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The RING for gypsy moth control: Topical application of fragment of its nuclear polyhedrosis virus anti-apoptosis gene as insecticide

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ABSTRACT

Numerous studies suggest a cellular origin for the *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) anti-apoptosis genes IAPs, thus opening a possibility to use the fragments of these genes for modulation of host metabolism. We report here the strong insecticidal and metabolic effect of single-stranded antisense DNA fragment from RING (really interesting new gene) domain of gypsy moth LdMNPV IAP-3 gene: specifically, on reduction of biomass (by 35%) and survival of *L. dispar* caterpillars. The treatment with this DNA fragment leads to a significantly higher mortality rates of female insects (1.7 fold) accompanied with the signs of apoptosis. Additionally, we show increased expression of host IAP-1, caspase-4 and gelsolin genes in eggs laid by survived females treated with RING DNA fragment accompanied with calcium and magnesium imbalance, indicating that the strong stress reactions and metabolic effects are not confined to treated insects but likely led to apoptosis in eggs too. The proposed new approach for insect pest management, which can be considered as advancement of "microbial pesticides", is based on the application of the specific virus DNA, exploiting the knowledge about virus–pest interactions and putting it to the benefit of mankind.

1. Introduction

In recent decades scientists came to the idea that nucleic acids, a "language" of nature, should be used as a tool for different practical purposes. The field of insecticides' creation and insect pest management is not an exception. Due to insecticide resistance emerging with the time for every new pesticide preparation [1,2] and constant growth of world population, additional pest management tools are needed to confront future agricultural demands. The post-genomic approaches are one of the responses to this impending problem. Recent studies with our newly proposed DNA insecticides [3,4] as well as RNA interference (RNAi) techniques [5,6] and genetically modified crops [5,7] show a good potential of post-genomic approaches for insect pest management.

It seems very attractive to use oligonucleotides as insecticides since they can work selectively, they are subjects of natural biodegradation in ecosystems in contrast to majority of chemical insecticides [8,9], and commercial synthesis of nucleic acids in vitro becomes more and more affordable.

Unfortunately, RNAi as a tool for insect pest control in Lepidoptera (moths and butterflies) has many times proven to be difficult to achieve [10], leading to some complications with applicability of this approach to be used on a large scale. Also limited knowledge about the selectivity of preparations on the base of RNA has hampered their use for insect pest control. For example, the potential hazards posed by RNAi-based pesticides and genetically modified crops to non-target organisms include off-target gene silencing, silencing the target gene in unintended organisms, immune stimulation, and saturation of the RNAi machinery [5]. Overcoming the mentioned problems, our recent studies show that DNA insecticides designed for gypsy moth caterpillars can be selective, and thus non-harmful both for non-target insects such as black cutworm and tobacco hornworm [3,4,11], and plants such as wheat [12], oak and apple tree [13], what paves the way to the creation of selective insecticides that are well-tailored to target pest insects [3]. Of note, we chose gypsy moth *Lymantria dispar* as a target model insect for our investigations since gypsy moth caterpillars damage over 500 plant species and cause substantial economic loss. The gypsy moth larvae are voracious feeders, consuming a total of about 1 m^2 of foliage over

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their caterpillar stage [14,15]. During population outbreaks which last 1–3 years, larvae may defoliate host trees completely and then switch to cereal crops and even vegetables.

Hence, DNA insecticides are a novel preparation against gypsy moth, (*L. dispar*, serious insect pest of forests and fruit trees [15]), based on DNA sense or antisense fragments of the anti-apoptotic gene of gypsy moth nuclear polyhedrosis virus. The present study is based on our preliminary results where topical application of a solution with two single-stranded DNA (ssDNA) fragments from BIR (baculoviral IAP repeat) and RING (really interesting new gene) domains of LdMNPV (*L. dispar* multicapsid nuclear polyhedrosis virus) IAP-3 (inhibitor of apoptosis) gene induces a significantly higher mortality of gypsy moth caterpillars in comparison with the application of the control solutions [3]. In this paper we identify and characterize active at picomolar concentrations RING DNA fragment as acting molecule with the promising insecticidal potential.

The idea of DNA insecticides is coherent to the mode of action of antisense molecules [16–18], mRNA-antisense DNA hybrids [19] and mechanisms that resemble those of DNA interference [20] and RNA interference [21,22]. Our recent studies show that DNA insecticides might act through the inactivation of the mechanisms involved in post-transcriptional expression of insect anti-apoptosis genes [3,4]. Targeting genes for pest management that are inherently tied to a specific pair virus–host (for example, anti-apoptosis genes), may reduce the likelihood of target gene silencing in a non-target organism and, thus, to lower relative environmental risks. We suppose the advantage in the use of short (around 18 nucleotides long) insect-specific DNA insecticides, compared to described relatively long double-stranded RNA fragments, because 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 [5]. This problem is difficult to be solved to guarantee specificity of RNAi preparations for crop protection. In addition, gene silencing by feeding or injection of double-stranded RNA requires high concentrations of active substances for success [10]. This issue could be resolved with application of described here DNA insecticides on the base of short antisense DNA fragments.

Of note, the idea of DNA insecticides is unique and has its own peculiar features such as topical application, small size of oligonucleotides, picomolar concentrations of ssDNA and the concept of using viral anti-apoptosis genes, what differs from other known post-genomic approaches for insect pest management. Application of short ssDNA fragments as insecticides is our novel approach without analogy in literature. It could lead to the creation of selective, low-risk for agroecosystem food webs, affordable and relatively fast-acting DNA insecticides for control of lepidopteran pests at caterpillar stage.

2. Materials and methods

2.1. Study area

Caterpillars of gypsy moth *L. dispar* (Lepidoptera: Erebidae) were identified and collected in the forests of the Crimean peninsula in three different locations, namely Dubki (lat. 44.93272419, long. 34.03238297, alt. 302 m), Luchistoye (lat. 44.73612538, long. 34.3751049, alt. 399 m) and Lavrovoye (lat. 44.576835, long. 34.31060314, alt. 269 m) in May 2014. The aim to collect caterpillars from 3 locations was to make uniform biological material and obtain average susceptibility of gypsy moth caterpillars to DNA insecticides from Crimean forests.

2.2. Sequences of the applied DNA fragments

We designed DNA fragments according to the LdMNPV genome sequenced by Kuzio's group [23] and found in ICTVdb database (http://www.ictvonline.org). DNA fragments were synthesized by Metabion International AG (Germany). The sequences of the applied single-stranded DNA fragments were the following: 1) 5′-GCC GGC GGA ACT GGC CCA-3′ (134843-134860; sense chain; BIR domain); 2) 5′-CGA CGT GGT GGC ACG GCG-3′ (135159-135142; antisense chain; RING domain; experimental group, hereafter abbreviated as RING); 3) 5′-AAA AAA AAA AAA AAA AAA-3′ (poly(A) oligonucleotide); 4) 5′-GA AG GC AC TG-3′ (random oligonucleotide).

2.3. Treatment technique

In average, 20–25 2nd instar caterpillars from each location were used per each control and experimental groups for the treatment with DNA fragments. Each experiment was performed in 4 replicates (thus 80–100 caterpillars were included for each treatment group). A water solution with ssDNA fragment (10 pmol/μl, either poly(A), BIR or RING) was applied topically on caterpillars via fine spraying. We collected small drops of solution from the surface of 10 caterpillars and found approximately 0.2–0.3 μl of solution on each caterpillar after spraying (2–3 pmol of ssDNA per caterpillar). On the 7th and 14th day after the treatment the biomass of alive caterpillars was measured.

2.4. Insect rearing

Gypsy moth caterpillars were grown in Petri dishes on oak leaves (*Quercus robur*) at temperature 25 °C till pupation. Each pupa was weighed on the day of its formation. On emergence time, the numbers of male and female moths were counted. After that, male and female insects from each experimental group were placed in a separate jar where they mated and females laid eggs. Laboratory scales Axis BTU210 (Axis, Poland) with 1 mg discreteness were used to weigh caterpillars, pupae and egg masses.

2.5. Detection of the possible LdMNPV infection of L. dispar by PCR

Specific PCR condition and following primers for the LdMNPV p39 capsid protein gene were used for detection of possible LdMNPV contamination in gypsy moth caterpillars: 5′-ACG TTC TCG TTG AAC GTG CTG-3′ (forward primer), 5′-CTG GTG AAC CAC AAA ACC CTG-3′ (reverse primer) [24]. DNA was extracted using the “DNA-sorb-AM” (AmpliSens, Russia) and PCR reactions were performed using amplification kit “AmpliSens-200-1” (AmpliSens, Russia) following manufacturer's protocols. DNA was initially denatured for 3 min at 94 °C, followed by 5 cycles of 1 min denaturation at 94 °C, 1 min hybridization at 61 °C and 1 min elongation at 72 °C, followed by 30 cycles of 0.75 min denaturation at 94 °C, 0.75 min hybridization at 61 °C and 0.75 min elongation at 72 °C, and followed by a final elongation step at 72 °C for 5 min.

2.6. Detection of alkaline phosphatase activity

Each caterpillar was homogenized in distilled water, and alkaline phosphatase activity in whole homogenate was measurement by the Liquick Cor-ALP kit (PZ Cormay S.A., Poland) on the BS-3000 m semi-automatic biochemistry analyzer (Sinnowa, China) according to manufacturer's instructions.

2.7. Calcium and magnesium assay

For calcium and magnesium measurements 600 eggs of gypsy moth from different egg masses were used per each replicate in each group.

Measurements of calcium and magnesium concentrations in eggs laid by females from control group and females affected by DNA fragments, were performed by titrimetric method according to GOST standard protocol 23268.5-78 (GOST, the set of technical standards maintained by the Euro-Asian Council for Standardization, Metrology