Oberemok VV, Laikova KV, Zaitsev AS, Gushchin VA, Skorokhod OA.

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

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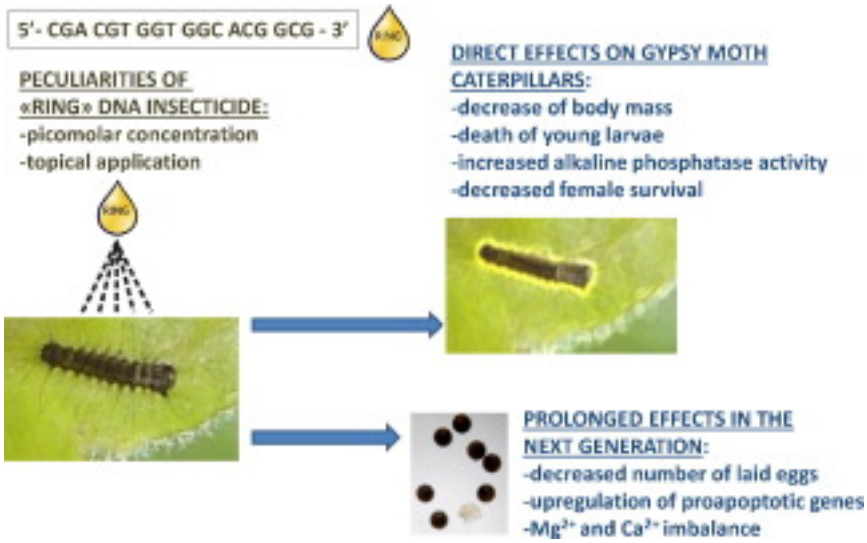
Highlights

- RING DNA insecticide works against gypsy moth caterpillars in picomolar range.
- RING DNA insecticide has both direct and prolonged insecticidal effects on the moth.
- The use of highly conservative RING sequence could resolve insecticide resistance.
- RING DNA insecticide is short oligonucleotide and could be easily synthesized.

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.

Graphical abstract



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Keywords

Gypsy moth *Lymantria dispar*
DNA insecticides
Lymantria dispar multicapsid nuclear polyhedrosis virus
Viral IAP genes
RING (really interesting new gene) domain
Pest management

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 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],[17], [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 thebiomass 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 and Certification). Specifically, for calcium assay, the distilled water (from 10 to 100 ml) was added to egg ash in a conical 250 ml flask, then the suspension was neutralized with hydrochloric acid (0.1 mol/l) in the presence of indicator methyl red until it turned to pink color. After, the extra of hydrochloric acid (1 ml) was added to solution, and it was boiled during 5 min with refluxcondenser to remove carbon dioxide (reflux condenser can be replaced by an inverted funnel). Then solution was cooled to 20 °C. By adding of 2 mol/l sodium hydroxide, solution pH was adjusted to 12–13. As a specific calcium indicator 1 ml of calconcarboxylic acid solution was added, and sample was slowly titrated with 0.05 mol/l trilon B (ethylenediaminetetraacetic acid, EDTA) until the color was changed from cherry to blue. Mass concentration of calcium ions, X, measured in mg/l, was calculated by the formula $X = V1 \cdot M \cdot 40.08 \cdot 1000 / V2$, where V1 is volume of trilon B added for titration, in ml; M — molar concentration of trilon B added for titration, in mol/l; 40.08 — molar mass of calcium, in g/mol; V2 — volume of distilled water added to ash, in ml.
For magnesium assay, the distilled water (100 ml) was added to egg ash in a conical 250 ml flask, pH was adjusted to 10 by adding of ammonia buffer solution (5 ml), and the specific indicator for sum of both magnesium and calcium Eriochrome Black T (sodium 1-[1-Hydroxynaphthylazo]-6-nitro-2-naphthol-4-sulfonate, 3 mg) was administered. Then the sample was slowly titrated with 0.05 mol/l trilon B until the color was changed from cherry to blue. The mass concentration of magnesium ions, X, in mg/l, was calculated from the difference of volumes of trilon B, consumed for a) the titration of sum of calcium and magnesium ions and b) separately only calcium ions by the formula:
$X = (V1 - V2) \cdot M \cdot 24.32 \cdot 1000 / V3$, where V1 — volume of trilon B added for titration of both calcium and magnesium, in ml; V2 — volume of trilon B added for calcium titration, in ml; M — molar concentration of trilon B added for titration, in mol/l; 24.32 — molar mass of magnesium, in g/mol; V3 — volume of distilled water added to ash, in ml.

2.8. Real time PCR quantification of expression of apoptotic and anti-apoptotic genes in the insect eggs

RNA extraction was carried out with PureLink® RNA Mini Kit (Ambion, Life technologies, USA) according to manufacturer's instructions. For extraction, insect eggs (60 mg, approx. 100 individual eggs) were ground by pistil in liquid nitrogen in 1.5 ml tube. Three independent extractions were carried out to produce replicates. Quality of extracted total RNA was assessed by loading (5 μl) of eluted volume into 1.5% agarose gel and running in TBE (Tris-borate-EDTA) buffer (10 V/cm) for 30 min. The quantity, intensity and pattern of RNA bands were equal in all experimental groups, confirming the quality and reproducibilityof RNA extraction from the insect material. For reverse transcription, the total RNA (5 μg) was annealed with oligo dT(18) primer and proceeded with RevertAid H Minus Reverse Transcriptase kit (Thermo Scientific, USA) according to manufacturer's instructions. Reaction was conducted at 42 °C for 60 min in Thermostat "Termite" (DNA Technology, Russia).

For quantitative real time PCR studies and amplification with gene specific primers the aliquot of obtained cDNA (0.5 μl) was used per each sample with addition of the following primers: (*L. dispar gene*, *sequence* 5′ > 3′): IAP1, forward CGCTGCAAGTAATGCTGAGG, reverse GCACACGCAACTACATGTCC; hemolin, forward GGGAATTGCAAGCACTCGTC, reverse AGAGTGCCATCAGGTGACAG; caspase 4, forward GCAGTCCATCGCACAGAAAC reverse TGATTAGCGTGGAGGTCGTG; actin, forward GGAAGTTGCTGCGTTGGTAG, reverse TCCCATTCCGACCATGACAC; gelsolin, forward GACTGGAGCGCAAGTTCAAG, reverse TGACGATGTGTCGTCCGAAC. The qPCRmix-HS SYBR (Evrogen, Russia) master mix was used according to manufacturer's instructions. A Step One Plus® thermocycler (Life technologies, USA) was used to set up amplification according to the following procedure: 1 min of initial denaturation at 95 °C, followed by 40 cycles with 10 s of denaturation at 95 °C, 20 s of annealing at 60 °C, 20 s of elongation at 72 °C. Finally all PCR products were melted to estimate specificity of amplification and presence of additional products.

2.9. Statistical analysis

We used non-parametric Pearson's chi-squared test (χ^2) with Yates's Correction and Mann–Whitney test to evaluate the significance of difference between the groups' means (Sofa Statistics 1.3.3 software).

3. Results

3.1. Mortality of caterpillars in different groups

After the treatment with antisense ssDNA fragment from RING domain of IAP-3 gene (RING group) a progressive mortality of caterpillars was observed (Fig. 1). Significance in mortality was observed on the 12th day after the treatment in comparison with water-treated group (Fig. 1), whereas treatment with BIR domain ssDNA fragment (BIR group) also led to mortality over the all control groups, but without significant difference (Fig. 1). The percentage of perished caterpillars continued to rise up to 14th day of the experiment, reaching in average 42.5 ± 23.2% of caterpillar individuals perished in the RING group, 13.8 ± 6.0% of individuals perished in the group treated with BIR, 9.0 ± 3.1% in poly(A), 9.5 ± 2.5% in the group treated with random oligonucleotide, and 7.5 ± 1.8% of individuals perished in the control group (Fig. 1). Statistical analysis with χ^2 test showed also that the total mortality rate of caterpillars from three locations for 4 replicates in RING group was significantly increased versus water control (χ^2 value = 6.8 on the 14th day, corresponds to $p < 0.01$), whereas BIR oligonucleotide has not demonstrated a significant insecticidal effect on caterpillars during the whole period of the experiment.

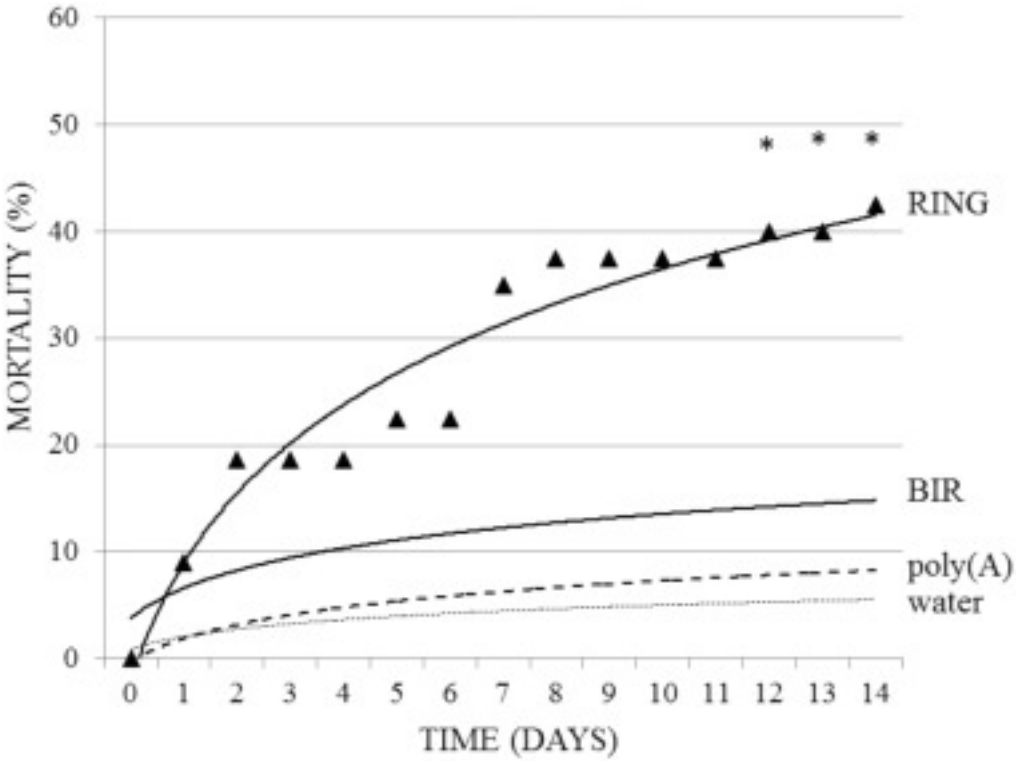

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Fig. 1. Increase of caterpillar mortality after RING ssDNA treatment. Logarithmic trend lines for mean mortality (%) in RING (solid line), BIR (solid line) and control groups (poly(A) and water, dashed and dotted lines respectively), and means for RING group (triangles) are plotted versus time after single treatment with DNA insecticide. Significance of difference between RING group and control is indicated by * when $p < 0.05$.

Importance of this result is in separate application of ssDNA from BIR and RING domains. The RING domain ssDNA fragment on alone is able to cause significant insecticidal activity, emphasizing that RING domain played dominant insecticidal role in a pair BIR + RING, applied in our previous experiments, probably acting as an antisense molecule [3], [4].

Generally, in caterpillars from RING-treated group, that were going to perish, we observed the loss of appetite, slowness of movement, dehydration and decrease of body size that corresponds to apoptotic patterns on the cell level [25] and on the organism level [26]. These signs possibly indicate apoptosis processes that lead to further death of the insect. We evaluated then the reduction of biomass of caterpillars as one of the apoptotic patterns [25].

3.2. Biomass reduction caused by the applied DNA fragments

On the 7th day after the treatment we noticed significant reduction in biomass of survived caterpillars from RING group in comparison with control (Fig. 2). The difference in mean biomass became more evident on the 14th day. Mean individual biomasses on the 14th day were 180.5 ± 27.4 mg, 157.9 ± 21.8 mg, and 105.7 ± 20.4 mg in control, BIR and RING groups respectively.

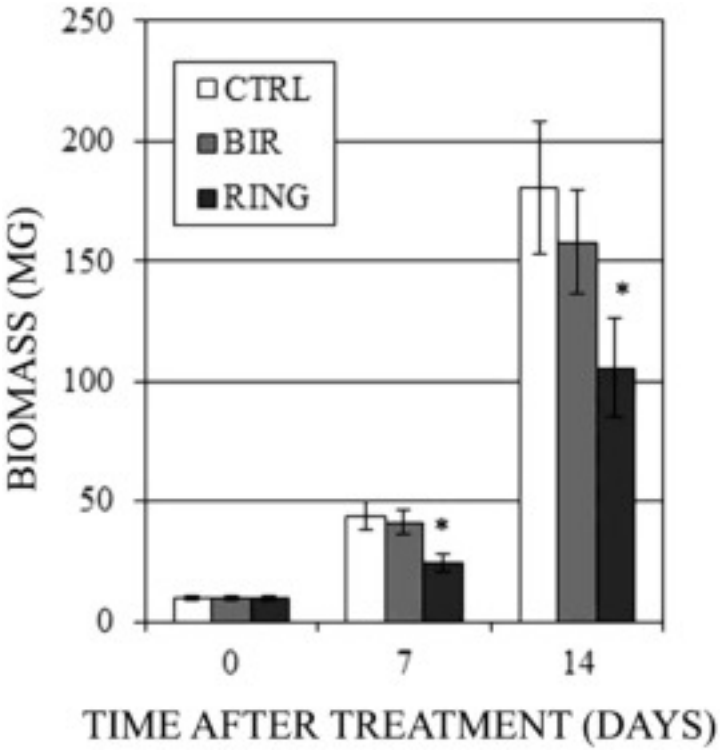

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Fig. 2. Caterpillar biomass reduction after treatment with RING ssDNA fragments. Biomass of caterpillar in milligrams is plotted for control water group (CTRL), BIR and RING groups before treatment (day 0) and on days 7 and 14 after single treatment with DNA insecticide. Means and SE are represented for 4 experimental replicates with caterpillars from 3 different locations. Significance of difference between RING group and CTRL is indicated by * when $p < 0.05$.

To date, it is the first evidence of significant influence of short antisense single-stranded viral DNA fragments on accumulation of insect biomass. Taking into consideration our previous results we think that antisense RING domain fragment might specifically act (whereas sense BIR domain fragment might not) through the blocking of the mechanisms involved in post-transcriptional expression of insect anti-apoptosis genes [3], [4]. Of note, in most studies "standard" amounts of double-stranded RNA applied to achieve RNAi varies between 1 and 100 μg (reviewed by Terenius in [10]). In our experiments we used 3 pmol of viral 18nucleotide long DNA fragments per caterpillar that corresponds to approximately 3 ng of ssDNA per mg of body mass. Thus, our DNA insecticide works in substantially lower concentrations and accordingly may be cheaper in comparison with RNA preparations forpest insect control.

We evaluated if significant reduction of biomass accumulation of gypsy moth caterpillars is associated with LdMNPV "contamination" of gypsy moth larvae. PCR for detection of a p39capsid gene region did not reveal the virus infection in the investigated caterpillars from all studied groups (data not shown). Thus, biomass reduction effect caused by the applied viral antisense DNA fragment depends only on a genotype of the host insect.

3.3. Increasing of alkaline phosphatase activity in response to RING ssDNA

After 24 h from the start of the treatment we evaluated alkaline phosphatase activity in caterpillars in different groups of treatment and found that only caterpillars from RING group had significantly elevated alkaline phosphatase activity in comparison with water control (Fig. 3), plausibly meaning the stress condition.

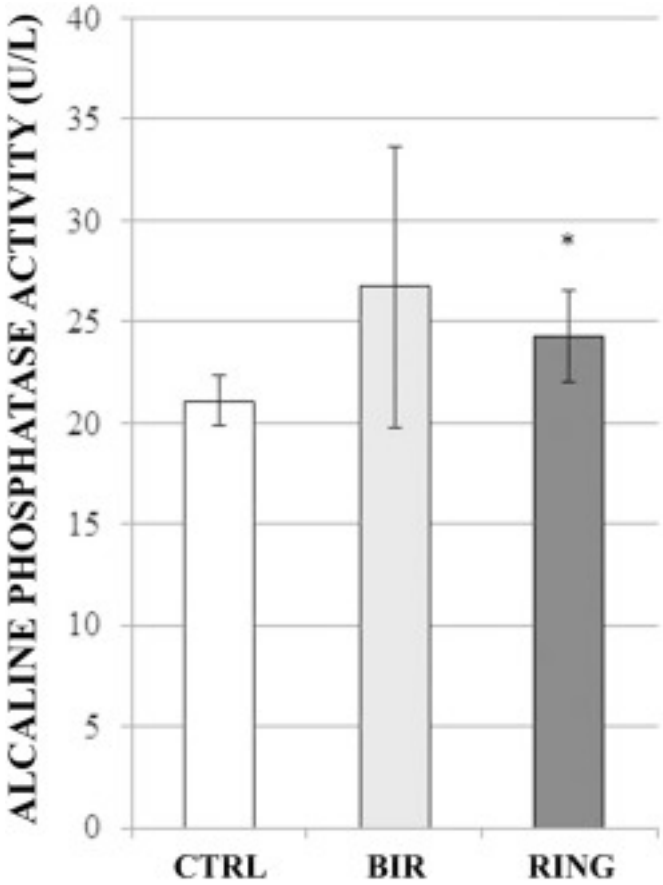

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Fig. 3. Alkaline phosphatase activity in larvae from different groups 24 h after treatment with BIR and RING DNA fragments. Means and SE are represented for 4 experimental replicates with caterpillars from 3 different locations. Significance of difference between RING group and control is indicated by * when $p < 0.05$.

3.4. Reduced pupal mass and the number of female imago survived after RING treatment

We next supposed that biomass reduction in different groups of the experiment could affect the ratio of survived female and male insects based on evidence that pupae and imago of gypsy moth females are substantially greater than those of males [27]. Thus, the biomass accumulation should be more critical for female insects, and observed weight loss of caterpillars induced by RING ssDNA may affect stronger female individuals.
Indeed, after pupation we noticed that the average size and mass of pupae was not the same in the different groups. While the average individual biomass of control pupae was in conventional range, insects treated with RING ssDNA fragment showed significantly decreased individual pupal mass (0.59 ± 0.07 g in RING vs 0.76 ± 0.06 g in control, Fig. 4).

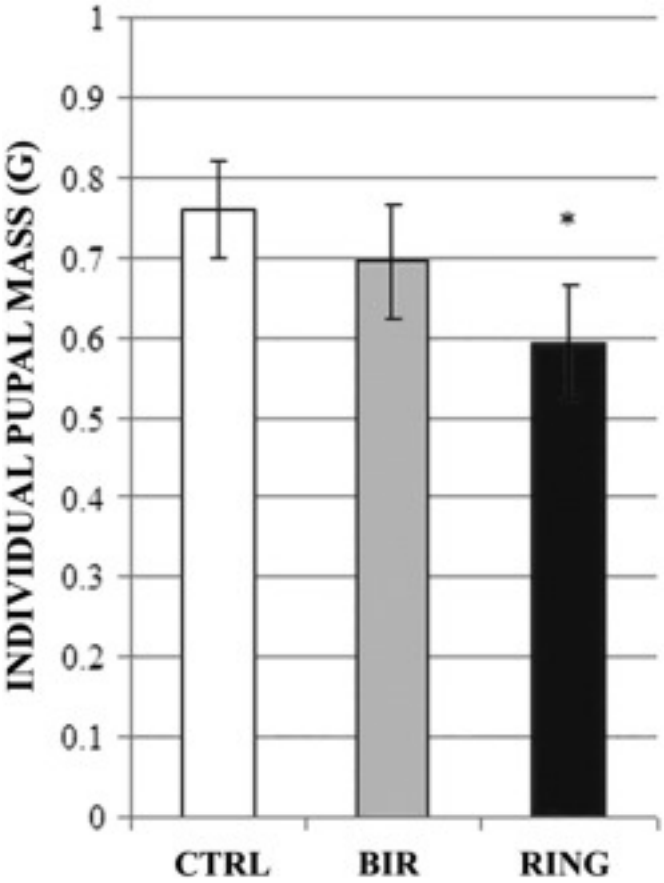

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Fig. 4. Decrease of pupal mass after RING ssDNA treatment. Individual pupal mass was measured in control group (CTRL, n = 28 pupae), BIR group (n = 23) and RING group (n = 21) after treatment (means and SE). Significance of difference between RING group and control is indicated by * when $p < 0.05$.

We calculated the number of imago of male and female insects after the pupal stage and found that in RING group the frequency of survived females was significantly 1.66 times lower in comparison with control group, whereas in BIR group it was 1.46 times lower versus control (Table 1). Males survived better the treatment with RING ssDNA [28].

Table 1. The frequency of female imago survived after DNA insecticide treatment in BIR (baculoviral IAP (inhibitor of apoptosis) repeat) and RING (really interesting new gene) groups.

	Control	BIR	RING
Female frequency	0.63	0.43	0.38*
The χ^2 value vs control	–	0.32 ($p = 0.57$)	4.09 ($p < 0.05$)

The χ^2 values for experimental groups versus water control group were calculated. Significance of difference versus control is indicated by * for $p < 0.05$.

3.5. Calcium and magnesium concentration in eggs are affected by RING ssDNA

To study if the stress reaction effects are confined to treated insects or extended also tooffspring (next Lymantria generation), we studied some regulatory processes in eggs, laid by affected Lymantria female imago. Calcium and magnesium involved processes may have pivotal role in egg development, thus the egg calcium and magnesium macroelement concentrations were measured.

We found significant increase in accumulation of calcium and magnesium in eggs laid by treated females in RING group in comparison with untreated control group and BIR group. The mass fraction of calcium measured in eggs laid by affected females in Control, BIR and RING groups were 0.50%, 0.46% and 0.78% of total ash egg mass respectively. The similar significant imbalance was detected for concentrations of magnesium in Control, BIR and RING groups, where mass fractions were 0.06%, 0.05% and 0.35% of total egg mass respectively (Fig. 5).

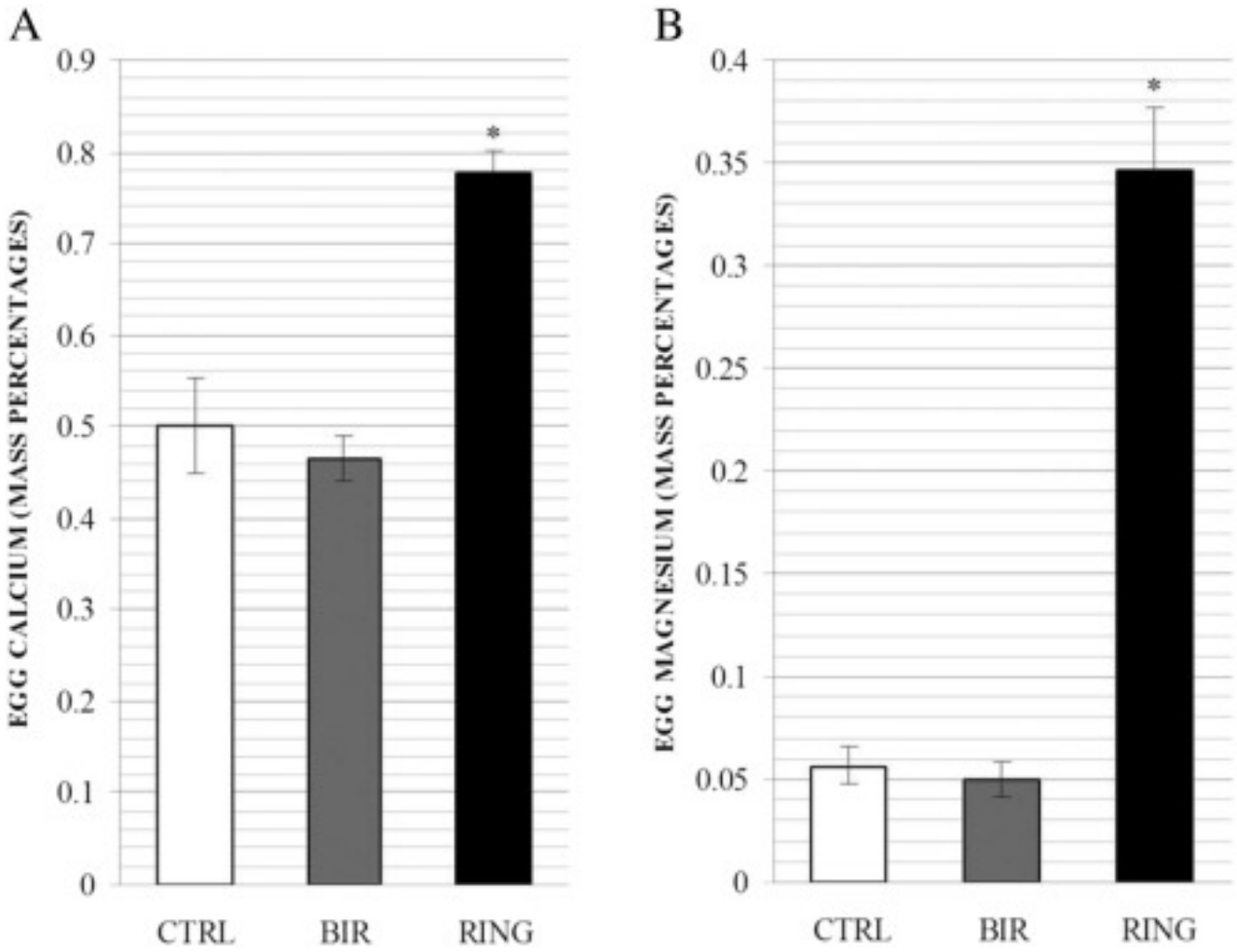

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Fig. 5. Concentration of calcium and magnesium in the gypsy moth eggs affected by RING ssDNA. Means and standard errors of mass fraction of calcium (A) and magnesium (B) in the eggs are presented for 3 replicates for each control (CTRL), BIR and RING groups. Significance of difference between RING group and CTRL is indicated by * when $p < 0.05$.

3.6. DNA insecticides affect the apoptotic and anti-apoptotic gene expression in the next generation of *L. dispar*

To learn more about long-term effect of single application of ssDNA fragments on the subsequent generation we studied the expression in eggs of some important Lymantria genes, involved in cellular stress response and apoptotic/anti-apoptotic reactions: caspase-4(European Nucleotide Archive reference is HQ328971), IAP-1 (AB758122.1), gelsolin(JQ787871.1) and hemolin (AF453868). For these experiments RNA was extracted from eggs that were laid by survived females from different groups. As a control housekeeping gene we used actin (AF182715.1). In RING group we observed the increase in IAP-1 and caspase-4 gene expression (by 16 ± 7% and 90 ± 44% respectively), and strong expression increase in gelsolin mRNA (79 ± 42 times) (Fig. 6). These results show that single treatment of *L. dispar* larvae with ssDNA fragments of LdMNPV IAP-3 gene is not limited to direct action on caterpillars, but leads to increased expression of important genes involved in various regulations, including apoptotic processes in eggs.

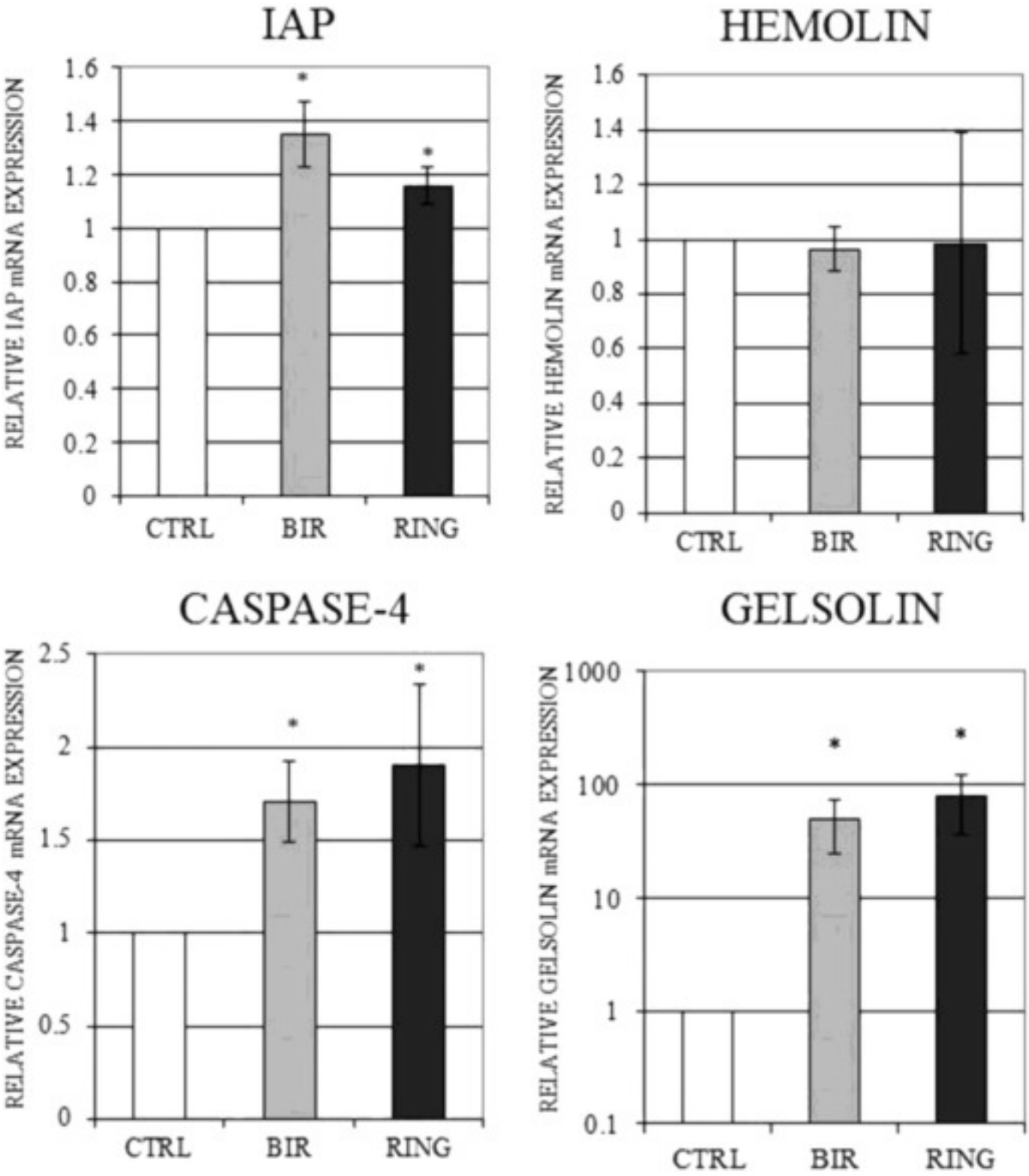

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Fig. 6. Treatment of larvae with BIR and RING ssDNA leads to increased IAP, caspase-4 and gelsolinexpression in deposed eggs. Means and standard errors of mRNA expression are represented for 3 replicates relative to control group (CTRL). Significance of difference between BIR and RING groups vs CTRL is indicated by * when $p < 0.05$.

Interestingly, measured in eggs, we observed significant effect on expression of apoptotic/anti-apoptotic genes in both RING ssDNA and BIR ssDNA groups. This suggests to extend in future the study of involved genes' expression in

all insect development stages (caterpillar, pupa, imago, egg) for further understanding the insecticide features of RING fragment ssDNA versus BIR + RING fragments ssDNAs [3], [4].

4. Discussion

This study opens potential new routes for insect pest management on the base of short single-stranded antisense fragments of anti-apoptosis genes of insectnucleopolyhedroviruses. Beyond significant direct insecticidal activity of RING domainfragment, it substantially affects the biomass accumulation of caterpillars, increases femalemortality rate and leads to the increased expression of important cell stress genes in the eggs laid by female insects. Thus, DNA insecticide based on RING domain fragment possesses a long-term effect on the treated caterpillars and causes substantial difference in number of caterpillars that will appear in the next generation in comparison with BIR-treated group and control. Of interest, consequences of just one treatment with the viral antisense DNA fragment, that were observed during the whole life cycle and in the next generation ofgypsy moth, suggest that viral DNA has an ability to coordinate particular cellular pathways, not only being a part of whole viral genome, but also as short fragments of it. It is fundamental and important field of research in the light of rising interest in the interaction between host and DNA viruses in humans. Based on the observed significant role of ssDNA we can suppose the general existence of mechanisms of eukaryotic cell regulation by endogenous short ssDNA fragments, which require further investigation.

n our opinion, in the present study antisense RING fragment showed insecticidal effect due to the interference with the expression of gypsy moth IAP genes, which led to apoptosis of the insect cells and subsequent weight loss and death of caterpillars. Recently, we cloned one part of the gypsy moth genome with the fragments of BIR and RING domains of LdMNPV IAP-3 gene as primers, and it had an overlap with: i) the corresponding part of the LdMNPV IAP-3 gene and ii) *L. dispar* IAP-1 mRNA for an inhibitor of apoptosis protein with the high cover by query. It allowed us assuming that we cloned a part of gypsy moth anti-apoptosis gene [3]. That finding suggests that described here insecticidal effect of antisense RING domain fragment might act through the inactivation of the mechanisms involved in post-transcriptional expression of insect anti-apoptosis genes. Interesting, Yamada et al. [29]have found that LdMNPV IAP-3 gene induced apoptosis in Ld652Y cells in a transient expression assay. We think that RING domain fragment blocks a host IAP gene which is similar to LdMNPV IAP-3 gene and has the anti-apoptotic activity in gypsy moth. Moreover, it is potentially interesting to study the fragments of other single-stranded fragments of LdMNPV IAP genes as DNA insecticides, such as Lp-Apsup (*L. dispar* apoptosis suppressor) [30].
Ions of several metals such as calcium and magnesium play an important role during apoptosis [31]. In cells that undergo activation or excitation, calcium is released from the

endoplasmic/sarcoplasmic reticulum to activate calcium-dependent kinases andphosphatases, thereby regulating numerous cellular processes, including apoptosis andautophagy. In the case of apoptosis, endogenous ligands or pharmacological agents induced prolonged cytosolic calcium elevation, which in turn could lead to cell death [32]. One of the major characteristics of apoptosis is the degradation of DNA after the activation of Ca/Mg dependent endonucleases, resulting in DNA cleavage with strand breaks [33]. In this study we found that insecticidal effect of RING domain fragment, apparently according to the apoptosis scenario, caused significant overall accumulation of calcium and magnesium ions in the eggs laid by the affected females, confirming that the single-stranded DNA fragment has strong effect on insect metabolism which is extended to eggs. Analysis of expression of genes, involved in apoptosis processes shows that both antisense RING and sense BIR fragments lead to increased expression of IAP-1, gelsolin andcaspase-4 genes in eggs. To explain observed phenomenon one of the hypotheses is that the response of a host cell on viral ssDNA fragments, is probably initiated through recognition of deoxyribose in structure of DNA. DNA in normally functioning cells is not found in cytoplasm and its appearance out of the nucleus may serve as a signal for stress reaction or immune response. We think that RING fragment acts both non-specifically and specifically (possibly as antisense molecule) whereas BIR fragment acts solely in non-specific manner, explaining the difference in activity described in Results section. It is not clear for now whether only these two BIR and RING ssDNA fragments (or other virus ssDNAs) are able to induce such time distant effect on expression of apoptotic and anti-apoptotic genes in eggs.

Another explanation of observed effects may be the action of ssDNA through activation of Toll-like insect receptors [34]. We found 4 CpG parts (the ligands for insect analog of Toll-like 9 receptor) in antisense RING fragment and 2 CpG parts in sense BIR fragment. Stimulation (or over-stimulation) of immune system of *L. dispar* larvae by CpG might lead to the decreased viability and premature death of the insect and requires further investigation. The field of insect immunity is under rising interest because of its importance for insect physiology, and can become a potential target for creation of new insecticides [35].

The way of DNA insecticides' application, the topical spraying, is effective and convenient, as shown here. In the literature, investigations on two insects – the gypsy moth *L. dispar*(works of V. Oberemok, starting from the description of method in 2008 in Ukrainian patent and in 2011 in his PhD thesis) and the mosquito *Aedes aegypti*[36] – provided the first demonstration that mortality of insects can be induced by topical application of both short single-stranded DNA (early investigations of V. Oberemok) and relatively long double-stranded RNA [36]. Additionally, the topical application of dsRNA was also demonstrated in the Asian corn borer *Ostrinia* furnacalis[22]. The effectiveness of topical

application of single-stranded DNA or double-stranded RNA was quite unexpected. Previously, for example, it had been thought that oral administration was the only possible way to deliver double-stranded RNAs to target tissues, other than injection, as the insect midgut is not protected by chitin. The induction of insect mortality by topical application of single-stranded DNA or double-stranded RNA could be explained by passage to interior tissues via the tracheal system that is not covered by chitinous exoskeleton [37] or via diffusion through soft thin cuticle over most of the body of young larvae. Hence, it might be impossible to use DNA insecticides against cryptic feeding insects and adult beetles because elytra could provide some protection from a contact insecticide. At the same time, DNA insecticides look very suitable for pest insect control of lepidopteran pests at caterpillar stage, especially during early larval instars, when exoskeleton of the insects is thin.
To avoid influence of LdMNPV on the experiments, we studied here only LdMNPV-free insects, thus viral DNA fragment interfered with host metabolic reactions that were wholly under the control of the genetic apparatus of the insect and not virus. Interestingly, our another study on LdMNPV-infected insects shows that RING domain fragment in the same concentration has faster significant insecticidal effect on I–III instar gypsy moth caterpillars infected with LdMNPV in comparison with controls composed of LdMNPV-free and LdMNPV-infected gypsy moths (under publication).

In this research RING fragment caused stress reaction and elevated alkaline phosphataseactivity in gypsy moth caterpillars in a manner similar to some other insecticides. It is known that insect cells react on stress factors, including different insecticides through non-specific elevation of alkaline phosphatase activity [38], [39], [40]. For example, activity of acid and alkaline phosphatases in cypermethrin treated beetles was significantly increased after 24 h exposure [38]. Thus, together with effect on viability of gypsy moth caterpillars and biomass accumulation, systemic biochemical alteration is an additional proof of DNA insecticide effectiveness.
Importantly, DNA insecticides could resolve or improve insecticide resistance problem. If we will use short single-stranded fragments of very conservative parts of insect host anti-apoptosis genes, resistance to the insecticides will grow slower because the potential mutations that change the target anti-apoptosis genes occur at very low rate in the conservative parts. Thus, if we are not able to stop genetic processes leading to insecticide resistance, we could slow down emergence of insecticide resistance by using DNA insecticides on the base of very conservative regions of functionally important genes, such as anti-apoptosis genes. This approach is of immense value if not revolutionary, and elaborations in this field may lead to a very safe and cheap agriculture sustained by the DNA insecticides.

Important further elaboration of DNA insecticides we see in the deepening in the comprehension of the mechanism of their action, additional reduction of the ssDNA concentration in the end-product and increasing of their effectiveness by ssDNA carriers using. We plan to establish if insect-specific DNA insecticides based on short antisense fragments of viral antiapoptosis genes will be effective for other species of phytophagous insects, and generally if the observed phenomenon is widely distributed among insects.

Conflict of interest

The authors declare no competing financial interests.

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