GENETIC HETEROGENEITY OF PHTHORIMAEA OPERCULELLA GRANULOVIRUS: RESTRICTION ANALYSIS OF WILD-TYPE ISOLATES AND CLONES OBTAINED *IN VITRO*

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Summary. – Genetic heterogeneity of a wild-type granulovirus (Tunisia isolate) of the potato tuber moth *Phthorimaea operculella* (Phthorimaea operculella granulovirus, Phop GV) has been studied. The heterogeneity was indicated by the presence of several submolar fragments in the profiles obtained by use of several restriction endonucleases. It was also demonstrated by variations in the restriction profile of the wild-type Tunisia isolate that had underwent since 1991 in our laboratory numerous passages *in vivo*. A comparison of the Tunisia isolate used in Egypt in the biological control programme with other PhopGV isolates indicated that it could not be related to any of the 3 genotypes previously defined. Five clones obtained from the Tunisia isolate *in vitro* were further grown both *in vitro* and *in vivo*. The restriction analysis of these clones demonstrated that none of them was identical to the parental wild type virus and to any other PhopGV geographic isolates. Genotypic differences between the clones were also shown. A 19 kbp *Bam*HI fragment absent in the original Tunisia isolate but present in its passages since 1995 at a submolar concentration, was always present at a molar concentration in its clones. The presence of this fragment reflects probably a selection of one or more variants present in the original isolate and its possible adaptation to the growth in our laboratory conditions.

Key words: potato tuber moth; *Phthorimaea operculella;* granulovirus; virus clones; cell line; genetic virus heterogeneity

Introduction

Similarly to nucleopolyhedroviruses (NPVs, genus *Nucleopolyhedrovirus*) (Knell and Summers, 1981), some granuloviruses (GVs, genus *Granulovirus*)) isolated in the field consist of mixtures of genetically heterogeneous variants (Crook *et al.*, 1985; Arif *et al.*, 1986; Smith and Crook, 1988). In the case of PhopGV (tentative species of genus *Granulovirus*), several isolates apparently reflecting their geographical origin have been previously reported (Vickers *et al.*, 1991; Kroschel *et al.*, 1996). These authors demonstrated that three distinct genotypes could be identified among nine isolates originating from several countries and different areas of Peru, respectively.

In Egypt, a Tunisia isolate of PhopGV was introduced in 1991 to start a biological control programme for *P. operculella*. Vickers *et al.* (1991) described this isolate and found that it was probably a mixture of two genotypes. As the absence of cell lines capable of supporting replication of GVs has hindered their cloning in particular and the progress in investigation of their molecular biology and genetics in general, the mixed genotypes hypothesis could not be proved. Until 1995, only the *Cydia pomonella* model has been available for the *in vitro* multiplication of GVs (Winstanley and Crook, 1993), and GV clones have been obtained *in vivo* only (Smith and Crook, 1988, 1993; Crook *et al.*, 1997).

Having worked on the *P. operculella* model for several years, we have established several cloned and uncloned cell lines derived from our first *P. operculella* cell line obtained in 1993 (Léry *et al.*, 1995) and have demonstrated GV DNA

Abbreviations: EDTA = ethylenediamine tetraacetate; GV = granulovirus; MGM = Modified Grace's Medium; NPV = nucleopolyhedrovirus; PhopGV = Phthorimaea operculella granulovirus virus; Pop-95 = ORS-Pop-95

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replication in them (Léry *et al.*, 1998). Then, we have achieved a complete and stable PhopGV multiplication *in vitro* that guaranteed permanent availability of this model (Léry *et al.*, 1995). In analysing the multiplication of PhopGV in these cell lines *in vitro*, we have observed a typical cytopathic effect (Léry *et al.*, 1997) that enabled a plaque assay and a cloning of PhopGV *in vitro*.

In the present paper, we compared restriction profiles of DNAs of the PhopGV Tunisia isolate and of other geographical PhopGV isolates. In order to determine the degree of heterogeneity of this isolate, we analysed changes of its DNA restriction profile during 3 years of passaging in our laboratory conditions. Furthermore, to prove the existence of several virus variants in the parental wild type isolate, we compared restriction profiles of DNAs of the original virus an of its clones.

Materials and Methods

Cells. ORS-Pop-95 (Pop-95) cell line (Léry *et al.*, 1997) between its 15th and 23th passage was used. It was cultivated in Modified Grace's Medium (MGM, Léry and Fédière, 1990) with 15% foetal bovine serum (FBS) at 19°C. The cells were routinely subcultured every 10 to 15 days and the cultures were seeded with 1 to 2 x 10^6 cells per a 25 cm^2 tissue culture flask.

Viruses. Three PhopGV isolates, namely Tunisia, Venezuela and Australia, isolated from the potato tuber moth *P. operculella*, were a gift from Dr. El-Bedewi, International Potato Center (IPC), Cairo. The Tunisia isolate has been used in the biological control programme in Egypt since 1991 and passaged in their laboratory in larvae of *P. operculella* of Egyptian origin also reared in their laboratory until 1993, then in our laboratory. The insects were infected as follows: a suspension was prepared from infected fourth-instar larvae in a Tris buffer pH 7.5 with Triton X-100; small potato tubers (25 to 30 g) were covered with this suspension and air-dried; *P. operculella* eggs were laid on small pieces of filter paper which were then pinned on the infected potato tubers. After hatching, the developed larvae were allowed to feed on the tubers and to get infected. The larvae were maintained in the dark at 27°C in separate plastic rearing boxes. Fully diseased fourth-instar larvae were harvested after 7 to 12 days. Three different *in vivo* passage samples of the Tunisia isolate taken in 1994, 1995 and 1996, and designated samples 1994, 1995 and 1996 were analysed in this study.

Virus purification from infected larvae. After 7 to 12 days of infection, white diseased larvae were collected and homogenised in 0.01 mol/l Tris.HCl pH 7.5 in a Potter-Elvehjem homogeniser. After three cycles of clarification by sonication and centrifugation at 800 x g for 5 mins, at 1,000 x g for 10 mins and at 1,250 x g for 20 mins, respectively, the last supernatant was centrifuged at 15,000 x g for 30 mins. The pellet containing virus was resuspended in 0.01 mol/l Tris.HCl pH 7.5, layered on a 30% – 70% (w/v) sucrose density gradient and centrifuged at 30,000 x g for 20 mins. The band containing granules was collected, concentrated as above and resuspended in TE buffer (0.01 mol/l Tris.HCl pH 7.5 and 1 mmol/l ethylenediamine tetraacetate (EDTA) pH 8.0) and stored at -20°C. The virus inclusion body concentration was determined spectrophotometrically using the following equations: A_{420} = 0.8 for a concentration of 0.1 mg of granules/ml, and 1 mg of granules = 6.0 x 10^{10} granules (Chang and Tanada, 1978). The supernatant was ultracentrifuged at 100,000 x g for 30 mins. The pellet was resuspended in Tris buffer overnight at 4°C, layered on a 20% – 50% (w/v) sucrose density gradient and centrifuged at 100,000 x g for 1 hr. The band containing virions was collected, concentrated as above and stored at -20°C.

DNA extraction. Purified granules were suspended in TE buffer and incubated at 37°C for 30 mins with an equal volume of 0.12 mol/l Na_2CO_3 pH 10.9. The sample was then treated with 2% Sarkosyl and 0.2 mg/ml proteinase K at 50°C for 2 hrs. The DNA was extracted using a standard phenol-chloroform-isoamyl alcohol protocol, ethanol precipitated (Summers and Smith, 1987), and resuspended in TE buffer. DNA concentration was determined from A_{260}.

Restriction analysis. The Tunisia isolate samples 1994, 1995 and 1996, the Venezuela and Australia isolates, and the clones obtained from the Tunisia isolate sample1995 were subjected to restriction analysis. Viral DNAs (1 – 2 μg) were digested at 37°C for 2 hrs with restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Xho*I according to the supplier's protocol (Boehringer). Electrophoresis of genome fragments was carried out on 0.75% – 1% agarose gels in TEP buffer (0.08 mol/l Tris.HCl, 0.08 mol/l phosphoric acid, 2 mmol/l EDTA, pH 8.0) at 60 V for 2 hrs. The gels were visualised and photographed on a UV-transilluminator. The size of the fragments was estimated by comparison with lambda DNA size markers in three successive assays.

In vitro cloning. The viral suspension used for the *in vitro* cloning was prepared from larvae infected with the Tunisia isolate sample 1995. Diseased but not dead larvae were homogenised and sonicated for 1 min in 2 ml of MGM without FBS. After two centrifugations at 5,000 x g for 20 mins and one at 15,000 x g for 30 mins, the supernatant diluted in MGM was ultrafiltered (0.45 μm pore size). Ten-fold dilutions (0.5 ml) of the filtrate were used for infection of 1-day-old Pop-95 cell cultures in 35 mm Petri dishes. After 4 hrs of contact at 19°C, the virus inocula were removed. The cells were overlaid with 1% low melting agarose in MGM with 10% FBS. After 16 days of incubation at 27°C and appearance of cytopathic effect, the plates were stained with MTT at 27°C for 4 hrs and the clones were picked up. They were diluted in 0.5 ml of MGM without FBS and recloned twice. Then the clones were grown in Petri dishes at 27°C with 2 ml of MGM with 15% FBS. After 2 weeks, the cells and supernatants were harvested and centrifuged at 13,000 rpm for 30 mins in Eppendorf tubes. Each pellet was checked for virus multiplication by a DNA dot blot hybridisation test and the supernatants were used to multiply the clones *in vitro*.

Multiplication of virus clones in cell cultures was performed in three passages. One-day-old Pop-95 cell cultures in 25 cm^2 flasks were used for infection. Aliquots (0.75 ml) of each supernatant diluted with the same volume of MGM without FBS were used as inocula. After 4 hrs of contact with the cells at 19°C, virus inocula were removed and replaced by 4 ml of fresh medium containing 15% FBS; the cells were incubated at 27°C for 2 weeks.

Virus purification from infected cell cultures. On day 15 p.i., the infected cells were scraped into the medium and the obtained

suspension was pooled and centrifuged at 400 x g for 15 mins. The pellet was treated with STE buffer (0.15 mol/l NaCl, 0.02 mol/l Tris.HCl, 1 mmol/l EDTA, 0.5% Aprotinine and 0.5% Nonidet P-40) at 0°C for 10 mins under agitation. The treated pellet and the supernatant were pooled, homogenised in a Potter-Elvehjem homogeniser, sonicated and centrifuged at 15,000 x g for 30 mins. The pelleted granules and virions were then purified as described above for the virus extracted from the larvae.

Multiplication of virus clones in larvae. Second- and third-instar germ-free larvae of *P. operculella* reared in our laboratory conditions were fed on small potatoes (16 g each for 25 larvae). The larvae were inoculated on the head capsule using a semi-purified granule suspension of cloned viruses produced *in vitro*. To prepare an inoculum, the pooled medium and cells from two infected tissue culture flasks were centrifuged at 800 x g for 5 mins. The pellet was treated with 1 ml of STE buffer as described above and the resulting supernatant was centrifuged at 15,000 x g for 30 mins. The pellet resuspended in 200 μl of 0.01 mol/l Tris.HCl was used as the inoculum. The infected larvae were kept at 27°C in plastic rearing boxes.

DNA dot blot hybridisation test. Granules or virions purified from infected cells and cellular extracts were used in the test. For this purpose, 1 ml of infected cell suspension (1 – 2 x 10^6 cells) or supernatant was pelleted by centrifugation at 13,000 rpm for 30 mins. The pellet resuspended in 100 μl of TE buffer was incubated at 37°C for 30 mins with an equal volume of 0.12 mol/l Na_2CO_3 pH 10.9, and then treated with 2% Sarkosyl and 0.2 mg/ml proteinase K at 50°C for 2 hrs. Various dilutions of the resulting suspension were subjected to the dot blot hybridisation test. A digoxygenin-labelled DNA probe was prepared from a cloned fragment of PhopGV DNA and the whole procedure was performed according to the supplier (Boehringer).

Results

Restriction analysis of DNA of PhopGV Tunisia isolate

To obtain restriction profile of the PhopGV maintained in our laboratory, DNA of the Tunisia isolate sample 1995 was digested with 9 restriction endonucleases, namely *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Xho*I, and the obtained fragments were separated by agarose gel electrophoresis (Fig. 1, Table 1). Besides distinct molar bands, also faint submolar bands were observed with two restriction endonucleases, *Bam*HI (19.0 kbp B band and 5.90 kbp K band)) and *Xho*I (20.0 kbp B band). Summing of sizes of the molar fragments gave similar results (mean 113.48 kbp).

Restriction analysis of different samples of PhopGV Tunisia isolate

Using the same restriction endonucleases, different samples of PhopGV Tunisia isolate passaged in our laboratory,

Table 1. Number and size of restriction fragments of PhopGV Tunisia isolate sample 1995 DNA

	Restriction endonuclease								
Fragment	*Bam*HI	*Eco*RI	*Hind*III	*Pst*I	*Eco*RV	*Sal*I	*Sma*I	*Bgl*II	*Xho*I
A	26.00	20.50	21.00	38.00	23.00	14.50	36.00	20.00	33.00
B	19.00*	20.50	14.00	38.00	15.50	9.60	36.00	13.80	20.00*
C	17.50	13.80	10.30	17.00	10.60	9.60	24.00	9.70	14.60
D	11.30	13.80	8.00	17.00	7.50	7.40	10.00	9.40	12.20
E	10.20	12.80	7.00	3.05	6.70	6.80	7.10	9.40	11.20
F	9.90	9.20	7.00	1.25	6.20	6.40		8.30	10.20
G	8.70	7.30	6.20		6.20	6.40		8.30	8.50
H	7.50	6.40	5.80		5.90	5.10		8.10	6.40
I	7.50	3.40	4.60		4.10	5.00		6.40	6.40
J	6.00	3.00	3.90		4.10	4.60		5.40	5.40
K	5.90*	2.80	3.60		4.00	4.50		5.10	3.85
L	2.85		3.40		3.80	4.20		4.60	1.70
M	2.75		3.20		3.30	4.20		3.80	
N	0.92		3.15		3.20	3.90		1.30	
O	0.83		2.50		3.05	3.80			
P	0.68		2.05		2.90	3.20			
Q	0.57		2.05		1.60	3.05			
R			1.50		1.50	2.50			
S			1.50			2.30			
T			1.30			2.15			
U			0.90			1.85			
V			0.85			1.55			
W						0.80			
Total	113.20	113.50	113.80	114.30	113.15	113.40	113.10	113.60	113.45

The values represent estimated sizes in kbp. *Distinct (molar) bands accompanied by additional faint (submolar) bands not taken in consideration.

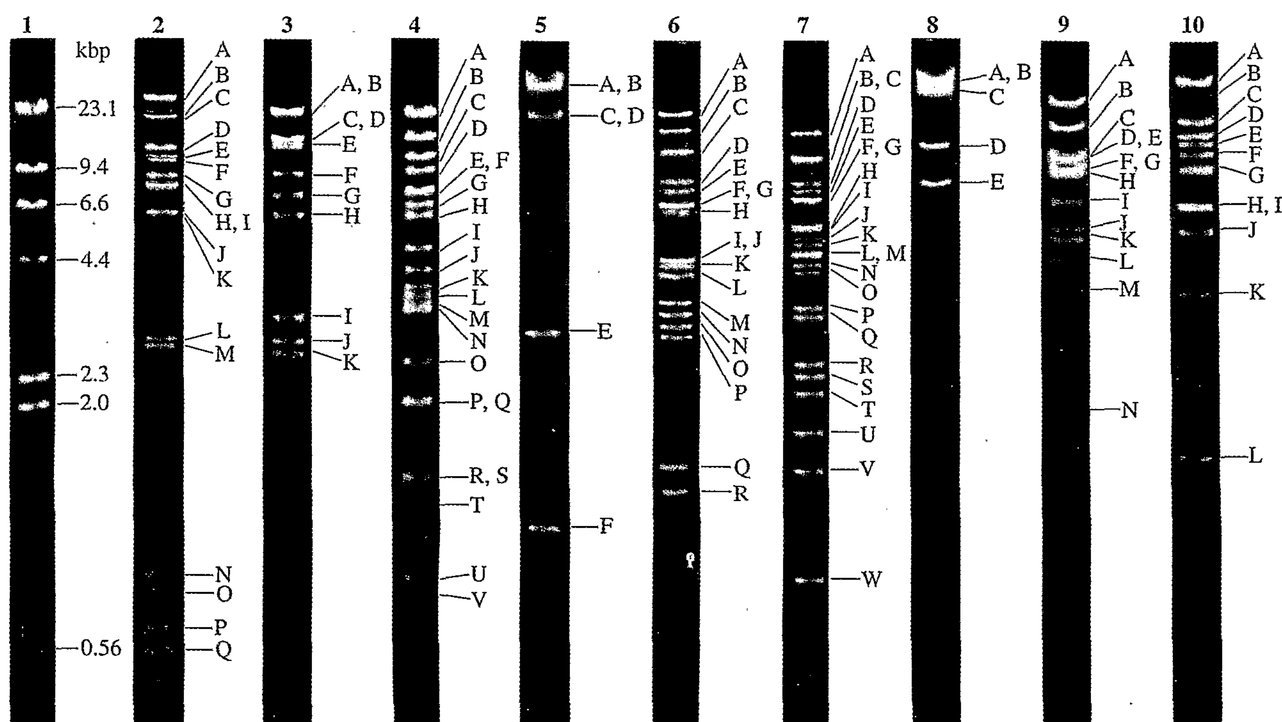

Fig. 1
Restriction profiles of PhopGV Tunisia isolate sample 1995 DNA
*Hind*III/lambda DNA size markers (lane 1), *Bam*HI (2), *Eco*RI (3), *Hind*III (4), *Pst*I (5), *Eco*RV (6), *Sal*I (7), *Sma*I (8), *Bgl*II (9) and *Xho*I (10).

namely Tunisia isolate samples 1994, 1995 and 1996 representing different passage samples taken in the respective years, were compared. The isolates Venezuela and Australia were also included in the comparison. Besides, data on the original Tunisia isolate as well as on Peru (Vickers *et al.,* 1991) and Yemen isolates (Kroschel *et al.,* 1996) were used for comparison. Three of nine restriction endonucleases used gave differences in the respective restriction patterns.

The results with *Bam*HI are listed in Table 2. When comparing the samples of the Tunisia isolate from 1994–1996, 2 closely situated faint bands of 6.00 kbp (J) and 5.90 kbp (K) of the same intensity were found in the 1994 sample. In the 1995 sample, the proportion of the submolar K band decreased and was none in the 1996 sample. On the contrary, the submolar J band was present at a molar concentration as far back as in the 1995 sample. After this date, a submolar 19.00 kbp B band appeared in the restriction profile of the isolate (Fig. 2). No submolar bands were found in the restriction profile of original Tunisia isolate by Vickers *et al.* (1991). These authors estimated the total size of DNA of this virus at 114.86 kbp, while we found a value of 113.2 without the submolar fragments. A comparison of restriction data on PhopGV isolates of other origin also revealed some differences. The Yemen (Kroschel *et al.*,1996) and Lima (Vickers *et al.*,1991) isolates were identical to the Tunisia isolate (Vickers *et al.*,1991). The B band was present at a molar concentration in both the Venezuela and Australia isolates. The submolar bands of 10.2, 8.7 and 5.9 kbp (E, G and K) present in the Australia isolate were absent in the Venezuela isolate (Fig. 2).

No differences could be detected in the *Sma*I restriction profiles of the 3 samples of the Tunisia isolate from 1994–1996, and of the Australia and Venezuela isolates. The profiles of these samples were different from those described previously (data not shown). In the original Tunisia isolate, Vickers *et al.* (1991) observed a submolar band of 26.0 kbp which was present in molar concentration in the Peru isolate, while a 7.5 kbp band found in the original Tunisia isolate was absent in the Peru isolate but was present in all the samples of the Tunisia isolate from 1994–1996 at molar concentrations. In contrast, a 6.2 kbp submolar band present in the Peru isolate was found as a double band in the original Tunisia isolate. All these bands were absent in the Yemen isolate.

Only few differences were observed in *Xho*I restriction profiles of the three samples of the Tunisia isolate from 1994–1996 (data not shown). Only a submolar 20.0 kbp band

B present in the samples until 1995, and also present in the Australia and Venezuela isolates, could differentiate the samples. This band was absent in the previously described PhopGV isolates. On the contrary, an additional band ranging in size from 3.5 to 4.3 kbp, absent in our samples, was present in these isolates (Table 3).

Characterisation of clones of PhopGV Tunisia isolate

After the third cycle of selection by plaque assay, 12 clones were subjected to DNA dot blot hybridisation test. Five of these clones that gave a strong hybridisation signal and corresponded to the most pathogenic clones were selected for multiplication *in vitro*. After three serial passages of the clones only small amounts of granules were obtained from them after purification. Therefore, the clones were then grown *in vivo* in germ-free laboratory-reared larvae of *P. operculella* and sufficient amounts of DNA for further restriction analysis were obtained in this way.

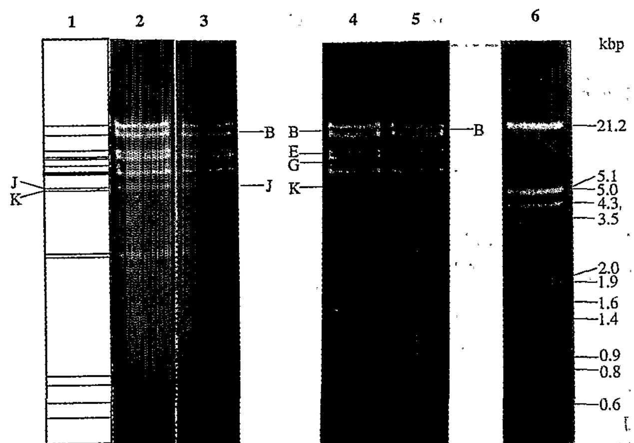

Fig. 2

BamHI restriction profiles of DNAs of various PhopGV isolates and samples

Tunisia isolate sample 1994 (lane 1), Tunisia isolate sample 1995 (2), Tunisia isolate sample 1996 (3), Australia isolate (4), Venezuela isolate (5) and (*Hind*III+*Eco*RI)/lambda DNA size markers (6).

Table 2. Comparison of *BamHI* restriction fragments of DNAs of various PhopGV isolates and samples

Fragment	PhopGV							
	Tunisia[a] isolate	Tunisia sample 1994	Tunisia sample 1995	Tunisia sample 1996	Yemen[b] isolate	Australia isolate	Venezuela isolate	Peru[a] isolate
A	25.00	26.00	26.00	26.00	26.00	26.00	26.00	25.00
B			19.00[c]	19.00[c]		19.00	19.00	
C	17.50	17.50	17.50	17.50	18.00	17.50	17.50	17.50
D	11.30	11.30	11.30	11.30	11.40	11.30	11.30	11.30
E	10.60	10.20	10.20	10.20	10.30	10.20[c]		10.60
F	10.20	9.90	9.90	9.90	9.80	9.90	9.90	10.20
G	9.00	8.70	8.70	8.70	8.40	8.70[c]		9.00
H	8.10	7.50	7.50	7.50	7.60	7.50	7.50	8.10
I	8.10	7.50	7.50	7.50	7.60	7.50	7.50	8.10
J	6.00	6.00[d]	6.00	6.00	6.10	6.00	6.00	6.00
K		5.90[d]	5.90[c]			5.90[c]		
L	3.00	2.85	2.85	2.85	2.90	2.85	2.85	3.00
M	2.85	2.75	2.75	2.75	2.80	2.75	2.75	2.85
N	0.96	0.92	0.92	0.92	1.05	0.92	0.92	0.96
O	0.88	0.83	0.83	0.83	0.95	0.83	0.83	0.80
P	0.74	0.68	0.68	0.68	0.75	0.68	0.68	0.74
Q	0.63	0.57	0.57	0.57	0.65	0.57	0.57	0.63
Total	114.86	113.15	113.20	113.20	114.30	113.30	113.30	114.86

The values represent estimated sizes in kbp.
[a]Data taken from Vickers *et al.* (1991). [b]Data taken from Kroschel *et al.* (1996). [c]Faint bands not taken in consideration. [d]Faint bands taken in consideration, as a unique band.

The production of granules *in vivo* gave different results in dependence on the clone. The granule yields varied from 1.82 x 10^{9} granules/larva (for the clones 4.2, 4.4 and 6.1) to 5.0 x 10^{9} granules/larva (for the clones 8.1 and 8.2). In comparison, using a viral suspension of 10^{8} granules/ml, the *in vivo* yields of the wild type sample 1995 were around 2 x 0^{10} granules/larva.

Each clone was digested with the restriction endonucleases used for the characterisation of various isolates and samples and was compared with the wild type sample 1995.

Table 3. Comparison of *XhoI* restriction fragments of DNAs of various PhopGV isolates and samples

Fragment	PhopGV							
	Tunisia[a] isolate	Tunisia sample 1994	Tunisia sample 1995	Tunisia sample 1996	Yemen isolate[b]	Australia isolate	Venezuela isolate	Peru isolate[a]
A	28.00	33.00	33.00	33.00	30.00	33.00	33.00	28.00
B				20.00[c]		20.00[c]	20.00[c]	
C	15.00	14.60	14.60	14.60	14.60	14.60	14.60	15.00
D	11.80	12.20	12.20	12.20	12.20	12.20	12.20	11.80
E	11.00	11.20	11.20	11.20	11.00	11.20	11.20	11.00
F	10.00	10.20	10.20	10.20	10.00	10.00	10.00	10.00
G	9.00	8.50	8.50	8.50	8.60	8.60	8.60	9.00
H	6.00	6.40	6.40	6.40	6.40	6.40	6.40	6.00
I	6.00	6.40	6.40	6.40	6.40	6.40	6.40	6.00
J	5.60	5.40	5.40	5.40	5.40	5.40	5.40	5.60
K	4.30	3.85	3.85	3.85	3.60	3.85	3.85	4.30
L	4.30				3.50			4.30
M	1.60	1.70	1.70	1.70	1.60	1.70	1.70	1.60
Total	112.60	113.25	113.20	113.20	113.30	113.20	113.20	112.60

The same legend as in Table 2.

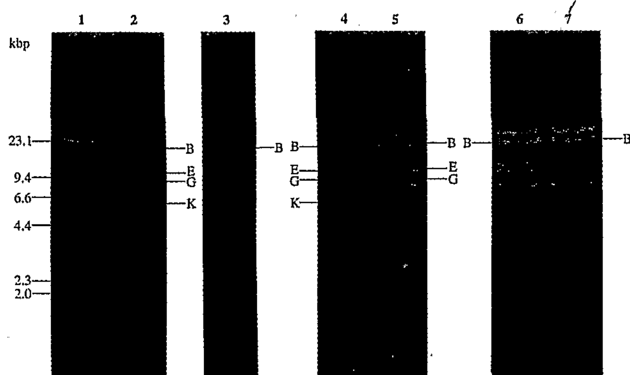

Fig. 3
***Bam*HI restriction profiles of DNAs of various clones of PhopGV**
*Hind*III/lambda DNA size markers (lane 1); Tunisia isolate sample 1995 (2), clone 4.2 (3), clone 4.4 (4), clone 6.1 (5), clone 8.1 (6) and clone 8.2 (7).

Three of these enzymes, *Bam*HI, *Sma*I and *Xho*I, showed differences among the clones.

Using *Bam*HI (Fig. 3), submolar bands allowed to distinguish 3 different profiles. In the clone 4.4, bands E (10.2 kbp), G (8.7 kbp) and K (5.9 kbp) were present. Bands E and G were also present in the clone 6.1. No submolar bands could be detected in the clones 4.2, 8.1 and 8.2. A comparison of the clones with the wild type sample 1995 (Table 4) indicated that all the clones were different from the virus from which they were derived. Regarding the profiles obtained with other isolates, the clones 4.2, 8.1 and 8.2, gave the same profiles as the Venezuela isolate, while all the other clones behaved differently.

Using *Sma*I (Fig. 4), no submolar bands could be detected in the clones as well as in the parental wild type virus. The clones presented differences in the largest fragments A, B, C and D. The clones 8.1 and 8.2 were identical and had 2 double bands of 26.0 and 22.0 kbp. The clones 4.4 and 6.1 were identical and presented 2 double fragments with molecular weights of 25.0 and 23.0 kbp. Finally the clone 4.2 had a unique quadruple band of 24.0 kbp. All the clones were different from the parental wild type virus (Table 5).

In *Xho*I profile, only the clone 6.1 was different from the others with a submolar band of 20.0 kbp, observed in the parental wild type virus and also in the Venezuela and Australia isolates. The other 4 clones were similar to the Tunisia isolate sample 1994.

Discussion

The estimated size of genome of the PhopGV Tunisia isolate sample 1995 from our laboratory of 113.48 kbp (sum of sizes of its fragments) is similar to that reported for the original Tunisia isolate (Vickers *et al.*, 1991). The submolar bands found in DNA restriction profiles of the sample 1995 were genuine because they were reproducible. Submolar bands are fairly typical for the majority of wild type baculoviruses (Easwaramoorthy and Cory, 1990) which usually exhibit some degree of heterogeneity (Knell and Summers,

Table 4. Comparison of *BamHI* restriction fragments of DNAs of various clones of PhopGV Tunisia isolate

	PhopGV				
Fragment	Tunisia isolate sample 1995	Clone 4.2	Clone 4.4	Clone 6.1	Clone 8
A	26.00	26.00	26.00	26.00	26.00
B	19.00[a]	19.00	19.00	19.00	19.00
C	17.50	17.50	17.50	17.50	17.50
D	11.30	11.30	11.30	11.30	11.30
E	10.20		10.20[a]	10.20[a]	
F	9.90	9.90	9.90	9.90	9.90
G	8.70		8.70[a]	8.70[a]	
H	7.50	7.50	7.50	7.50	7.50
I	7.50	7.50	7.50	7.50	7.50
J	6.00	6.00	6.00	6.00	6.00
K	5.90[a]		5.90[a]		
L	2.85	2.85	2.85	2.85	2.85
M	2.75	2.75	2.75	2.75	2.75
N	0.92	0.92	0.92	0.92	0.92
O	0.83	0.83	0.83	0.83	0.83
P	0.68	0.68	0.68	0.68	0.68
Q	0.57	0.57	0.57	0.57	0.57
Total	113.20	113.30	113.30	113.30	113.30

The values represent estimated sizes in kbp.
[a]Faint bands not taken in consideration.

Table 5. Comparison of *SmaI* restriction fragments of DNAs of various clones of PhopGV Tunisia isolate

	PhopGV				
Fragment	Tunisia isolate sample 1995	Clone 4.2	Clone 4.4	Clone 6.1	Clone 8
A	36.00	24.00	25.00	25.00	26.00
B	36.00	24.00	25.00	25.00	26.00
C	24.00	24.00	23.00	23.00	22.00
D		24.00	23.00	23.00	22.00
E	10.00	10.00	10.00	10.00	10.00
F	7.10	7.10	7.10	7.10	7.10
Total	113.10	113.10	113.10	113.10	113.10

The values represent estimated sizes in kbp.

1981; Crook, 1986). Thus, more than one genotypic variant was probably present in our Tunisia isolate sample 1995. Vickers *et al.* (1991) describing several geographical isolates of PhopGV distinguished 3 different genotypes with minor differences. The Tunisia isolate described by these authors was considered a mixture of 2 genotypes and, similarly to our observations, faint additional bands were present in restriction profiles of this isolate. Four of the restriction

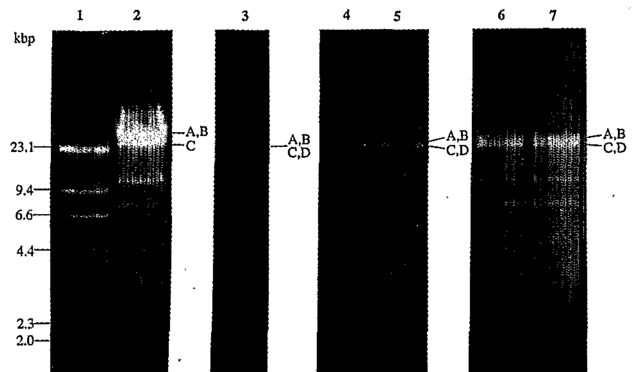

Fig. 4
***SmaI* restriction profiles of DNAs of various clones of PhopGV**
*Hind*III/lambda DNA size markers (lane 1), Tunisia isolate sample 1995 (2), clone 4.2 (3), clone 4.4 (4), clone 6.1 (5), clone 8.1 (6) and clone 8.2 (7).

endonucleases tested by these authors (*Sma*I, *Bgl*II, *Xho*I and *Pst*I) but not *Bam*HI showed differences among the isolates allowing to distinguish the three genotypes. None of these genotypes could be completely related to that of the samples of the Tunisia isolate studied by us which apparently represents another genotype. When we compared restriction profiles of our samples of the Tunisia isolate from 1994–1996, we could not detect any differences with *Bam*HI and *Xho*I. The restriction profiles of the Tunisia isolate sample 1994 were close to those of the original Tunisia isolate described by Vickers *et al.* (1991). This might represent (1) an adaptation of the original Tunisia isolate after the long time passaging in our laboratory which resulted in successive mutations (Crook *et al.*, 1985), (2) an *in vivo* recombination between the Tunisia isolate and a putative endogenous PhopGV (Smith and Crook, 1993) or (3), as recently demonstrated on *Cydia pomonella* and *Cryptophlebia leucotreta* (Jehle *et al.*, 1995), an interspecific recombination between the Tunisia isolate and another GV. The sum of estimated sizes of the *Bam*HI fragments E and G was equal to the estimated size of the *Bam*HI fragment B. This probably reflects the elimination of a *Bam*HI restriction site on the *Bam*HI fragment B in our samples under study. The presence of several faint bands in restriction profiles of the Australia isolate indicated its heterogeneity. With regard to the different restriction profiles of our Tunisia isolate samples from 1994–1996 one cannot exclude a possibility that the Australia isolate was in fact a result of contamination of the Tunisia isolate by the Venezuela isolate or *vice versa*.

The cloning of the Tunisia isolate apparently induced a selection, as it has been previously reported by several authors for an *in vivo* GV cloning (Smith and Crook, 1988; 1993). Significant differences were observed among the clones with those 3 restriction endonucleases (*Bam*HI, *Sma*I

and *Xho*I) which gave differences also among different PhopGV isolates. This indicates that probably more than two virus variants were present in the Tunisia isolate sample 1995 used for cloning. The sizes of the *Sma*I fragments A, B, C and D were different probably due to a modification of respective restriction sites. With *Bam*HI, no clone was obtained which contained the E and G fragments, and lacked the B fragment. This probably indicates a selection of one virus variant as the same fragment seemed to become predominant in restriction profiles of the more recent sample. Considering this possibility, it would be of interest to study also the other clones obtained which appeared less infectious *in vitro* and to compare their restriction profiles. The virus population of the Tunisia isolate sample 1995 from our laboratory is obviously heterogeneous; recombinations or mutations have probably occurred in this virus in our laboratory conditions and have been observed also in the PhopGV Peru isolate (Vickers *et al.*, 1991) as well as in other baculoviruses (Knell and Summers, 1981). Thus we can reasonably admit the presence of more than 3 genotypes in PhopGV with regard to its worldwide distribution. The GV clones obtained *in vitro* have an advantage over the clones obtained *in vivo* (Cherry and Summers, 1985). The use of the *in vitro* model also reduces the risk of transposon mutagenesis. During an *in vivo* infection, when virus replication takes place in various tissues, a higher number of different active transposons may be caught by viral DNA than during replication in a cell line (Jehle *et al.*, 1995). Our results on the increased production of the clones 8.1 and 8.2 suggest that they are more pathogenic than the uncloned wild type viruses. This tentative conclusion correlates with previous results that demonstrated that small genotypic differences between closely related GVs resulted in large differences in their virulence (Crook, 1981; Harvey and Volkman, 1983). Essentially the same results were obtained with other NPVs (Allaway and Payne, 1983; Cherry and Summers, 1985). Further studies should be performed to analyse more accurately the impact of the different clones *in vivo*. A comparison of the clones multiplied *in vivo* and *in vitro* would allow us to establish that their growth *in vivo* did not modify their restriction profiles and other properties. This should be taken in account provided PhopGV clones will be used extensively as viral pesticide.

In conclusion, we analysed changes in the PhopGV Tunisia isolate during its passaging in our laboratory in 1994–1996, and using its clones obtained *in vitro*, we proved the existence of several variants in the original wild virus. One of those variants could be more adaptable to the gowth in our laboratory conditions. These results open also the way and form the necessary baseline for further studies on GVs, especially on the *P. operculella*/PhopGV system. The major aim of such studies should be a follow-up of uncontrolled populations of GVs already used in Egypt and several other countries.

References

Allaway GP, Payne CC (1983): A biochemical and biological comparison of the three European isolates of nuclear polyhedrosis viruses from *Agrotis segetum*. *Arch. Virol.* **75**, 43–54.

Arif BM, Guangyu Z, Jamieson P (1986): A comparison of three granulosis viruses isolated from *Choristoneura* spp. *J. Invertebr. Pathol.* **48**, 180–186.

Chang PM, Tanada Y (1978): Serological study on the transmission of a granulosis virus of the armyworm, *Pseudaletia unipuncta* (Lepidoptera: Noctuidae), to other lepidopterous species. *J. Invertebr. Pathol.* **31**, 106–117.

Cherry CL, Summers MD (1985): Genotypic variation among wild isolates of two nuclear polyhedrosis viruses isolates from *Spodoptera littoralis*. *J. Invertebr. Pathol.* **46**, 289–295.

Crook NE (1981): A comparison of the granulosis viruses from *Pieris brassicae* and *Pieris rapae*. *Virology* **115**, 173–181.

Crook NE (1986): Restriction enzyme analysis of granulosis viruses isolated from *Artogeia rapae*. *J. Gen. Virol.* **67**, 781–787.

Crook NE, James JD, Smith IRL, Winstanley D (1997): Comprehensive physical map of the *Cydia pomonella* granulosis genome and sequence-analysis of the granulin gene region. *J. Gen. Virol.* **78**, 965–974.

Crook NE, Spencer RA, Payne CC, Leisy DJ (1985): Variation in *Cydia pomonella* granulosis virus isolates and physical maps of the DNA from three variants. *J. Gen. Virol.* **66**, 2423–2430.

Easwaramoorthy S, Cory JS (1990): Characterization of the DNA of granulosis viruses isolated from two closely related moths, *Chilo infuscatellus* and *Chilo sacchariphagus indicus*. *Arch. Virol.* **110**, 113–119.

Harvey JP, Volkman LE (1983): Biochemical and biological variation of *Cydia pomonella* (codling moth) granulosis virus. *Virology* **124**, 21–34.

Jehle JA, Fritsch E, Nickel A, Huber J, Backhaus H (1995): TC14.7: A novel lepidopteran transposon found in *Cydia pomonella* granulosis virus. *Virology* **207**, 369–379.

Knell JD, Summers MD (1981): Investigation of genetic heterogeneity in wild isolates of *Spodoptera frugiperda* polyhedrosis virus by restriction endonuclease analysis of plaque-purified variants. *Virology* **112**, 190–197.

Kroschel J, Fritsch E, Huber J (1996): Biological control of the potato tuber moth (*Phthorimaea operculella*, Zeller) in the Republic of Yemen using granulosis virus: Biochemical characterization, pathogenicity and stability of the virus. *Biocont. Sc. Technol.* **6**, 207–216.

Léry X, Fédière G (1990): Effect of different amino acids and vitamins on lepidopteran cell cultures. *J. Invertebr. Pathol.* **55**, 47–51.

Léry X, Abol-Ela S, Giannotti J (1995): Multiplication of the potato tuber moth granulosis virus in *Phthorimaea*

operculella cell cultures. *Egypt. J. Biol. P. Cont.* **5**, 107–109.

Léry X, Zeddam JL, Giannotti J, Croizier L, Fédière G, Abol-Ela S (1995): Establishment of a cell line derived from embryos of the potato tuber moth *Phthorimaea operculella* (Zeller). *In Vitro Cell. Develop. Biol.* **31**, 836–839.

Léry X, Giannotti J, Taha A, Ravalec M, Abol-Ela S (1998): Multiplication of a granulosis virus isolated from the potato tuber moth in a new established cell line of *Phthorimaea operculella. In Vitro Cell. Develop. Biol.* **33**, 640–646.

Léry X, El-Tarras A, Abol-Ela S, Giannotti J (1998): Yield and activity of *Autographa californica* nucleopolyhedrovirus and *Phthorimaea operculella* granulosis virus in cloned and uncloned cell lines of *Phthorimaea operculella. Cytotechnology* (in press).

Smith IR, Crook NE (1988): *In vivo* isolation of baculovirus genotypes. *Virology* **166**, 240–244.

Smith IR, Crook NE (1993): Characterization of new baculovirus genotypes arising from inoculation of *Pieris brassicae* with granulosis viruses. *J. Gen. Virol.* **74**, 415–424.

Summers MD, Smith GE (1987): A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric. Exp. St. Bull.* **1555**, 1–37.

Vickers JM, Cory JS, Entwistle PF (1991): DNA characterization of eight geographic isolates of granulosis virus from the potato tuber moth (*Phthorimaea operculella*) (Lepidoptera, Gelechiidae). *J. Invertebr. Pathol.* **57**, 334–342.

Winstanley D, Crook NE (1993): Replication of *Cydia pomonella* granulosis virus in cell cultures. *J. Gen. Virol.* **74**, 1599–1609.