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Characterization of the deoxyuridine triphosphatase gene of *Ophiusa disjungens* nucleopolyhedrovirus

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Summary. – *Ophiusa disjungens* is one of the main insect pests that attack *Myrtaceae* species. Nucleopolyhedroviruses (NPVs) of the *Baculoviridae* family have been used for decades as biological pesticides to control insect pests. A new NPV, named *Ophiusa disjungens* nucleopolyhedrovirus (OpdiNPV), was recently isolated from OpdiNPV-infected *O. disjungens* larvae. In this study, a *Pst*I fragment of OpdiNPV genome containing the deoxyuridine triphosphatase (dUTPase) gene was successfully cloned, sequenced and analyzed. Upstream of a 402 bp long ORF of the dUTPase gene, encoding a 133 aa long protein, typical transcription promoter boxes CAGT and TATA were found. The dUTPase was first expressed in his-tagged form in *Escherichia coli* as a 35.5 kDa protein. Then it was successfully expressed in insect *Trichoplusia ni* (Tn) cells in the form of an EGFP-fusion protein. It first appeared (at 24 hrs post infection (p.i.)) in the cell nucleus, but later (at 72 hrs p.i.) it was excluded from the nucleus and diffusely scattered all over the cell. These findings may serve as basis for development of engineered OpdiNPVs as biopesticides to control *O. disjungens* and other Lepidoptera insects.

Keywords: Ophiusa disjungens; nucleopolyhedrovirus; dUTPase

Introduction

Insect viruses, especially NPVs, which infect arthropods and consist of rod-shaped virions that contain large double-stranded, supercoiled DNA genomes ranging in size from 88 to 165 kilobase pairs (Blissard and Rohrmann, 1990), are considered potential biocontrol agents of the larvae of many Lepidoptera. NPVs typically produce two virion phenotypes of progeny virus: occlusion-derived virus and budded virus. The occlusion/derived virus transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas the budded virus causes systemic infection within the host (Keddie *et al*., 1989). The two viral forms are essential for natural propagation of NPVs (Yang *et al*., 2009). NPVs can be isolated from infected larvae, then purified and reproduced to control insect pests. NPVs are now used for expression of heterologous genes. Furthermore, the development of cell culture techniques have made large-scale production of genetically modified NPVs for improved viral pesticides possible.

Ophiusa disjungens (Walker) (previously known as *Anua indiscriminata*), is a moth of the *Noctuidae* family. It is found in Southeast Asia and the South Pacific Ocean, including China, Thailand, Japan, Tonga, New South Wales, and Queensland. The larvae feed on various *Myrtaceae* species, including *Eucalyptus spp*., *Syncarpia glomulifera* and *Psidium guajava.* A NPV was found in the larvae of *A. indiscriminata* and denominated as AiNPV (Li *et al*., 2007). However, *A. indiscriminata* is a junior synonym for *O. disjungens*, so we renamed AiNPV as OpdiNPV in this paper. The OpdiNPV polyhedron is irregular in shape and the median lethal concentration against the larvae of *O. disjungens* was 3.4×10^4 polyhedral inclusion bodies per milliliter (Li *et al*., 2007). Although the morphology and bioassay of O. disjungens

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Abbreviations: dUTPase = deoxyuridine triphosphatase; NPV(s) = nucleopolyhedrovirus(es); OpdiNPV = *Ophiusa disjungens* nucleopolyhedrovirus; p.i. = post infection

nucleopolyhedrovirus have been reported, little is known about its genetics.

dUTPase plays an essential role in nucleotide biosynthesis. dUTPase is present not only in eukaryotes and prokaryotes, but also in several virus families (Broyles, 1993; Elder *et al*., 1992; Williams, *et al*., 2005; Wohlrab and Francke, 1980; Liu and Yang, 2005). More attention has been paid to the dUTPase in mammalian viruses (Broyles, 1993; Elder *et al*., 1992; Oliveros *et al*., 1999; Wohlrab and Francke, 1980; Weiss *et al*., 1997). However, little is known about the characteristics of the NPV dUTPase. In this study, the dUTPase gene of OpdiNPV was cloned and sequenced. Nucleotide and amino acid sequences were analyzed. The gene was expressed in *E. coli* and insect Tn cells as well. In the latter system, also the subcellular localization of the expressed dUTPase was investigated. The potential implication of this research will help to develop the virus that could be used as a bio-pesticide or an engineered pesticide.

Materials and Methods

Virus and cells. Trichoplusia ni (Tn) cells were maintained at 27°C in TC-100 insect medium supplemented with 10% (v/v) fetal bovine serum (Gibco, USA). An *O. disjungens* nucleopolyhedrovirus isolate was obtained from the infected *O. disjungens* larvae with nucleopolyhedrosis symptoms. The occlusion bodies were purified according to standard procedures (Christian *et al*., 2001; Lin *et al*., 2002).

Cloning and sequencing. Virus DNA was digested with *Pst*I (TaKaRa, Japan) at 37°C for 2 hrs. The *Pst*I-K fragment of OpdiNPV DNA was cloned into pUC18 (TaKaRa) plasmid vector and sequenced. Primers F (5′-CGGGATCCATGCATCGCTTGAGATA-3′) and R (5′-CCCAAGCTTTTAACATCATCCGCGT -3′) (*Bam*HI and *Hind*III sites are underlined) were designed according to the sequence of *Pst*I-K fragment to amplify the dUTPase gene by PCR.

Sequence and phylogenetic analyses. The DNA sequence of the dUTPase genes were analyzed with the aid of the DNASTAR (DNASTAR Inc., USA) and BioEdit software (Hall, 1999). Sequence blast search was performed using the National Center for Biotechnology Information (NCBI) BLAST searching services (Altschul *et al*., 1990). The internet service of PROSITE was used to analyze the domains in the deduced amino acid sequence of the dUTPase gene. GeneDoc software was used for homology shading among the aligned sequences (Genetics Computer Group Inc., USA). A phylogenetic tree was constructed by the N-J method in MEGA software (Tamura *et al*., 2007).

Expression of dUTPase in E. coli. The dUTPase gene was amplified using the gene-specific primer pair F and R described above. The PCR product of dUTPase gene was cloned into the expression vector pET-32a (Novagen, USA) as a *Bam*HI-*Hind*III fragment to generate a plasmid of pET-32-DUT, in which dUTPase gene is fused with His-tag at the C terminus. The generated fusion plasmid was transformed into *E. coli* BL21 cells for expression analysis.

Expression of dUTPase in Tn cells. The dUTPase gene was cloned into a transfer vector of pFastBacEGFP (Invitrogen, USA) to generate a donor plasmid of pEGFP-DUT in which dUTPase gene is fused with an EGFP tag at the N-terminal. The donor plasmid was transformed into a competent *E. coli* strain of DH10Bac cells (Invitrogen, USA) to generate the recombinant *Autographa californica* multiple nucleopolyhedrovirus bacmid (AcMNPV) DNA. Lipofectamine 2000 (Invitrogen, USA) was used to transfect the reconstructed bacmid DNA into Tn cells. The cells were incubated at 27°C for 5 days post transfection. The transfection supernatant was harvested and named as vEGFP-DUT. Tn cells were infected with the vEGFP-DUT at a multiplicity of infection of 10 and then incubated at 27°C for approximately 3 days.

Western blot analysis. *E. coli* cells were washed twice with PBS (pH 6.2). The extracts from the cells were subjected to 12% SDS-PAGE, blotted to nitrocellulose membranes, and incubated with antiHis-MAb (Abcam, USA). After washing and incubation with a secondary antibody IgG-HRP (Abcam, USA), the blots were visualized using a SuperSignal HRP-DAB chemiluminescent substrate (Pierce, USA).

Confocal fluorescence microscopy. The transfected Tn cells were examined for EGFP- DUT expression using a confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany) at 24 and 72 hrs p.i.

Results and Discussion

Sequence and phylogenetic analysis

*Pst*I-K fragments of OpdiNPV genome is composed of 3492 base pairs (bp). Sequence analysis of the genomic fragment revealed the presence of a dUTPase gene which is 402 bp long, encoding a polypeptide of 133 amino acids (aa) with a predicted molecular mass of 15.1 kDa. The deduced isolectric point of this protein is 9.38. The early transcription motifs CAGT and a TATA boxes are located at -32–29 and -9–6 nt upstream of the translational start codon ATG respectively (Fig. 1), suggesting that the dUTPase gene may be an early gene of OpdiNPV. Similarly, the dUTPase gene of the Rana grylio virus (RGV), which begins to transcribe and translate as early as 4 hrs p.i., was classified as an early viral gene during the *in vitro* infection (Zhao *et al*., 2007). No polyadenylation signal sites were found within the noncoding region downstream of the translation termination codon, which is similar to *Helicoverpa armigera* single nucleopolyhedrovirus ORF1 (Wu *et al*. 2001).

The functional motifs of deduced amino acid sequence of OpdiNPV dUTPase genes included two protein kinase C phosphorylation sites (SLR and TVKSDK, located at aa 46–

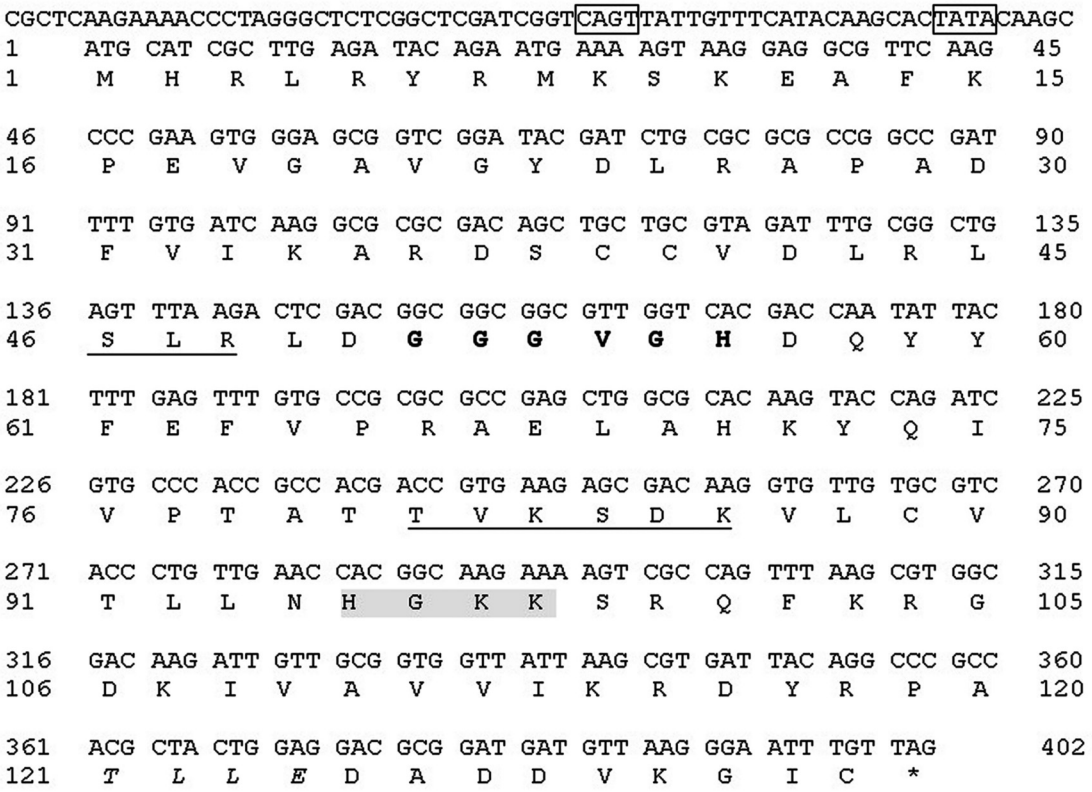

Fig. 1

Complete nucleotide and amino acid sequences for *O. disjungens* nucleopolyhedrovirus dUTPase

The protein kinase C phosphorylation sites (underlined), the casein kinase II phosphorylation sites (italicized), the N-myristoylation site (bold) and the amidation site (shaded) are shown. The CAGT and TATA boxes are boxed.

48 and 81–86, respectively), a casein kinase II phosphorylation site (TLLE, located at aa 121–124), a N-myristoylation site (GGGVGH, located at aa 51–56), and an amidation site (HGKK, located at aa 95–98, Fig. 1).

Sequence alignment with other NPVs, showed that the dUTPase of OpdiNPV shared amino acid sequence identities ranging from 28% to 50%. OpdiNPV dUTPase showed 35% and 40% identity to *Spodoptera frugiperda* multiple nucleopolyhedrovirus and *Spodoptera exigua* multiple nucleopolyhedrovirus dUTPase, respectively (Fig. 2). The phylogenetic analysis confirmed that OpdiNPV was most closely related to *Orgyia leucostigma* nucleopolyhedrovirus and *Euproctis pseudoconspersa* nucleopolyhedrovirus. It is more distantly related to other NPVs, such as *Agrotis segetum* nucleopolyhedrovirus, *Spodoptera frugiperda* multiple nucleopolyhedrovirus, *Spodoptera exigua* multiple nucleopolyhedrovirus, *Mamestra configurata* nucleopolyhedrovirus (A and B), *Helicoverpa armigera* multiple nucleopolyhedrovirus, *Agrotis ipsilon* multiple nucleopolyhedrovirus, *Spodoptera litura* nucleopolyhedrovirus; and C*hrysodeixis chalcites* nucleopolyhedrovirus.

Expression of dUTPase in E. coli

Over-expression of the dUTPase gene was analyzed with SDS-PAGE using total protein extracts from host cells. Fig. 3 shows the protein electrophoresis profile of dUTPase (C-terminal His-tagged form) and Western blot analysis. The recombinant dUTPase protein with natural molecular mass of 15.1 kDa migrated as 35.5 kDa protein, due to the presence of the His-tag (20.4 kDa). Western blot analysis also showed that the molecular mass of the expressed protein detected with a monoclonal antiHis-antibody was identical to the deduced molecular mass of 35.5 kDa of the OpdiNPV dUTPase gene with His-tag.

Expression of dUTPase in Tn cells

Subcellular localization of OpdiNPV dUTPase was determined by EGFP-DUT fusion protein expression with EGFP as a visual marker. The EGFP-DUT fusion gene was inserted into AcMNPV bacmid and produced vEGFP-DUT (data not shown). The EGFP-DUT construct was transfected

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                                20                  40                  60                  80
OpdiNPV  :  MHRLRYRMKSKEAFKPEVGAVGYDLRAPADFVIKARDSCCVDLRLSLRLDGGGVGHDQYYFEFVPRAELAHKYQIVPTATTVK  :  83
AiMNPV   :  mgghvlkykksaaafaprratagaagydlctpndfvvkardfvvvdtgvaielppnmyaqiksrsgnavkyqivaaagvidnd  :  83
AsNPV    :  maervlkytkspgafaprmasegaagydlytphdfvikardsmlidtgvciemppnmyaqiksrsgnavkhqivaaagvidnd  :  83
CcNPV    :  mstankrsrlsiedcsdneyyhqeqhenmyfkklnkdaiapqratlgsagydlytptdvclkpqkwtvvdigiaiqlppkryg  :  83
EpNPV    :  mnyvksacaytckiklapnaimpklatpesagydlqtpsdfvikakdscivdlrikiqlpsgiyakienhskmtmlkhrivva  :  83
HaMNPV   :  magrvikfkksinafpphmatagsagydlrtpvdfvikardkhivdvgvaielpydtyaqvtgrsglafyhqivvgagvidnd  :  83
McNPV-A  :  magrvikfkksvnafvpqmatagsagydlrtpvdfvikardkhivdigvaielpydtyaqvksrsglafhhqivvgagvidnd  :  83
McNPV-B  :  magrvikfkksveafvpqmatagsagydlrtpvdfvikardkhivdigvaielpydtyaqvksrsglafhhqivvgagvidnd  :  83
OlNPV    :  mnackliaprafapqlatdgsagydlrapedfvikardsctvdtglaielprglyakiesksglafkhqivaagvvdndyr  :  83
SeMNPV   :  msgqvlrfkktsksaytprmasdgaagydlhtpvdfvikakdsilvnteiaiqlpprmyaqiksrsgnaakyqvvaaagvidn  :  83
SfMNPV   :  mtikmntlkftkspngytprmatagaagydlytpvdfiikpgrqiiidtevsiqlpegtyaqiasrsgnavkyevvvlagvid  :  83
SlNPV    :  mtikmntlkftkspngytprmatagaagydlytpvdfiikpgrqiiidtevsiqlpegtyaqiasrsgnavkyevvvlagvid  :  83
            M

                          100                 120                 140                 160
OpdiNPV  :  SDKVLCVTLLNHGKKSRQFKRGDKIVAVVIKRDYRPATLLEDADDVKGIC---------------------------------  :  133
AiMNPV   :  yrgtlcvllfnhgkksrqfrrgdriaqfivrnyhtlplkecdelssterningfgstgr-------------------------  :  142
AsNPV    :  yrgtlsvllfnhgkksrqfrrgdriaqfivrqyyklplkeceqlshtkrdangfgstgr-------------------------  :  142
CcNPV    :  riaersglatkhgigiqagiidtdyrgpvgvclinrskkeynfkkgdkiaqmiiesyhtpnvimvdelddtdrgsggfgstgq  :  166
EpNPV    :  ggivdndhdgslqvvlfnhgkksrhfkrgdkiaqmilkkycivpfsrinefdfelkyddtyv---------------------  :  145
HaMNPV   :  yrgtinvllfnhgkksrafnllrpnvlytgh-----------------------------------------------------  :  114
McNPV-A  :  yrgtinvllfnhgkksrtfkrgdkiaqmivhqycklplietdelsttqrdtngfgstgr-------------------------  :  142
McNPV-B  :  yrgtinvllfnhgkksrtfkrgdkiaqmivhqycklplietnqlsttqrdtngfgstgr-------------------------  :  142
OlNPV    :  gkicvilmnhgkrsrqfkrgdkiaqmvlhkyytvpmveadvlssteracngfgstgr-------------------------  :  140
SeMNPV   :  dyrgplkvllfnhgkksrqfrrgdriaqfivrtyyklpleevdeltvterdaggfgstgq-------------------------  :  143
SfMNPV   :  ndyrgsikvllrnlgkknrqfqrgdriaqliinnyykfsweqvdelcdtdrgeqgfgstgq-----------------------  :  144
SlNPV    :  ndyrgsikvllrnlgkknrqfqrgdriaqliinnyykfsweqvdelcdtdrgeqgfgstgq-----------------------  :  144
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Fig. 2

Alignment of amino acid sequences of dUTPases of various baculoviruses

Shading indicates homology.

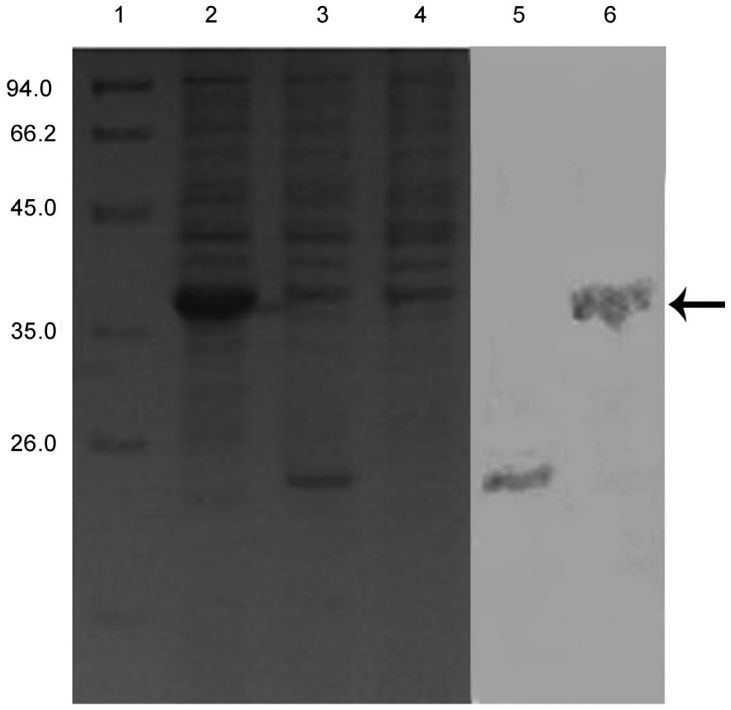

Fig. 3

Expression of his-tagged *O. disjungens* nucleopolyhedrovirus dUTPase in *E. coli*

SDS-PAGE (lanes 1–4) and Western blot analysis (lanes 5–6). Protein size markers (lane 1), negative controls (lanes 3, 4, and 5), expression of his-tagged dUTPase (lanes 2 and 6).

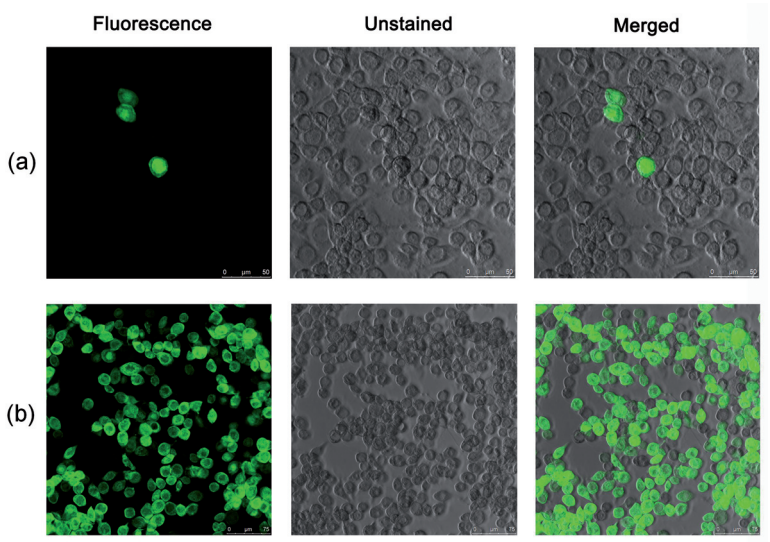

Fig. 4

Subcellular localization of dUTPase in Tn cells

Confocal fluorescence microscopy, cells at 24 (a) and 72 (b) hrs p.i.

into Tn cells. Green fluorescence was observed mainly in the nucleus of the transfected cells 24 hrs p.i. and was diffusely scattered and excluded from the nucleus 72 hrs p.i. (Fig. 4). This result indicates that the EGFP-DUT fusion protein was localized in the nucleus at first, and then expanded throughout the transfected cells. Using the same methods, Zhao (2007) showed that the EGFP-DUT fusion protein was localized in the cytoplasm in *Rana grylio* virus-infected cells. Immunofluorescence also confirmed dUTPase cytoplasm localization. Muha *et al.* (2009) showed the nuclear localization signal movements of *Drosophila melanogaster* dUTPase isoforms (23 kDa and 21 kDa) during nuclear cleavage. During interphase, the 23 kDa isoform is located within the nuclear space while the 21 kDa isoform is diffusely scattered and is excluded from the nuclei. As the nuclei enters mitosis, the 21 kDa dUTPase shows a localization shift to the karyoplasm, meanwhile the 23 kDa dUTPase starts to diffuse from the nuclear space. During metaphase, the 21 kDa dUTPase remains near the chromosomes, and most of the 23 kDa dUTPase is scattered in the cytoplasm. By the end of cytokinesis, the embryo regains the state of interphase when 23 kDa is in nucleus and 21 kDa dUTPase is in cytoplasm (Muha *et al.*, 2009).

Previous studies have shown that dUTPase is critical for virus replication by controlling the cellular ratios of dUTP to dTTP (Oliveros *et al.*, 1999, Turelli *et.al.*, 1996). Mutation of viral dUTPase results in substitution in the virus genome and reduces productive infection, neurovirulence, neuroinvasiveness and reactivation from latency (Lichenstein *et al.*, 1995; Oliveros *et al.*, 1999; Payne and Elder, 2001; Pyles *et al.*, 1992; Turelli *et al.*, 1996, 1997; Gong *et al.*, 2010). These reports showed the functions of dUTPase gene in viral infection, but they also indicated that dUTPase gene could be genetically modified to find some important characteristics of the dUTPase gene. Our study on the molecular structure and the subcellular localization of dUTPase gene would help to use *O. disjungens* nucleopolyhedrovirus as a bio-pesticide or to manipulate dUTPase by gene engineering to improve the control effects of OpdiNPV on *O. disjungens* and other Lepidoptera.

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