

DNA Insecticides: Application of the Iap-2 Gene Single-stranded Fragments from Three Different Nucleopolyhedroviruses against Second Instar Gypsy Moth Larvae

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Abstract We discovered that the external application of a solution containing one single-stranded DNA fragment, 18 nucleotides long, either from the BIR domain of the AcMNPV IAP-2 gene or the RING domain of the TnSNPV IAP-2 gene, induces a significantly higher mortality of gypsy moth larvae compared to a solution of distilled water. The results show the insecticidal potential of viral DNA fragments that can be used to create safe, relatively inexpensive and fast-acting insecticides to control quantity of pest insect populations. In our opinion, insecticidal effect may depend on the specific sequence of double and triple hydrogen bonds in a single-stranded DNA fragment or on a sequence of a few nitrogenous bases at specific sites of an oligonucleotide. The results suggest that small single-stranded viral DNA fragments may have practical applications as DNA insecticides.

Keywords DNA Insecticides, Anti-Apoptotic Gene IAP-2, Gypsy Moth, Nucleopolyhedrovirus, Pest Control

1. Introduction

One of the main reasons why viral preparations have not found wide practical application is because their production is based on the cultivation of large numbers of host insects. This technology is time and labour intensive, which makes it expensive to produce the amount of virus required to control insect pest populations. Beyond the economic cost of production, baculoviruses take longer to kill their targets than chemical insecticides do [12]. A large field of research is devoted to improving baculoviruses by increasing their infection rate through genetic modifications [6]. An alternative to this trend may be a technology based on the application of single-stranded viral DNA fragments

possessing insecticidal activity [13, 14, 15, 18].

The creation of effective biological preparations based on small fragments of DNA is promising due to the mode of action of viral DNA in the host cells, the large variability and specificity and uniqueness of the sequences, and a relatively high chemical stability. The concept that underlies the creation of DNA insecticides is following. Exogenously introduced single-stranded DNA fragments of a virus that coincide with genes of a host cell should influence its biochemical activity in a manner similar to antisense molecules [2] and by mechanisms that resemble those of DNA and RNA interference [4, 9]. DNA insecticides may also be made based on the many genes that play important roles in a host cell's life, e.g. anti-apoptosis genes.

Baculoviruses have two classes of anti-apoptosis genes, p35 and IAP genes that can block apoptosis, the process of programmed cell death, through the induction of various signals in a phylogenetically wide range of organisms [1, 11]. It is known that anti-apoptotic baculoviral genes (IAP genes) are homologous to genes found in their hosts [5]. This fact predicts their ability to influence the host apoptosis/anti-apoptosis system and cause apoptosis. Thus, if anti-apoptosis genes of a virus are homologous to the host anti-apoptosis genes, then the application of single-stranded fragments of viral anti-apoptosis genes should interfere in the biochemical reactions of the gypsy moth cells (for example, mechanisms characteristic for antisense oligonucleotides) that will lead to the blocking of anti-apoptosis proteins synthesis. When many cells undergo apoptosis, an insect dies.

One possible and convenient method of applying DNA insecticides is by external application characteristic of contact insecticides. It is known, that the presence of developed epicuticle limits to some extent the permeability of an insect's exoskeleton for majority of insecticides. Nevertheless, chlororganical, organophosphate and other

contact insecticides easily penetrating the epicuticle through the most permeable areas [20] that are unprotected with cuticle and wax, such as the spiracles of the respiratory system. Single-stranded DNA molecules have both hydrophilic (polar sugar-phosphate backbone) and hydrophobic (nitrogenous bases) regions [21] that allow them penetration through the polar and non-polar parts of insect tissues. Nitrogenous bases, being hydrophobic, tend to face the central axis of the double helix, pointing away from the surrounding aqueous environment. In the case of single-stranded DNA, the nitrogenous bases tend to interact with hydrophobic molecules of the surrounding environment (wax of epicuticle, triacylglycerides of cytoplasmic membrane, etc.). Experiments on RNA interference have shown that negatively charged double-stranded RNA fragments are able to penetrate through the cuticle of the round worms [10, 19] and insects [22].

Thus, it is possible to create fast-acting, safe and relatively inexpensive DNA insecticides to control phyllophagous insect populations. Our previous research work on gypsy moth showed a significant insecticidal effect of single-stranded fragments from the IAP-3 gene of *Lymantria dispar* multicapsid nucleopolyhedrosis virus [13, 14, 15, 18]. In this paper, we examine the possibility of applying DNA insecticides, based on fragments of IAP-2 gene of different viruses, against gypsy moth larvae.

2. Material and Methods

Two DNA fragments from relatively conserved baculovirus inhibitor of apoptosis repeat (BIR) (sense chain) and highly conserved really interesting new gen (RING) (antisense chain) domains of anti-apoptotic gene IAP-2 of nuclear polyhedrosis viruses of gypsy moth (*Lymantria dispar* (Lepidoptera: Erebidiae)) – LdMNPV, cabbage looper (*Trichoplusia ni* (Lepidoptera: Noctuidae)) – TnSNPV and alfalfa looper (*Autographa californica* (Lepidoptera: Noctuidae)) – AcMNPV were chosen for our experiments. Both domains have significant similarities, especially in the RING domain, with analogous fragments of anti-apoptotic genes of other known nucleopolyhedroviruses. Each of the IAP-2 gene fragments tested consists of 18 nucleotides and were diluted in distilled water to a concentration of 100 pmol/μl. The DNA sequences of the six fragments tested were obtained from the International Committee on Taxonomy of Viruses database and synthesized by Eurofins MWG Operon (Germany) with HPSF-clearance. Distilled water was used as a negative control. The larvae were reared from egg masses collected in the field. Second-instar gypsy moth larvae from different egg masses were randomized and used for the experiments. Each larva was externally treated with approximately 1 μl aliquot of either distilled water or a solution containing DNA fragments with pipette, and then placed into a vessel for cultivation. Larvae were reared on fresh birch leaves. Each variant of experiment was performed in three replicates with 25-30 individuals of gypsy

moth per each variant. Statistical analysis was performed using Pearson's χ^2 -test with Yates's correction comparing the controls with each DNA fragment tested. Pairwise comparisons were also conducted between pairs of treatments.

3. Results

Fragments of anti-apoptotic gene IAP-2 of nuclear polyhedrosis viruses (NPV) of gypsy moth, cabbage looper and alfalfa looper were found to have different effects on the viability of gypsy moth caterpillars. Both the BIR domain of the AcMNPV IAP-2 gene and the RING domain of the TnSNPV IAP-2 gene had significant insecticidal effects. Surprisingly, fragments of RING and BIR domains of the LdMNPV IAP-2 gene did not have a significant effect on the gypsy moth larvae tested (Figure 1, Table 1).

Table 1. Values of χ^2 -test for different pairs of the experiment

Variants	Value of χ^2 , df=1
LdMNPVIAP-2 BIR/control	0.19
LdMNPVIAP-2 RING/control	2.78
TnSNPVIAP-2 BIR/control	0.19
TnSNPVIAP-2 RING/control	4.36*
AcMNPVIAP-2 BIR/control	7.41**
AcMNPVIAP-2 RING/control	2.39

*P<0,05; **P<0,01; df = 1

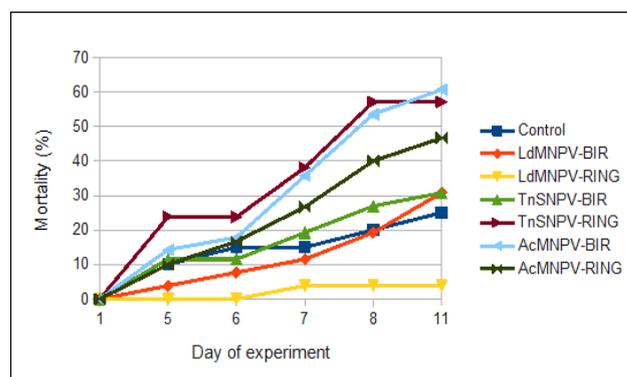


Figure 1. Dynamics of mortality of caterpillars in different groups of experiment

In similar experiments with LdMNPV IAP-3 gene, significant differences in mortality between experimental (BIR+RING) and control groups were found [13, 14, 15, 18]. Analogous fragments of the BIR and RING domains of the LdMNPV IAP-2 gene do not possess insecticidal effect for gypsy moth larvae. One possible explanation is the difference in structures between IAP-2 and IAP-3 genes (Table 2). The level of similarity between BIR and RING domains of IAP-2 and IAP-3 genes is 44% and 72%, respectively.

Table 2. Level of similarity between fragments of BIR and RING domains of IAP-2 and IAP-3 genes

Gene fragment	Sequence	Level of similarity
LdMNPV _{IAP-2} BIR	5'-CGCAAACGCCCTGGCCGC-3'	8 nucleotides, 44%
LdMNPV _{IAP-3} BIR	5'-GCC GGC GGA ACTGGCCCA-3'	
LdMNPV _{IAP-2} RING	5'-ACACGTGCC GGCACGGCA-3'	13 nucleotides, 72%
LdMNPV _{IAP-3} RING	5'-CGACGTGGTGGCACGGCG-3'	

Table 3. Level of similarity among the oligonucleotides tested and insecticidal effect

Oligonucleotide	Level of similarity between each oligonucleotide and AcMNPV _{IAP-2} BIR 5'-GTT GAC ATG TTG GCT CGT-3'	Larval mortality, %
LdMNPV _{IAP-2} RING	5'-ACA CGT GCC GGC ACG GCA-3'	3.9
TnSNPV _{IAP-2} RING	5'-TGA CGT GTT TAC AAG GCA-3'	57.1
AcMNPV _{IAP-2} RING	5'-CCA GGT GAC GGC ACG GCA-3'	46.7
LdMNPV _{IAP-2} BIR	5'-CGCAAACGCCCTGGCCGC-3'	30.8
TnSNPV _{IAP-2} BIR	5'-CGG CAA CTT TTT GGC TGT-3'	30.8
AcMNPV _{IAP-2} BIR	5'-GTTGACATGTTGGCTCGT-3'	60.7

Table 4. Level of similarity by sequences of double and triple bonds between fragments of BIR and RING domains of IAP-2 genes from *Lymantria dispar*, *Trichoplusia ni* and *Autographa californica* nucleopolyhedroviruses

Single-strandedDNA fragment	Sequence of double and triple hydrogen bonds in a fragment
LdMNPV _{IAP-2} , BIR	5'-333 222 333 332 333 333-3'
TnSNPV _{IAP-2} , BIR	5'-333 322 322 222 333 232-3'
AcMNPV _{IAP-2} , BIR	5'-322 323 223 223 332 332-3'
LdMNPV _{IAP-2} , RING	5'-232 332 333 333 233 332-3'
TnSNPV _{IAP-2} , RING	5'-232 332 322 223 223 332-3'
AcMNPV _{IAP-2} , RING	5'-332 332 323 333 233 332-3'

The experiment revealed some interesting peculiarities in the insecticidal activity of different oligonucleotides depending on their structure (Table 3). For example, AcMNPV_{IAP-2} BIR and TnSNPV_{IAP-2} RING fragments with very different sequences (16 nucleotides are different), had similar significant levels of insecticidal activity (60.7% and 57.1% respectively) (Table 3).

In contrast, AcMNPV_{IAP-2} RING and LdMNPV_{IAP-2} RING, with very similar sequences (only 3 different nucleotides), had very different levels of insecticidal activity against gypsy moth larvae (46.7% and 3.9 %, respectively).

Our results suggest that insecticidal activity may depend on the localization of only a few nucleotides at specific sites of the oligonucleotide. If this hypothesis is right, then this phenomenon may lead to a less expensive method for producing DNA insecticides, where synthesis of only a few nucleotides would be required to obtain a commercial product.

Another possible hypothesis for the differences in insecticidal activities is related to the orientation of hydrogen bonds in single-stranded DNA fragments. DNA fragments

from AcMNPV_{IAP-2}, BIR and TnSNPV_{IAP-2}, RING domains both have fairly long identical sequences of double and triple hydrogen bonds that can be formed by A (or T) and G (or C) nitrogenous bases (Table 4). These sequences could be interacting with target molecules, such as enzymes, that play an important role in a cell's life.

Total mortality caused by the oligonucleotides is not significant when compared with distilled water (control) ($\chi^2=1.33$; $df=1$). This implies only certain sequences have insecticidal activity and emphasizes the fact that cells selectively "respond" to specific oligonucleotides.

4. Discussion

The results show that DNA insecticides may have a potential in regulating pest populations. The mode of operation of DNA insecticides inside the cell is not clear, since the BIR fragment of the AcMNPV IAP-2 gene that showed the most significant mortality compared with the control belongs to the part of the sense strand of the viral genome (Table 5).

Table 5. Categorization of fragments as sense (+) or antisense (–) strands

DNA fragment	Sequence	Category
TnSNPV _{IAP-2} BIR	5'-CGG CAA CTT TTT GGC TGT-3'	+
AcMNPV _{IAP-2} BIR	5'-GTT GAC ATG TTG GCT CGT-3'	+
LdMNPV _{IAP-2} BIR	5'-CGC AAA CGC CCT GGC CGC-3'	–
TnSNPV _{IAP-2} RING	5'-TGA CGT GTT TAC AAG GCA-3'	–
AcMNPV _{IAP-2} RING	5'-CCA GGT GAC GGC ACG GCA-3'	–
LdMNPV _{IAP-2} RING	5'-ACA CGT GCC GGC ACG GCA-3'	+

The described situation is not typical for RNA interference and antisense oligonucleotides. The possible explanation may be given by SDTHB hypothesis (sequences based on double and triple hydrogen bonds) and off-target effects. This hypothesis makes meaningful sense, since the hydrogen bond is probably the most important bond that supports the interaction between molecules in a living cell. Beyond the classical pairing A/T(U) and G/C, in nucleic acids in a cell, there can also exist interactions between single-stranded nucleic acids and enzymes or structural proteins that based on definite sequences of double and triple hydrogen bonds found in a single-stranded RNA or DNA fragment. We term this phenomenon as SDTHB hypothesis. This idea is quite similar to Morse alphabet with its dashes and dots, and gives us a deeper look at possible interactions between single-stranded fragments of nucleic acids and other molecules than only the classical pairing of A/T(U) and G/C does because it works just for nucleic acids.

Concerning off-target effects, it is known that various RNAi and microRNA reagents, which differ in length and structure, often cause non-sequence-specific immune responses, in addition to triggering the intended sequence-specific effects [16]. This effect depends mostly on the reagent length, its structure, chemical modification, concentration, and cellular localization, rather than on its specific sequence, which may be involved in off-target effects [3, 7]. There are two possible hypotheses that could explain this phenomenon. The first hypothesis is that AcMNPV_{IAP-2} BIR is an antisense fragment for another important gene(s) of the cell but not for anti-apoptosis genes. The second hypothesis is that ssDNA fragments do not interact with pre-mRNA or mRNA of anti-apoptosis genes but interact with one or more proteins that play an important role in a cell life, such as the NOD2 (nucleotide oligomerization domain) [17] and NLRP3 (NOD-like receptor proteins) [8] sensors that belong to the NOD and NOD-like receptors, recognizing single-stranded and double-stranded RNA, respectively. NLRP3 and NOD2, in turn, stimulate the production of proinflammatory cytokines and interferons (IFNs) that leads to the inhibition of cell division and growth and eventually to apoptosis [16].

Well-known among bioinformatics programs BLAST (Basic Local Alignment Search Tool) [23] gives the maximum score for the *Lymantria dispar* clone Ld10 chorion protein mRNA with AcMNPV_{IAP-2} BIR (72% similarity as a minus strand) and for the vitellogenin gene of gypsy moth (plus strand) with TnSNPV_{IAP-2} RING (83% similarity as a minus strand). It is difficult to predict if the vitellogenin gene or the gene of chorion protein are involved in the insecticidal effect since the genome of gypsy moth has not been sequenced yet. The exact mechanism of action of AcMNPV_{IAP-2} BIR and TnSNPV_{IAP-2} RING fragments action is not clear and requires further studying. Although the mode of operation of DNA insecticides remains a “black box”, preparations based on single-stranded DNA fragments have a good potential for the practical application.

Regulation of insect pest populations in agrobiocenoses and artificial plantations is very important. Controlling of pest populations requires a safe, effective and inexpensive method. DNA insecticides could offer commercial products having the best characteristics of current insecticides: the low cost and speed of action of chemical insecticides combined with the safety of baculoviral preparations.

The main disadvantages of DNA insecticides are the necessity of contacting the insect cuticle and the relatively high cost of oligonucleotides production technology. These need to be overcome if DNA insecticides are to be used commercially on a large scale. The obvious advantages of DNA insecticides, such as speed of action and safety, make them promising means of insect control that appear ideal for practical application against phyllophagous insects. However, 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.

5. Conclusions

The external application of a water solution containing one 18 nucleotides long single-stranded DNA fragment, either from BIR domain of AcMNPV IAP-2 gene or RING

domain of TnSNPV IAP-2 gene, causes significantly higher mortality of gypsy moth second-instar larvae in comparison with the control in laboratory experiments. The results indicate that viral DNA fragments have the potential to create safe, inexpensive and fast-acting DNA insecticides to control pest insect populations.

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