POLYMERASE CHAIN REACTION ASSAY FOR CYDIA POMONELLA GRANULOVIRUS DETECTION IN *CYDIA POMONELLA* POPULATION

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Summary. – The polymerase chain reaction (PCR) assay was successfully used to identify Cydia pomonella granulovirus (CpGV) in larvae of *Cydia pomonella L.* (codling moth). PCR with the primers CpGV-2A/CpGV-2B and CpGV-3A/CpGV-3B was found suitable for detection of CpGV. The primers Cp-I/Cp-II and Cp-III/Cp-IV were able to identify the transposable element TCp3.2 in *C. pomonella* larvae. The presence of CpGV in the larvae from orchards,which had been infected with CpGV was tested during 2 years post infection. (p.i.). CpGV was found in as many as 15% of the surviving larvae 1 year p.i. in one location. The virus was not detected in CpGV-infected orchards 2 years p.i. or in natural *C. pomonella* populations. This result suggests a poor persistence of CpGV in surviving *C. pomonella* individuals and its slow spread in a natural host population. One the other hand, the presence of a transposable element, transposon TCp3.2 may correlate with virus redistribution in this insect population.

Key words: Cydia pomonella; codling moth; Cydia pomonella granulovirus; PCR; transposon

Introduction

Preparations based on baculoviruses represent a possible means of fruit protection. At present, CpGV based on microbial agents are frequently used in the biological control of *Cydia pomonella* in fruit orchards (Huber, 1986). CpGV (the genus *Granulovirus*, the family *Baculoviridae*) is highly pathogenic for *C. pomonella* (Crook, 1991). Single CpGV virions are occluded within a granular protein capsule or occlusion body. After ingestion of CpGV host, the granulin dissolves in the alkaline midgut and the virions are released. The virions initiate the infection of midgut epithelial cells; replicate and systemically spread throughout the major body tissues including the tracheal matrix, epidermis, and fat body, which leads to the death of the host (Federici, 1997; Thiem, 1997).

CpGV consists of a double-stranded circular DNA of 123,500 bp (CpGV-M1, a cloned Mexican isolate, Luque *et al*., 2001). Recently, a mutant of CpGV-M, CpGV-MCp4 was isolated from *C. pomonella* and characterized (Jehle *et al*., 1995). The transposable element TCp3.2 was identified in CpGV-MCp4, which originated from the genome of *C. pomonella* and was inserted into the viral genome during infection of host larvae (Jehle *et al*., 1998). TCp3.2 was found integrated into a non-coding region of CpGV genome in between two ORFs, lef-2 and 35Ra or 41 and 42 (Luque *et al*., 2001; Jehle *et al*., 1997; Arends and Jehle, 2002).

The detection of CpGV in *C. pomonella* larvae facilitated the evaluation of the frequency of occurrence of CpGV in natural population and its efficiency as microbial agents. The ELISA-based tools are very effective for routine detection of CpGV in larvae (Crook and Payne, 1980), but at low virus concentrations this method is of no use. Furthermore, a cross reaction with other baculoviruses may also occur (McCarthy and Henchal, 1983). Recent developments in molecular biological techniques have

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Abbreviations: CpGV = Cydia pomonella granulovirus; DEPC = diethyl pyrocarbonate; EDTA = ethylenediamine tetraacetate; GIB = granular inclusion bodies; LdMNPV = Lymantria dispar ncleopolyhedrovirus; PCR = polymerase chain reaction; SDS = sodium dodecyl sulfate

Table 1. Primers

Primer	Sequence (5'-3')	Nucleotide position	Product size (bp)	Genome
Sense CpGV 26-27-2A	TAAGTCTTGGCGGTAGTAGG	21311–21320	250	
Antisense CpGV 26-27-2B	GAGACAGTTCGAGAAGGTCA	21541–21560		Complete sequence
Sense CpGV 26-27-3A	GACCTTCTCGAACTGTCTCA	21542–21561	248	of CpGV genome (U53466)*
Antisense CpGV 26-27-3B	CCTTGACTATCTACGGCAAC	21770–21789		
Sense TCp3.2 Cp1	ACCCAAGAACCCAGCAAAGA	800–819	303	
Antisense TCp3.2 Cp2	TGCGGTTTGGTAAGCGTCTA	1083–1102		Transposon TCp3.2
Sense TCp3.2 Cp3	AATGGCTGCCTTTACGAATG	975–994	450	of CpGV (Z96154)*
Antisense TCp3.2 Cp4	GCAAAATAAACGCGAAAAGC	1405–1424		

*GenBank Acc. No.

facilitated the analysis and sequencing of the CpGV genome (Crook *et al*., 1997; Jehle *et al*., 1998; Luque *et al*., 2001), which have permitted the development of PCR-based tools for the detection of CpGV in *C. pomonella* larvae.

This paper reports on the development of a PCR assay for the detection of CpGV in *C. pomonella* larvae, which could be used to identify the frequency of CpGV in the insect populations infected with CpGV as well as in natural populations. The frequency of the CpGV transposable element TCp3.2 in host population is also evaluated to identify the possible role of this element in host-virus interaction.

Materials and Methods

C. pomonella and virus. A laboratory colony of *C. pomonella* originated from Russia and was maintained at ZD Chelčice since 1992. The CpGV M strain (Tanada, 1964) originating from the BBA Institute for Biological Control, Darmstadt, Germany was used. The virus preparations were made according to the formula developed at the ZD Chelčice.

Infection of C. pomonella larvae. Ten day-old *C. pomonella* larvae were fed a semi-synthetic diet sprayed with a suspension of CpGV at a rate 600 granular inclusion bodies (GIB) per cm^2. After 7 to 8 days the infected larvae were frozen at -20°C for later analysis. As a negative control uninfected larvae from the laboratory colony were used.

Field trials with CpGV were carried out in an experimental apple orchard in Prague-Ruzyně (0.34 ha, central Bohemia) and a commercial apple orchard in Velké Bílovice (2.09 ha, southern Moravia). In Velké Bílovice the cv. Jonathan and in Ruzyně cvs Šampion and Denár were used. The virus was applied as a spray using the mist blowers Hardi LE 465 (500 l/ha) and Agri-Master (400 l/ha) to plots in Ruzyně and Velké Bílovice, respectively. The applications were timed to coincide as close as possible with the periods when *C. pomonella* larvae were hatching. The plots were treated with CpGV from 1997 to 2000 in Velké Bílovice and from 1998 to 2000 in Ruzyně. The rates of CpGV applied in 1999 and 2000 varied from 0.26 x 10^{13} GIB/ha to 1.00 x 10^{13} GIB/ha (Table 2). The highest dose of CpGV was sprayed in the first two applications. The two chemically treated plots located close to CpGV-treated plots were used for the observation of virus spread in neighbor plots. Chlorpyrifosmethyl, phosalone, fenitrothion, fenoxycarb and diflubenzuron were applied in Velké Bílovice and teflubenzuron in Ruzyně. The *C. pomonella* larvae were collected from the orchards in the year of treatment with CpGV and in the following two years. The larvae from orchards, which were not treated with CpGV, Bulhary (20 km from Velké Bílovice) and Horoměřice (15 km from Ruzyně) were collected in 2001 and 2002 as untreated controls. Twenty larvae were collected from paper belt traps distributed randomly in experimental plots in all orchards except Velké Bílovice in 2000, where the larvae (also 20) were collected from damaged fruit at the end of the development of the second *C. pomonella* generation. From each belt,

Table 2. Description of experimental plots

Locality	Plot	Size of plot (ha)	Number of CpGV or insecticides spraying against 1st and 2nd CM generation and cumulative dose of CpGV per each generation (x 10^{13} capsules/ha)							
			1st gen.	Dose	2nd gen.	Dose	1st gen.	Dose	2nd gen.	Dose
			1999				2000			
Velké Bílovice	CpGV	2.09	3	1.743	2	1.463	3	1.993	2	1.523
	Chemically treated	2.00	9	–	1	–	8	–	2	–
Ruzyně	Non-treated control	0.34	–	–	-	–	–	–	–	–
	CpGV	0.34	3	2.289	0	–	3	2.07	2	1.07
	Chemically treated	0.34	3	–	0	–	3	–	2	–

Table 3. Percentage of *C. pomonella* larvae with CpGV and transposon TCp3.2

Locality	Plot	Number of larvae with CpGV/number of tested larvae (% of larvae with CpGV)				Number of larvae with transposon TCp3.2/number of tested larvae (% of larvae with transposon TCp3.2)			
		1999	2000	2001	2002	1999	2000	2001	2002
Velké Bílovice	CpGV	0/20 (0)	0/20 (0)	NT	0/20 (0)	8/20 (40)	12/20 (60)	NT	19/20 (95)
	Chemical treatment	0/20 (0)	0/20 (0)	NT	NT	13/20 (65)	14/20 (70)	NT	NT
Ruzyně	CpGV	NT	3/20 (15)	0/20 (0)	0/20 (0)	NT	5/20 (25)	5/20 (25)	19/20 (95)
	Chemical treatment	NT	0/20 (0)	0/20 (0)	0/20 (0)	NT	6/20 (30)	6/18 (33)	11/20 (55)
	Non-treated control	NT	0/20 (0)	0/20 (0)	0/20 (0)	NT	3/20 (15)	3/20 (15)	14/20 (70)
Horoměřice	Non-treated orchard	NT	NT	0/20 (0)	0/20 (0)	NT	NT	1/16 (6.25)	8/20 (40)
Bulhary	Non-treated orchard	NT	NT	NT	0/20 (0)	NT	NT	NT	15/20 (75)

NT = not tested.

a single larva was used for virus detection. The larvae were stored at -20°C until used in PCR.

PCR primer. Two pairs of primers including CpGV-2A/CpGV-2B (250 bp) and CpGV-3A/CpGV-3B (248 bp) (Table 1) were designed from the Cp26-Cp27 part of the complete sequence of CpGV-M1 genome (Luque *et al*., 2001). Two others pairs of primers including Cp-I/Cp-II (303 bp) and Cp-III/Cp-IV (450 bp) were designed from the sequence of the transposon TCp3.2 of the CpGV-MCp4 mutant as described by Jehle *et al*. (1998). The primers were optimized by the software program Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.).

Extraction of total DNA. A single *C. pomonella* larva was ground in a mortar with pestle in an extraction buffer (100 mmol/l Tris-HCl pH 8.0 containing 50 mmol/l ethylenediamine tetraacetate (EDTA) and 1% sodium dodecyl sulfate (SDS)). The homogenate (700 µl) was transferred to sterile centrifuge tubes and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) for 10 mins by vortexing. Then it was centrifuged at 10,000 x g for 5 mins. The aqueous phase was collected into new sterile centrifuge tube and the whole process was repeated. The aqueous phase (500 µl) was then mixed with a triple volume of ice-cold ethanol (absolute 3 99.8% v/v) and 100 µl of 10 mol/l LiCl, and the mixture was kept at -20°C for 45 mins to precipitate nucleic acids. The latter were collected by centrifugation at 20,000 x g for 8 mins and washed twice in 70% ethanol (v/v) for 10 mins at room temperature. Then it was again centrifuged at 20,000 x g for 8 mins, the ethanol was removed and the pellet was dried under vacuum, resuspended in 50 µl of sterile deionised water and stored at -20°C until used.

Amplification of DNA. The PCR reaction mixture consisted of 2.5 µl of 10x PCR buffer (15 mmol/l $MgCl_2$), 2.5 U of Taq DNA polymerase (0.5 µl), 10 pmoles of the upstream and downstream primers each (0.4 µl), 50 µmol/l dNTPs (0.2 µl) and 5 µl of 5x Q solution, all components from the Qiagen kit. The mixture was adjusted to 24 µl with sterile deionised water and 1 µl of total DNA was added. The reaction was carried out in a thermocycler (MJ Research) as follows: one step at 94°C for 2 mins (initial denaturation), 28 cycles of 3 steps: 94°C for 40 secs (denaturation), 55°C (the primers CpGV-2A/CpGV-2B or CpGV-3A/CpGV-3B) for 30 secs or 61°C (the primers Cp-I/Cp-II or Cp-III/Cp-IV) for 45 secs (annealing), and 72°C for 1 min (polymerization), and a final step at 72°C for 10 mins (elongation).

Agarose gel (1.5%) electrophoresis. Aliquots of PCR products were electrophoresed in the TBE buffer (90 mmol/l Tris-borate and 2 mmol/l EDTA) at 120 V for 45 mins. Bands were stained with ethidum bromide (0.5 µg/ml) and visualized under a UV transilluminator. A 100 bp ladder (MBI Fermentas) was used as DNA size marker.

Results

Primer pairs for detection of CpGV and transposon TCp3.2 by PCR

Both primer pairs used were specific for the detection of CpGV-infected *C. pomonella* larvae from a laboratory colony. A 250 bp fragment for the primer pair CpGV-2A/CpGV-2B and a 248 bp fragment for the primer pair CpGV-3A/CpGV-3B were recorded. No bands of the same molecular size were observed in non-infected larvae from a laboratory colony (Fig. 1, Table 3).

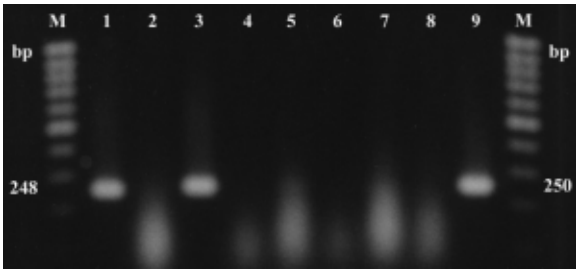

Fig. 1

Agarose gel electrophoresis of PCR products of CpGV from *C. pomonella* larvae

DNA size marker, 100 bp ladder (lanes M); CpGV-infected and uninfected larvae from the laboratory colony, the primer pair CpGV-3A/CpGV-3B (lanes 1 and 2, respectively); larvae from the CpGV-treated plots in Ruzyně and Velké Bílovice, the primer pair CpGV-2A/CpGV-2B (lanes 3 and 4, respectively); larvae from the CpGV-untreated orchards in Bulhary and Horoměřice, respectively (lanes 5 and 6, respectively); chemical control (lane 7); CpGV-uninfected and infected larvae from the laboratory colony, the primer pair CpGV-2A/CpGV-2B (lanes 8 and 9, respectively).

Any significant difference was not recorded between the primer pairs used for the detection of CpGV; the primer pair CpGV-2A/CpGV-2B was used in all subsequent tests.

Also the transposon TCp3.2 was detected successfully by PCR. Both the primer pairs used were specific for the detection of the transposon. The 303 bp and 450 bp PCR fragments were detected with both the primer pairs Cp-I/Cp-II and Cp-III/Cp-IV in the *C. pomonella* larvae from the laboratory colonies infected with CpGV. Therefore, in all subsequent tests only the primer pair Cp-III/Cp-IV was used. The transposon was not detected in uninfected larvae from the laboratory colony (Fig. 2, Table 3). The specificity of the amplified products was ascertained by restriction analysis (data not shown).

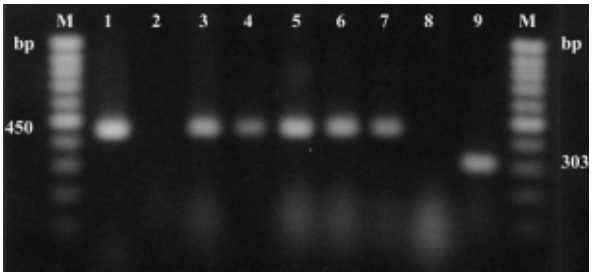

Fig. 2

Agarose gel electrophoresis of PCR products of transposon TCp3.2 from *C. pomonella* larvae

DNA size marker, 100 bp ladder (lanes M); CpGV-infected and uninfected larvae from the laboratory colony, the primer pair Cp-III/Cp-IV (lanes 1 and 2, respectively); larvae from the CpGV-treated plots in Ruzyně and Velké Bílovice (lanes 3 and 4, respectively); larvae from the CpGV-untreated orchards in Bulhary and Horoměřice (lanes 5 and 6, respectively); chemical control (lane 7); CpGV-uninfected and infected larvae from the laboratory colony, the primer pair Cp-I/Cp-II) (lanes 8 and 9, respectively).

Field trials on CpGV

The percentage of *C. pomonella* larvae positive for CpGV and transposon TCp3.2 from orchards subjected to different treatments to control CpGV and transposon TCp3.2 is summarized in Table 3. The same larvae were used for the detection of CpGV and the transposon. CpGV was detected in 15% of the *C. pomonella* larvae from the CpGV-treated plot in Ruzyně in 2000. On the other hand, the virus was not detected in the larvae from other CpGV-treated plots in 1999–2002. The transposon was also detected in the CpGV-positive larvae. In the larvae from both the chemically treated and untreated orchards no CpGV was detected.

The average efficiency of CpGV treatment in the reduction of *C. pomonella* population density in 1998–2000 was 89.9% and 93.8% in Velké Bílovice and in Ruzyně, respectively. The average number of larvae in paper belt traps per tree on the CpGV-treated plots ranged from 2.2 in Ruzyně to 3.0 in Velké Bílovice. On the other hand, the number of larvae on the untreated control localities Bulhary and Horoměřice reached 34.4 and 36.8 larvae per tree, respectively.

In the years when CpGV has been applied the percentage of the larvae with the transposon TCp3.2 in Velké Bílovice ranged from 40% to 70% and in Ruzyně from 15% to 30%. In the years following the CpGV treatment, the percentage of larvae with the transposon was in most cases even higher (Table 3). As in Ruzyně and Velké Bílovice, the percentage of larvae with the transposon recorded in outlying untreated orchards was 6.25% (2001) and 40% (2002) in Horoměřice and 75% (2002) in Bulhary (Table 3). This result indicates that the presence of the transposon TCp3.2 in a certain *C. pomonella* populations is independent of the mode of pest management used in the orchards.

Discussion

The PCR method described here was shown to be effective for detecting CpGV in *C. pomonella* larvae, which amplifies target DNA fragments. The primers for CpGV detection were selected from the Cp26 to Cp27 part of the genome, which have no homologies in other baculoviruses (Luque *et al*., 2001). The primers used for the detection of CpGV allowed infected larvae to be distinguished from healthy ones (Fig. 1, Table 3). On the other hand, the primers derived from the sequence of transposon TCp3.2 enabled simultaneous detecting of the CpGV-MCp4 mutant and the transposon in the *C. pomonella* genome (Fig. 2, Table 3).

The CpGV was present in only 15% of the surviving *C. pomonella* larvae in CpGV-treated orchards. In comparison with similar results from other geographical regions (Eastwell *et al*., 1999; Biache *et al*., 1999) it is the lowest frequency of CpGV in larvae from CpGV-treated orchards. Biache *et al*. (1999) have reported that from 40% to 70% of the larvae of *C. pomonella* had CpGV after different protocols of CpGV application and pointed out that this frequency decreased within a few years of the CpGV treatment. CpGV either persists in populations of *C. pomonella* from the previous season (Huber and Dickler, 1977) or is transferred transovarially from one generation to the next (Etzel and Falcon, 1976). This type of transovarial transfer was not proved in our study. In accordance with the results of Biache *et al*. (1999) we recorded CpGV in a small part of a *C. pomonella* population that survived the CpGV treatment. The persistence of CpGV among the surviving *C. pomonella* is low. This fast elimination of CpGV from the treated populations and its total absence in close and distant populations indicates a slow spread and poor preservation of this strain of CpGV in the surviving insect

populations. However, the high mortality of *C. pomonella* in the CpGV-treated plot demonstrated high efficiency of this virus preparation in reducing the insect population density in fruit orchards. One the other hand, e.g. in the case of *Lymantria dispar* infected with Lymantria dispar ncleopolyherdrovirus (LdMNPV), the mortality of larvae is low, whereas the frequency of virus persistence in the surviving insect population is high (Cunningham *et al*., 1991).

We detected transposon TCp3.2 only in *C. pomonella* larvae from laboratory colony infected with CpGV. In natural populations, regardless of the pest control practised in the orchards (CpGV-treated, untreated, chemical control), the transposon was found more or less frequently. Again, it was not present in uninfected *C. pomonella* larvae from the laboratory colony. Hence, the variability in the presence of the transposon in natural *C. pomonella* populations indicates its independent distribution in different local populations. Furthermore, the relatively high content of the transposon and low virus persistence in the surviving *C. pomonella* population suggest that it somehow prevents the CpGV persistence in surviving individuals. So far, the role of this transposon in CpGV or in host population has not yet been well understood.

Nevertheless, the PCR assay described here is an effective tool for identifying CpGV in *C. pomonella* larvae, which could be used for routine evaluation of the efficiency of CpGV as a microbial agent of pest management strategy for *C. pomonella* in fruit orchards.

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