between 1 and 100 μg [27], although in a few species, high levels of silencing can be achieved by the application of low amounts of dsRNA, less than 10 ng per mg of tissue [36,37]. For comparison, in experiments with DNA insecticides we use topically 3-30 pmol of viral 18-nucleotide-long DNA fragments per *Lymantria dispar* caterpillar, which corresponds to approximately 3-30 ng of DNA (per 0.7-12 mg of tissue of a caterpillar). Thus, ssDNA insecticides work in substantially lower concentrations and accordingly may be cheaper compared to RNA preparations for insect pest control.

We are also aware of the advantages in the use of short (around 18 nucleotides long) insect-specific DNA insecticides in comparison to the RNAi approach, because relatively long dsRNA is cleaved in cells into numerous, unpredictable and short (21-23 nucleotides) siRNAs that have abundant direct sequence matches throughout the genomes of most non-target organisms [38]. This problem is difficult to solve to guarantee the specificity of RNAi preparations for crop protection.

In our opinion, topical application of DNA insecticides is a convenient way of insect pest control. Though it might be impossible to use DNA insecticides against cryptic feeding insects and adult beetles because their elytra could provide some protection from contact with the insecticide, DNA insecticides appear to be very suitable for the control of lepidopteran pests such as gypsy moth at the caterpillar stage, especially during the early larval instar stages when the exoskeleton of the insect is thin.

Importantly, DNA insecticides could resolve or improve the problem of insecticide resistance. If we use short single-stranded fragments of very conservative parts of insect host IAP genes (for example, conservative RING-domain fragments), resistance to the insecticides will develop more slowly because potential mutations that change target IAP genes occur at a very low rate in conserved regions. Thus, if we are not able to stop the genetic processes leading to insecticide resistance, we could slow-down the emergence of insecticide resistance by using DNA insecticides based on the very conservative regions of functionally important genes, such as IAP genes. This approach is of immense value, if not revolutionary, and elaborations in this field may lead to very safe and cheap agriculture sustained by DNA insecticides.

CONCLUSION

Investigations in the field of application of post-genomic approaches for insect pest management, including those related to the RNAi approach, such as DNA insecticides, deserve attention and detailed study. Research of this kind may provide valuable information on how current attempts in this direction may be rearranged and supplemented for the production of safe and cost-efficient preparations for gypsy moth and other insect pest control based on nucleic acids, particularly ssDNA and dsRNA. Taking into consideration the advantages of DNA insecticides, we think they could combine the best properties of modern agents of gypsy moth control, namely rapid action, the affordability of chemical insecticides and the safety of biological preparations. Further mprovement of DNA insecticides would include establishing more precise mechanism of their action and extending their use to controlling other serious insect pests.

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