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A small molecule for a big transformation: topical application of a 20-nucleotide-long antisense fragment of the DIAP-2 gene inhibits the development of *Drosophila melanogaster* female imagos

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Abstract: Several genes have been identified to play important roles associated with sex selection in *Drosophila melanogaster*. An essential part is attributed to the sex-lethal gene that depends on the expression of the X:A (number of chromosomes to autosomes) ratio signal controlling both sex selection and dosage compensation processes in *D. melanogaster*. Interestingly, for sex selection in *D. melanogaster* there are no documented data addressing the role of the inhibitor of apoptosis (IAP) genes and their signaling influence on this biological process. In this study, we found that topical application of a 20-nucleotide-long antisense DNA fragment (oligoDIAP-2) from the death-associated inhibitor of apoptosis (DIAP)-2 gene interferes with *D. melanogaster* development and significantly decreases the number of female imagos and their biomass. We show that the applied antisense oligoDIAP-2 fragment downregulates the target DIAP-2 gene whose normal concentration is necessary for the development of female *D. melanogaster*. These data correspond to the results on downregulation of the target host IAP-Z gene of *Lymantria dispar* L. female imagos after topical treatment with an 18-nucleotide-long antisense DNA fragment from the *L. dispar* multicapsid nuclear polyhedrosis virus IAP-3 gene at the larval stage. The observed novel phenomenon linking the downregulation of insect IAP genes and the low rate of female imago development could have practical application, especially in insect pest control and molecular pathology.

Key words: inhibitor of apoptosis (IAP) genes; *Drosophila melanogaster* DIAP-2 gene; sex determination; developmental biology; DNA insecticides

INTRODUCTION

The *Drosophila* genome encodes the baculovirus inhibitor of apoptosis protein repeat (BIR) and really interesting new gene (RING) domain-containing IAP family members, (DIAP)-1 and DIAP-2 proteins; ectopic expression of either protein inhibits apoptosis. DIAP-1 protein is required continuously in many cells to inhibit the apical caspase Dronc and effector caspases activated by Dronc, such as Drice [1]. It has been suggested that DIAP-2 protein might also be an important inhibitor of apoptosis [2,3]. RNAi-mediated knockdown of the DIAP-2 gene in the S2 cell-line has been reported to result in increased susceptibility to stress-induced apoptosis. Several RNAi-based studies in S2 cells have also provided evidence that DIAP-2 protein is required for the innate immune response to infection with Gram-negative bacteria. DIAP-2 protein may identify a point of convergence between apoptosis and immune signaling pathways [4]. Notably, DIAP-2 protein has three BIR (DIAP-1 has two BIR domains) and one RING domain. The BIR domain mediates protein-protein interaction with caspase and IAP antagonists. The presence of several BIR domains increases the flexibility and potency of caspase inhibition [5] and may account for other functions [6] of DIAP-2 protein in *Drosophila melanogaster* cells. It was also shown that DIAP-2 null mutant animals develop normally and are fully viable, suggesting that DIAP-2 is dispensable for proper development [3], but with the question of "whether the down-regulation or

absence of DIAP-2 gene can affect the ratio of developed male and female imagos".

Although IAPs are multifunctional signaling proteins that affect diverse biological processes [3], there are no published data addressing their role in the sex determination of *D. melanogaster*. The mechanism of sex differentiation or determination is initiated by dimerization of proteins expressed by the sisterless gene encoded in the X chromosome and the deadpan gene encoded in the autosomes, respectively [7]. This results in homodimer and heterodimer products that depend on the ratio of X chromosomes and autosomes in the cell. Sequential development continues with the activation of regulatory genes, such as the sex lethal gene (Sxl) which can occur in early or late stage, the transformer gene (tra) and the double-sex gene (dsx) after which splicing occurs, alternately or by default, to produce dsx-f (double-sex-female)-specific or dsx-m (double-sex-male)-specific proteins, respectively, for onward female and male *D. melanogaster* development [8,9].

In the course of investigations with DNA insecticides on *L. dispar* larvae [10-13], we found that the topical application of the antisense (18-nucleotide-long) oligoRING fragment from the *Lymantria dispar* multicapsid nuclear polyhedrosis virus (LdMNPV) IAP-3 gene increased the mortality rate of insects and caused a significant decrease (1.66-fold) in the number of developed females when compared to the control group [12]. It was also shown that in baculovirus-free *L. dispar* larvae, oligoRING acts as antisense RNase H-dependent oligonucleotides [14], inducing degradation of target mRNA of the host IAP-Z gene (homologous to LdMNPV IAP-3 gene), followed by subsequent downregulation of target protein expression.

The present study investigates this pattern in order to determine whether the phenomenon is widely distributed among other insects, particularly in *D. melanogaster*. We topically applied a 20-nucleotide-long antisense DNA fragment (oligoDIAP-2) from the DIAP-2 gene to *D. melanogaster* larvae to investigate if it leads to a low rate of female imago development.

MATERIALS AND METHODS

Treatment technique

D. melanogaster

Twenty 2nd-3rd instar larvae were placed into snap-cap Eppendorf tubes per 1 replicate; they were further immersed into treatment solutions containing oligonucleotides (20 pmol/μL) for 20 min. The larvae were transferred into vials with fresh culture media and observed for 10 days until the imago emerged. The experiment was repeated in 9 replicates. The morphology and number of emerged imagos were analyzed under a binocular microscope.

L. dispar

On average, 20-25 2nd instar larvae from four locations in the Crimean forest were used per each control and experimental group for treatment with DNA fragments. Each experiment was performed in 4 replicates. A water solution with a single-stranded (ss) DNA fragment (10 pmol/μL, either BIR or RING) was applied topically to larvae via fine spraying. We collected small drops of solution from the surface of 10 larvae and found approximately 0.2-0.3 μL of solution on each larva after spraying (2-3 pmol of ssDNA per larva).

Insect rearing

D. melanogaster

A total of 540 2nd-3rd instar larvae of wild-type strain (Simferopol, Crimea), maintained on baker's yeast-based forage under a 12:12 h light/dark cycle maintained at 25-27°C and 55-60% relative humidity were used. Laboratory scales Axis BTU210 (Axis, Poland) with 1 mg discreteness were used to weigh the female imagos. *D. melanogaster* is a sexually dimorphic species, in which males and females can be easily distinguished on the basis of several morphological differences. The adult male abdomen is rounded and the posterior segments of the abdomen are entirely dark and shiny. Females have an abdomen with a pointed tip and the coloration of the posterior segments varies from pale to almost entirely dark.

L. dispar

After treatment with oligoDNAs, *L. dispar* larvae were grown in Petri dishes on oak leaves (*Quercus robur* L.*)* at 25°C until pupation. On emergence of imago from pupae, female moths were taken for host IAP-Z gene expression analysis with real-time (RT) quantitative reverse transcription (PCR). *L. dispar* is a sexually dimorphic species, in which males and females can be easily distinguished on the basis of several morphological differences. Adult males are light brown with dark brown wings while adult females are slightly larger than males and are almost white with a few dark bands on the wings.

Sequences of the applied DNA fragments

D. melanogaster.

ssDNA oligonucleotides from sense and antisense regions of the DIAP-2 gene were designed according to the information with regard to the *D. melanogaster* death-associated inhibitor of apoptosis 2 (DIAP-2) transcript variant B mRNA, with NCBI reference sequence: NM_176182.2 (https://www.ncbi.nlm.nih.gov), synthesized by Evrogen (Russia). The synthesized ssDNA fragments were as follows: (1) 5′-ATA TGC ATT CTC CAA TAA AT-3′ (1963-1982; sense region), and (2) 5′-ATT TAT TGG AGA ATG CAT AT-3′ (1963-1982; antisense region). The oligonucleotides were diluted with distilled water to a concentration of 20 pmol/μL. The insect larvae were divided into 3 groups: "sense", "antisense" (treated with oligonucleotides from DIAP-2 gene), and the control group (treated with distilled water).

L. dispar.

DNA fragments were synthesized by Evrogen (Russia). The sequences of the applied ssDNA fragments from LdMNPV IAP-3 gene were as follows [12]: 5′-GCC GGC GGA ACT GGC CCA-3′ (LdMNPV strain 3054, BIR domain, sense strand; http://www.ictvonline.org) and 5′-CGA CGT GGT GGC ACG GCG-3′ (LdMNPV strain 3054, RING domain, antisense strand; http://www.ictvonline.org).

Gene expression analysis by RT-PCR

RNA extraction was carried out with a PureLink® RNA Mini kit (Ambion, Life technologies, USA) according to the manufacturer's instructions. Prior to extraction, the imagos were ground by pestle in liquid nitrogen in a 1.5 mL tube. Six independent extractions were carried out to produce replicates per each variant. The quality of extracted total RNA was assessed by loading of eluted volume (5 μL) into 1.5% agarose gel and running in Tris-borate-EDTA (TBE) buffer at 10 V/cm for 30 min. The quantity, intensity and pattern of RNA bands were equal in all experimental groups, confirming the quality and reproducibility of the RNA extraction from the insect material. For reverse transcription, the total RNA (5 μg) was annealed with oligo-dT(18) primer and processed with the RevertAid H Minus Reverse Transcriptase kit (Thermo Scientific, USA) according to the manufacturer's instructions. The reaction was performed at 42°C for 60 min in Thermostat "Termite" (DNA Technology, Russia).

D. melanogaster

For quantification of *D. melanogaster* DIAP-2 gene expression, forward 5′-TGA AAT GAC CTT ATA TAG AT-3′ and reverse 5′-ATT TAT TGG AGA ATG CAT AT-3′ primers were applied. A LightCycler® 96 instrument by Roche (Switzerland) and qPCRmix-H SYBR reagents by Evrogen (Russia) were used, according to the following procedure: 10 min initial denaturation at 95°C, followed by 40 cycles with 10 s of denaturation at 95°C, 20 s annealing at 60°C, 20 s elongation at 72°C. Finally, all PCR products were melted to estimate the specificity of the amplification and the presence of additional products.

L. dispar

For quantification of the *L. dispar* IAP-Z gene (sequenced recently) which has more than 90% similarity with the LdMNPV IAP-3 gene, forward 5′-AGG CCC GTG TCG CCG GTC-3′ (oligoIAP-Z) and reverse 5′-CGA CGT GGT GGC ACG GCG-3′ (oligoRING) primers were used. The qPCRmix-HS SYBR (Evrogen, Russia) master mix was used according to manufac-

turer's instructions. A LightCycler® 96 instrument by Roche (Switzerland) was used to set up amplification according to the following procedure: 10 min of initial denaturation at 95°C, followed by 30 cycles of 10 s of denaturation at 95°C, 10 s of annealing at 60°C, 16 s of elongation at 72°C. Finally, all PCR products were melted to estimate the specificity of the amplification and the presence of additional products.

Data analysis

Data analysis was performed using the nonparametric Chi-square test with Yates's correction, followed by a Student's t-test for group comparison. Data are represented as mean±standard error. The analysis was carried out with Microsoft Excel 2007 and STATISTICA 7.

RESULTS AND DISCUSSION

A significant difference in mortality between oligoDNA-treated *D. melanogaster* individuals and control was not detected (Fig. 1A). Mortality was slightly higher in both the sense (increased by 13.1%) and antisense groups (15%) in comparison with the control. However, among the total number of survived imagos, the percentage of developed *D. melanogaster* females was significantly lower in the antisense group (31.2%) in comparison with the control (55.1%) ($p<0.01$) (Fig. 1B). Thus, topical application of the antisense fragment from the DIAP-2 gene significantly decreased the development of female imagos. We also found a significant decrease ($p<0.01$) in the biomass of *D. melanogaster* female imagos in the group treated with antisense oligonucleotide in comparison with the control (0.75-1.23 mg) (Fig. 2). This corresponds with the results obtained for *L. dispar* larvae that were topically treated with the antisense oligoRING fragment of the LdMNPV IAP-3 gene [12].

In *D. melanogaster* female imagos, the antisense fragment of the DIAP-2 gene caused a 4.96±0.06-fold significantly stronger downregulation of the target DIAP-2 gene when compared with the control ($p<0.01$) (Fig. 3). Thus, the antisense fragment from the DIAP-2 gene acted as an antisense RNase H-dependent oligonucleotide whose action was followed by downregulation of target protein expression [14].

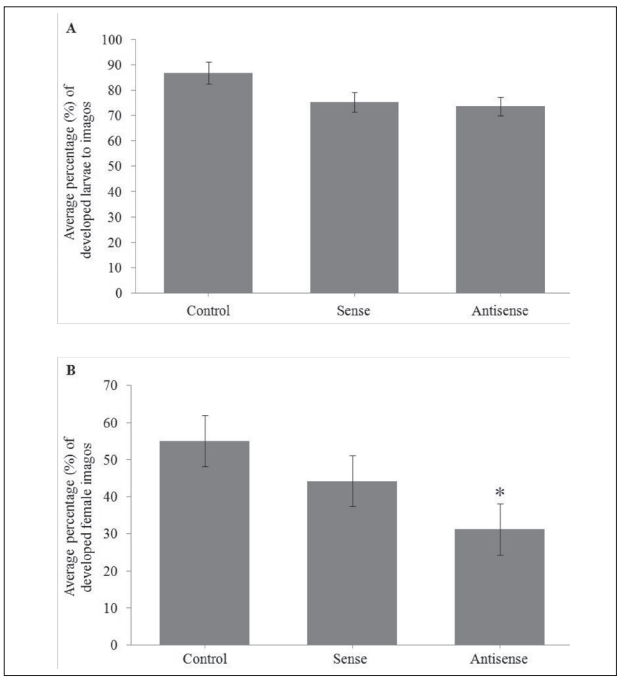

Fig. 1. Average percentage of developed imagos. **A** – total number of developed imagos (males + females) from oligoDNA-treated groups did not show significant difference in survival as compared to the control. The mean and SE are for 9 replicates. **B** – antisense fragment from DIAP-2 gene induces decreased rates of *D. melanogaster* female development in comparison with the control. The mean and SE are for 9 replicates. The significance of difference between the control and antisense DIAP-2 group is marked with an asterisk when $p<0.01$.

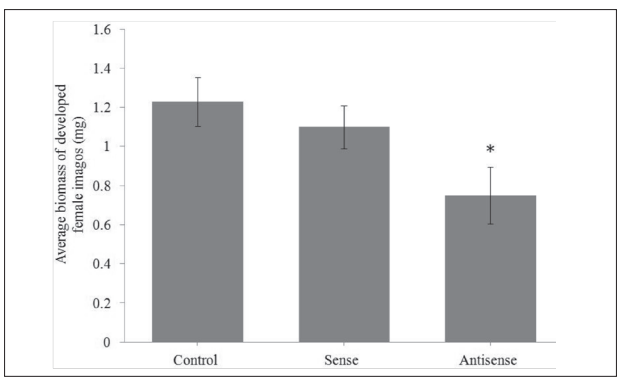

Fig. 2. Antisense fragment from DIAP-2 gene decreased the biomass of *D. melanogaster* female imagos as compared to the control. The mean and SE are for 9 replicates. The significance of difference between the control and antisense DIAP-2 group is marked with an asterisk when $p<0.01$.

Notably, at the pupal stage, the target DIAP-2 gene was not significantly downregulated in insects from both groups treated with oligonucleotides. Similar results were obtained for host IAP-Z gene (homologous to

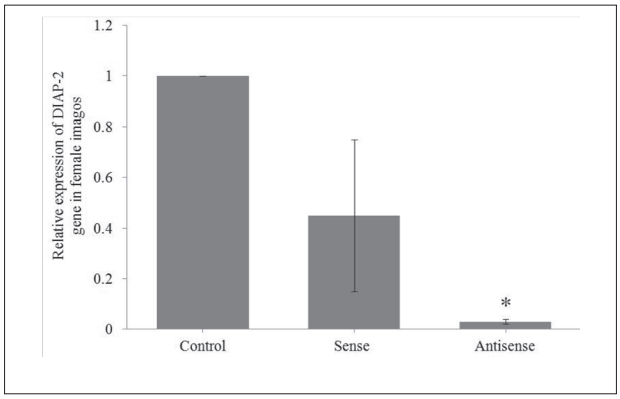

Fig. 3. Treatment of *D. melanogaster* larvae with an antisense fragment from the DIAP-2 gene leads to significant downregulation of target DIAP-2 gene expression in female imagos. The mean and SE of mRNA expression are for 6 replicates relative to the control. The significance of difference between the antisense DIAP-2 group vs. the water-treated control is indicated by an asterisk when $p<0.01$.

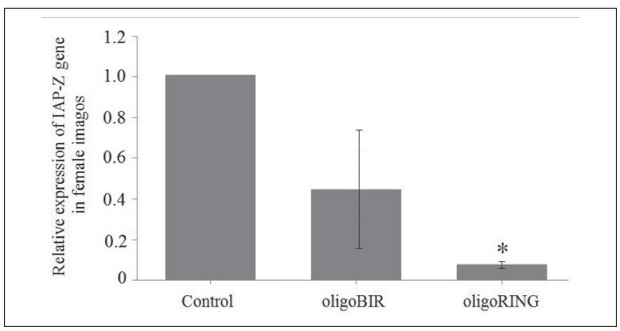

Fig. 4. Relative expression of IAP-Z gene in *L. dispar* female imagos in response to topical application of ssDNA. An asterisk (*) is present when $p<0.01$; expression of the IAP-Z gene in the control group (water) was taken as 1 (100%). The mean and SE are for 6 replicates.

LdMNPV IAP-3 gene) expression in *L. dispar* female imagos after topical application of ssDNA fragments, which is presented in Fig. 4.

Here we report for the first time that topical treatment of *L. dispar* larvae with the antisense RING-domain fragment of its nuclear polyhedrosis virus (oligoRING) leads to a 12.06±1.48-fold significant downregulation of the target host IAP-Z gene in *L. dispar* female imagos when compared with the water-treated control at about 75 days posttreatment. The data indicate that the fragment of the LdMNPV IAP-3 gene 5′-CGA CGT GGT GGC ACG GCG-3′ (oligoRING) acts as an antisense RNase H-dependent oligonucleotide, inducing the degradation of target mRNA of the *L. dispar* IAP-Z gene. The obtained results on the downregulation of the *L. dispar* IAP-Z gene in response to the oligoRING insecticide is of potentially great interest for species-specific plant protection approaches [12].

Genes that have been identified as playing crucial roles in sex selection and determination in *D. melanogaster* are evolutionary conservative [15]; however, no direct link to IAP genes in sex determination in *D. melanogaster* and other insects, including *L. dispar*, has been established. The concept of development and survival from Drosophila embryo to adult involves several processes, with a network of cell proliferation, differentiation and apoptosis events [16-18] in which both DIAP-1 and DIAP-2 genes play important roles. For the first time, we show that our ssDNA antisense oligoDIAP (20-nucleotides-long) fragment is capable of interfering with sex selection in *D. melanogaster* with a high efficiency. The results obtained in this study pave the way to a better understanding of other functional complexities played out by the DIAP-2 gene in *D. melanogaster*, especially with the use of ssDNA antisense fragments. Sex selection in *D. melanogaster* is guided by a cascade of events that lead to a decision-making cardinal point that involves the dsx (doublesex) gene, a major gene that concludes the regulatory events [19-22] and directs the cell to synthesize either male- or female-specific proteins. Several factors can disrupt or inhibit the functions of the dsx gene and sex determination, which can include sex-specific sensitivity to environmental variation [23], multiple forms of Sxl proteins [24] that can also be expressed in this process, as well as some mechanisms of evolutionary transitions between genetic sex-determining systems [25]. We suggest that the topical application of the antisense oligoDIAP-2 fragment (5′-ATT TAT TGG AGA ATG CAT AT-3′) triggers silencing of the dsx gene at some point in the cascade of cell reactions, which inhibits the synthesis of specific proteins required for the development of female imagos. Obviously, the first step in this process is initiated by the downregulation of the target DIAP-2 gene, followed by a decrease in biomass accumulation, which is more critical for the development of female imagos since they are larger than male imagos. We suggest that the antisense oligoDIAP-2 fragment interfered with the morphological make-up of the genetic females of *D. melanogaster* and that it stimulated the development

of phenotypic males. This corresponds to the results obtained for *L. dispar* after treatment with the oligoRING fragment [12]. The phenomenon of intersex is well-known and occurs in both insect species [26].

In today's post-genomic era, antisense oligonucleotides are facing a renaissance, and they could be applied as an effective and species-specific tool for controlling insect pests. Antisense oligoIAP fragments, termed in our investigations as "DNA insecticides" [13,27,28], are powerful tools that can increase our understanding of the functions of IAPs that contain conservative RING domains. RING domains function as modules for ubiquitination that can additionally regulate gene expression, and which play a role in the sex determination of several animals [29]. Insect antisense oligoIAP fragments have the potential to harness insect populations [30] and can be applied in molecular pathology, studies in developmental biology and other areas of the fundamental biology of insects.

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