LETTER TO THE EDITOR

DETECTION OF ADOXOPHYES ORANA GRANULOVIRUS IN *ADOXOPHYES ORANA* BY PCR

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Received February 13, 2008; accepted July 3, 2008

Key words: AdorGV; DCR; RFLP

Viruses of the family *Baculoviridae* comprise a group of viruses that are pathogenic to insects that belong for the most part to the order *Lepidoptera*. These viruses put forward a novel approach to a pest control (*1, 2*). Adoxophyes orana granulovirus (AdorGV) is a "slow" virus belonging to the genus *Granulovirus* that usually induces host death in the final instar, regardless of when the host was infected (*3*). AdorGV is pathogenic to the summer fruit tortrix moth, *Adoxophyes orana*, a pest of more than 50 plant species from various families including fruits, forest trees, and ornamentals. *A. orana* is considered as an important pest particularly for apple and pear cultivation in the temperate zone. AdorGV is currently used in several countries in Europe as a bio-control agent for this insect (*4*). The detection of AdorGV in treated insect larvae will permit the evaluation of its presence in natural insect population as well as its efficiency as a specific anti-insect agent. We have made use of the complete sequence of AdorGV genome to develop PCR-based tools for the detection of AdorGV in the *A. orana* larvae (*5*). The PCR assay was confirmed by restriction fragment and sequence analysis.

AdorGV preparation Capex® (Andermatt Biocontrol, Switzerland) was used as an AdorGV-positive control for PCR optimization. Laboratory clones of *A. orana* larvae originating from a natural insect population served as negative, uninfected controls. Surviving *A. orana* larvae from Capex®-treated apple orchards were used for detection of AdorGV genome by PCR. Genomic DNA from individual *A. orana* larvae and from the Capex® AdorGV preparations was isolated using the DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. Two pairs of primers were designed using multiple sequence alignments in the Clustal-X program in order to amplify DNA sequences in granulin, the coding region of a gene highly conserved across granuloviruses (e.g. AdorGV-AF547984, CrleGV-NC_005068, PlxyGV-NC_002593, AgseGV-NC_005839) (*6*). Agrotis segetum granulovirus (AgseGV) is a newly proposed member of the genus *Granulovirus* (*7*). DNA from individual *A. orana* larvae was amplified using the primer pair AdorGra Forward/ AdorGra Reverse that targeted a 620 bp fragment of the AdorGV granulin gene and with the primer pair Granulin Forward/Granulin Reverse that targeted a 376 bp genome DNA fragment highly conserved in granuloviruses (Table 1). Taq™ polymerase (TaKaRa) was used for DNA amplification. The PCR reaction mixture consisted of 2.5 µl of 10x buffer (15 mmol/l $MgCl_2$), 2.5 U of Taq polymerase, 10 pmol of the forward and reverse primers (0.6 µl each) and 50 µmol/l dNTPs. The mixture was adjusted to 24 µl with sterile deionized water and 1 µl of total DNA was added. The reaction was carried out as follows: one step at 94°C for 2 mins (initial denaturation), 30 cycles of 3 steps: 94°C for 20 secs (denaturation), 55°C for 30 secs (annealing), and 72°C for 1 min (polymerization), and a final step at 72°C for 10 mins

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Abbreviations: AdorGV = Adoxophyes orana granulovirus; AgseGV = Agrotis segetum granulovirus; CpGV = Cydia pomonella granulovirus; CrleGV = Cryptophlebia leucotreta granulovirus, PlxyGV = Plutella xylostella granulovirus; RFLP = restriction fragment length polymorphism

Table 1. Primers for AdorGV detection

Primer name	Nucleotide sequence (5´-3´)	Positions
AdorGraF	GCACCACCTGTGTTATCGACAATC	41–64
AdorGraR	GACTTCGGCAGACGTGGTAC	641–660
GranulinF	GGGATATAACAAATCTTTGCG	3–23
GranulinR	GAACCACAGGTCCATGATCTC	349–369

(elongation). PCR amplicons were purified with the QIAquick gel extraction kit (Qiagen) and sequenced directly using the AdorGra Reverse and AdorGra Forward primers. Nucleotide and amino acid sequences were analyzed using the software packages Deambulum (http://www.infobiogen.fr/deamblum) and MEGA4 (*8*). The PCR fragments were also cleaved by *Hind*III (Promega) at 37°C for 4 hrs according to the manufacturer's instructions. The restriction fragments profile was analyzed with 2% agarose gel electrophoresis followed by ethidum bromide staining of DNA.

PCR assay described here has proved to be specific for the detection of AdorGV in *A. orana.* The primer pair AdorGraF/AdorGraR specifically amplifies a 620 bp fragment of granulin gene of AdorGV. Using this assay we detected AdorGV in larvae of *A. orana* from the virus treated orchards, Libčany and Slaný. Furthermore, AdorGV was not detected in the larvae of *A. orana* from natural population not treated with virus (Fig. 1b). The primer pair GranulinF/GranulinR is generically specific and amplifies a 367 bp fragment of granulin gene of both AdorGV and CpGV (Fig. 1a).

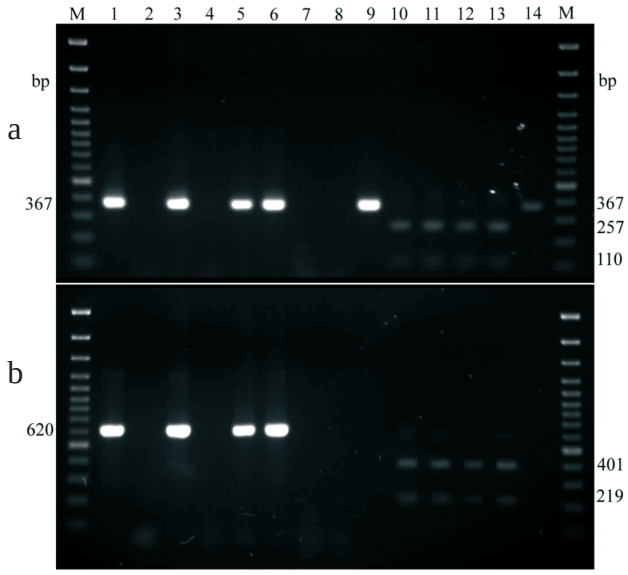

Fig. 1

AdorGV detection in *A. orana* larvae by PCR (1–9) and RFLP analysis (10–14)

Amplification of 367 bp fragment (panel A) and 620 bp fragment (panel B). DNA size marker 100 bp plus (M), virus-positive control-Capex1 (1), virus-free larvae (2), virus-positive and virus-free larvae from orchard Slaný treated with Capex, respectively (3, 4), virus-positive larvae from orchard Libčany treated with Capex: AdorG-L2, AdorG-L9 (5, 6), natural population of virus-free larvae (7, 8), CpGV detected in *Cydia pomonella* larvae (9). *Hind*III restriction profile of 367 bp product (10–14, panel A) and 620 bp product (10–13, panel B).

The specificity of the PCR assay was confirmed by restriction fragment length polymorphism (RFLP) analysis of PCR fragments using the *Hind*II restriction enzyme. The *Hind*III site in the granulin gene was found with the software program pDRAW32 (Acaclone, http://www.geocities.com/acaclone). The reliability of this PCR-based detection was also demonstrated by sequencing of the PCR amplicons. GenBank Acc. Nos for Capex1 (EU107361), AdorG-L2 (EU107362), and AdorG-L9 (EU107363). Analysis of these sequences gave a predicted 100% identity with reference virus isolates of AdorGV (AF547984, AY706658 and AY706657) (data not shown). Using PCR and RFLP analysis we have detected AdorGV in surviving *A. orana* larvae from orchards treated with the Capex® AdorGV preparation. AdorGV is a slow-killing virus and its persistence in surviving larvae of *A. orana* was more pronounced than that of a fast-killing virus like Cydia pomonella granulovirus (CpGV) in *Cydia pomonella* (*3, 9*). Hence, AdorGV may spread in orchards through horizontal transmission between different larval instars or vertical transmission to progeny. This flexibility could enhance the efficiency of this virus for the pest management.

This PCR assay together with RFLP and sequence analysis is a reliable tool for the detection and identification of AdorGV in insect larvae. The protocol described might be an effective tool for evaluating virus persistence in surviving larvae after virus treatment as well as for searching naturally occurring viruses in *A. orana* populations. For the first time, this work describes the PCR detection of AdorGV in *A. orana* larvae.

Acknowledgements. This work was supported by the project MZE 0002700603 from the Ministry of Agriculture, Czech Republic.

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