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RAPID COMMUNICATION

Insecticidal activity of three 10–12 nucleotides long antisense sequences from 5.8S ribosomal RNA gene of gypsy moth *Lymantria dispar* L. against its larvae

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Abstract

5.8S ribosomal RNA plays an important role in protein synthesis and eukaryotic ribosome translocation. Contact DNA insecticides based on antisense fragments of 5.8S ribosomal RNA gene of gypsy moth *Lymantria dispar* L. showed prospective insecticidal activity on its larvae. The most pronounced insecticidal effect was found for antisense fragments 10 and 11 nucleotides long (oligoRIBO-10 and oligoRIBO-11), whereas 12 nucleotides long fragment (oligoRIBO-12) caused the lowest level of insect mortality. This data corresponds to results obtained earlier using rabbit reticulocyte and wheat germ extracts, where maximum inhibition of protein synthesis was observed when a relevant oligomer 10-11 nucleotides long was used, whilst longer chain lengths resulted in reduced inhibition. Using oligoRIBO-11 fragment we have shown penetration of antisense oligonucleotides to insect cells through insects' exoskeletons. MALDI technique registered the penetration of the oligoRIBO-11 fragment into insect cells after 30 min and a significant response of insect cells to the applied oligonucleotide after 60 min, which indicates not only that the oligonucleotide enters the insect cells, but also the synthesis of new substances in response to the applied DNA fragment. Contact DNA insecticides developed from the L. dispar 5.8S ribosomal RNA gene provide a novel biotechnology for plant protection using unmodified antisense oligonucleotides.

Keywords: antisense oligonucleotides, DNA insecticides, gypsy moth, insect pest control, *Lymantria dispar*, 5.8S ribosomal RNA

Gypsy moth (*Lymantria dispar* L.; Lepidoptera: Erebidae) larvae are voracious feeders, able to consume more than 1 m^2 of foliage per larva during the caterpillar stage (Chen *et al.* 2013; Grayson *et al.* 2015). Larvae favor oak, but also feed on the foliage of 500 other plant species, including some conifers. Defoliation caused by gypsy moth larvae and the subsequent lack of carbohydrates weakens trees, which makes them more susceptible to borers, micropathogens, and drought. If a healthy tree is defoliated, the tree may re-leaf during the summer, but with smaller leaves (Petrovskii and McKay 2010). An already stressed tree defoliated by gypsy moth larvae may partially or totally die as a result of defoliation, although the impact may not be seen for many years. During surges in gypsy moth populations (outbreaks), which can last 1–3 years, larvae are capable of completely defoliating their host trees, after which they move on to cereal crops and even vegetables. The population densities of the gypsy moth are able to reach outbreak levels that can cause considerable economic losses in forests in Europe, Asia, Africa, North America (Alalouni *et al.* 2013), and even New Zealand (Pitt *et al.* 2004).

Biological control of the gypsy moth is based on the use of *Lymantria dispar* multiple nucleopolyhedrovirus (baculovirus preparations). Unfortunately, the use of selective baculovirus preparations in forestry and agriculture is not always successful. This failure can be explained by the fact that the occurrence of artificial epizootics depends not only on environmental, but also genetic factors, particularly genetic resistance to the applied micropathogen (Asser *et al.* 2007). Among non-selective chemical insecticides, pyrethroids, organophosphorus compounds, carbamates, chitin synthesis inhibitors, and neonicotinoids are most often used for gypsy moth control. As an alternative, over the past decade, there has been increased attention paid to the development of insecticides based on unmodified nucleic acid fragments, in particular antisense DNA fragments (Oberemok *et al.* 2017a; Oberemok *et al.* 2018) and double-stranded RNA fragments (Wang *et al.* 2011; Gu and Knipple 2013). These next-generation control agents are able to combine the best characteristics of modern insecticides: the affordability and swift action of chemical insecticides coupled with the selectivity of biological preparations. *In vitro* nucleic acid synthesis technologies are becoming less expensive, which means that in the future, the affordability of DNA insecticides and RNA preparations will become comparable to that of chemical insecticides. It should be noted that antisense DNA-based insecticides are the only nucleic acid preparations currently being developed for gypsy moth control. A post-genomic approach to regulating the number of leaf-eating insects based on the use of antisense oligonucleotides has great potential to become commercially viable in the near future.

For our most recent experiments with DNA insecticides, we chose to use the 5.8S rRNA gene, since successful application of its fragments as antisense oligonucleotides has been well documented. Studies on the inhibition of protein synthesis by specific anti 5.8S rRNA oligonucleotides have suggested that this RNA plays an important role in eukaryotic ribosome function, although the molecular basis for the involvement of 5.8 S rRNA in this process remains unclear. Speculation regarding the function of this sequence has focused on determining if it plays a role in tRNA binding (Lo *et al.* 1987; Abou-Elela and Nazar 1997;) and ribosome translocation (Graifer *et al.* 2005), at least in a universally conserved GAAC sequence region common to all 5.8S RNAs (Nazar 1982), since the region surrounding it is often species specific (Abou-Elela and Nazar 1997). Significant and reproducible inhibition has been produced using rabbit reticulocytes with several different unmodified DNA oligonucleotides; among these, the most inhibitory were specific for the universally conserved 5′-GAAC-3′ sequence. In the experiments carried out using rabbit reticulocyte extract, maximum inhibition was observed when an oligomer 10–11 nucleotides long was used; longer chain lengths resulted in reduced inhibition. A similar reduction was observed with wheat germ extract. With each type of extract, mutated sequences (even single-nucleotide mutations) significantly reduced the level of inhibition (Walker *et al.* 1990), providing a basis for the selectivity of action. As a result, we designed 10–12 nucleotide long antisense oligonucleotides (5′-TGCGTTCGAA-3′ – oligoRIBO-10; 5′-TGCGTTCGAAA-3′ – oligoRIBO-11; 5′-TGCGTTCGAAAT-3′ – oligoRIBO-12) from the *L. dispar* 5.8S ribosomal RNA gene that includes the universally conserved antisense 5′-GTTC-3′ sequence and applied it as a contact DNA insecticide in our experiments. Recently, we proposed novel biotechnology to protect plants from insect pests using DNA insecticide with improved insecticidal activity based on a new antisense oligoRIBO-11 sequence from the 5.8S ribosomal RNA gene. This investigational oligoRIBO-11 insecticide causes higher mortality among both *L. dispar* larvae grown in the lab and those collected from the forest (Oberemok *et al.* 2019). Additionally, it is more affordable and faster acting than our pioneer preparations based on longer antisense fragments of anti-apoptosis genes of the baculovirus-host system (Oberemok *et al.* 2017b), which makes it a prospective candidate for use in the development of a ready-to-use preparation (Oberemok *et al.* 2019). In this rapid communication, we will compare the insecticidal potential of three different 10–12 nucleotide long antisense sequences from the 5.8S ribosomal RNA gene of the gypsy moth *L. dispar* against its larvae. Also using the MALDI (matrix-assisted laser desorption/ionization) technique, we provided for the first time evidence of the penetration of the oligoRIBO-11 fragment into insect cells through the insects' exoskeletons.

Table 1. Mortality of *Lymantria dispar* larvae (shown as a percentage)

Day	Control	oligoRIBO-10	oligoRIBO-11	oligoRIBO-12
3rd	5.42 ± 1.05	12.92 ± 5.24*	9.18 ± 6.1	6.68 ± 2.45
6th	13.33 ± 3.4	29.17 ± 10.64*	26.68 ± 11.58*	23.32 ± 7.08*

*significant difference for $p < 0.05$

The mortality of the topically treated *L. dispar* larvae (30 pmol · larva^{-1}) reared in the lab (origin of egg masses: Tyumen, Russia) on a wheat germ-based insect artificial diet increased significantly on the 3rd day after treatment only in the oligoRIBO-10 group ($\chi^2 = 7.23$, $p < 0.01$, $N = 480$, $df = 1$) compared with the mortality of larvae in the control (water-treated) group (Table 1). In the groups treated with water, oligoRIBO-10, oligoRIBO-11, and oligoRIBO-12, we observed larval deaths of 5.42, 12.92, 9.18, and 6.68%, respectively. On the 6th day after treatment, we observed a statistically significant increase in insect mortality caused by oligoRIBO-10, oligoRIBO-11, and oligoRIBO-12, compared to the mortality seen in the control (water-treated) group ($\chi^2 = 19.21$, $p < 0.01$, $N = 480$, $df = 1$; $\chi^2 = 14.41$, $p < 0.01$, $N = 480$, df = 1; and $\chi^2 = 8.85$, $p < 0.01$, $N = 480$, $df = 1$, respectively). In the groups treated with water, oligoRIBO-10, oligoRIBO-11, and oligoRIBO-12, we observed larval deaths of 13.33, 29.17, 26.68, and 23.32%, respectively.

Thus, the most pronounced insecticidal effect was observed for antisense fragments 10 and 11 nucleotides long (oligoRIBO-10 and oligoRIBO-11), whereas a fragment 12 nucleotides long (oligoRIBO-12) caused the lowest level of insect mortality. These data correspond to results obtained earlier using rabbit reticulocyte and wheat germ extracts, where maximum inhibition of protein synthesis was observed when a relevant oligomer 10–11 nucleotides long was used; reduced inhibition resulted when oligos with longer chain lengths were used (Walker *et al.* 1990). Of note, one of the most pressing challenges when creating DNA insecticides against gypsy moth is the need to increase insect mortality, which at the moment is no more than 40–50% (Oberemok *et al.* 2019), compared to the mortality seen in the control, which generally does not exceed 10%. Fortunately, on other insect pests, we have obtained much more promising results, which indicate the great potential of this direction of research. In particular, we have shown a 90–100% mortality of the *Unaspis euonymi* Comstock under the action of a DNA insecticide based on an antisense fragment of 28S ribosomal RNA gene of this insect pest (unpublished data).

We used the oligoRIBO-11 fragment (5’-TGCGT TCGAAA-3’) (0.9 nmol · larva^{-1}) to demonstrate penetration of antisense oligonucleotides through the integuments into the cells of gypsy moth larvae. Studies were then conducted using the MALDI technique and a Bruker Microflex MALDI-TOF (Bruker, USA). To date, mass spectrometry is the most highly sensitive physico-chemical method available for analyzing oligonucleotides and proteins. After the oligoRIBO-11 fragment was applied to the larvae, MALDI registered the penetration of the fragment into the insect cells at 30 min post-treatment (peak penetration at 3360.1 Da) and detected a significant response to the applied oligonucleotide 60 min post-treatment (Fig. 1). In the control group, no peak characteristic of the oligoRIBO-11 fragment was found.

In addition, in the control group, the profile of the recorded peaks (peaks at 2558.2 Da, 2582.2 Da, and 2910.5 Da) differed noticeably from those of the experimental groups. This indicates that the oligonucleotide not only entered the insect cells, but also that synthesis of new substances in response to the applied DNA fragment occurred. Many new peaks were obtained in the diagram for samples 60 min post-treatment with the oligoRIBO-11 fragment (peaks at 2935.8 Da,

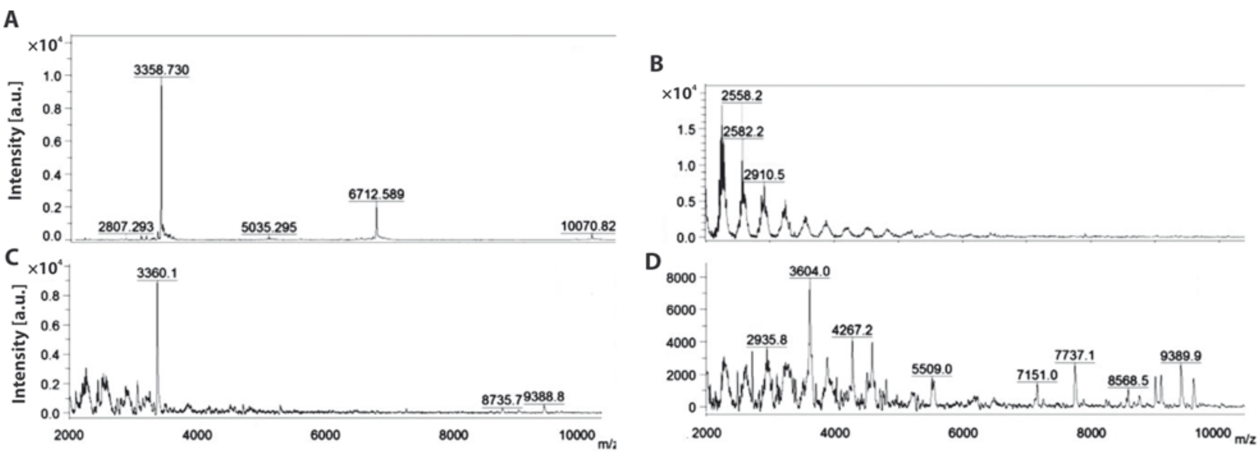

Fig. 1. Peak diagram of the substances found in homogenate samples of tissues from individual gypsy moth larvae after contact treatment with oligoRIBO-11 fragment (0.9 nmol · larva^{-1}): A – standard oligoRIBO-11 fragment (3358.73 ± 10 Da); B – control; C, D – 30 and 60 min after the drop containing the oligoRIBO-11 fragment has dried, respectively. Analysis of all samples was carried out after 7 consistent washes of the larvae with a solution of water and 70% alcohol. The experiment was carried out three times. Diagrams detailing the characteristics of the groups are presented for each group in the experiment. Samples were pre-desalted on Illustra™ NAP™ -5 G-25 Sephadex ™ columns according to manufacturer's instructions (GE Healthcare Life Sciences, USA) and then concentrated

3604 Da, 4267.2 Da, 5509 Da, 7151 Da, 7737.1 Da, 8568.5 Da, and 9389.9 Da). Studies using a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, USA) showed that, by their nature, a significant number of *de novo* registered substances belong to oligonucleotides with a predominantly higher molecular weight than that of the oligoRIBO-11 fragment. Their size ranged from 3238 to 7151 Da. In the control treated with water, there were absolutely no fractions of oligonucleotides ranging in size from 3238 to 7151 Da. Obviously, the oligoRIBO-11 fragment itself is a primer for such a synthesis of the new oligonucleotides by polymerase. This provides compelling evidence that short antisense DNA fragments (DNA insecticides) are able to penetrate the integuments of the gypsy moth larvae, triggering an active cell response. Thus, contact DNA insecticides developed from the *L. dispar* 5.8S ribosomal RNA gene penetrate the insects' exoskeletons and provide novel biotechnology for plant protection using unmodified antisense oligonucleotides.

Following certification, DNA insecticides will occupy a niche for well-tailored and affordable preparations on the current plant protection product market. In some cases, such as when attempting to control secretive insects and adult beetles, it may be impossible to use DNA insecticides because elytra may provide some protection from contact insecticides. Nevertheless, DNA insecticides appear to be excellent candidates for insect pest control of non-secretive lepidopteran pests at the larval stage, especially during early larval instars, when the insects' exoskeletons are thin (Oberemok *et al*. 2018).

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