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CHARACTERIZATION OF A NUCLEOPOLYHEDROVIRUS ISOLATED FROM THE LABOLATORY REARING OF THE BEET ARMYWORM *SPODOPTERA EXIGUA* (HBN.) IN POLAND

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Abstract: A nucleopolyhedrovirus isolated from the beet armyworm *Spodoptera exigua* (Polish laboratory culture), SeMNPV (P), morphologically similar to the viral bioinsecticide virus Spod-X^R, was characterized molecularly and biologically. Phylogenetic analysis based on three conserved baculovirus genes, *polh*, *lef-8* and *pif-2*, showed the highest homology of SeMNPV (P) to *Mamestra brassicae* (Mb) MNPV and *M. configurata* (Maco) MNPV, and much less to SeMNPV (Spod-X^R). These findings were confirmed by genomic DNA restriction profile analyses. Bioassays revealed that SeMNPV isolated from the commercial bioinsecticide Spod-X^R was the most infectious for *S. exigua*, while the infectivity of SeMNPV (P) and MbMNPV was significantly lower. These data suggest that SeMNPV (P) is a variant of MbMNPV.

Key words: nucleopolyhedrovirus, genomics, bioactivity, *Spodoptera exigua, Mamestra brassicae*

INTRODUCTION

The beet armyworm *Spodoptera exigua* (Hbn.) (*Lepidoptera, Noctuidae*) is a serious pest of vegetables and ornamental crops in greenhouses. It was accidentally introduced in Europe from Florida in the 1970s. Since the insect showed resistance to a number of chemical insecticides, there was a pressing need to find alternative control methods (Smits 1987), which is still the case today. Many pathogens were isolated from this pest insect, but the most potential appeared to be provided by baculoviruses although *Bacillus thuringiensis* also performs reasonably well. Nucleopolyhedroviruses (NPV) isolated from *Spodoptera exigua* differed both biologically and genetically (Vlak et al. 1981; Gelernter and Federici 1986; Muñoz et al. 1999; Murillo et al. 2001). Biological and molecular characterization, in particular infectivity evaluation of different isolates is essential for their effective practical use.

For years viruses were characterized based on morphology and biological activity against their hosts. This has caused confusion in nomenclature, as baculoviruses are named after the host from which they were isolated. The same virus that infects several hosts could be given different names; also viruses isolated from the same host species may represent different virus species (Cory and Myers 2003; Jakubowska et al. 2005b). Application of molecular methods such as restriction enzyme and sequence analysis allows quick and simple identification of baculovirus isolates. Recently presented reports show considerable genetic variation within one insect/virus isolate and emphasize the value of analyzing virus from individual insects, rather than from a group (Graham et al. 2004). Sequence information enriches the analysis and the availability of good molecular markers allowing solid classification and naming of baculoviruses.

Using both a biological and molecular approach we have characterized a nucleopolyhedrovirus isolated from a laboratory culture of *S. exigua*. This virus is morphologically similar to *Spodoptera exigua* (Se) MNPV from a commercially available bioagent, Spod-X^R. We therefore have provisionally named the isolate SeMNPV (P). The aim of the current study was to characterize and compare biologically and phylogenetically both isolates SeMNPV (P) and SeMNPV (Spod-X^R), and to evaluate their biological activity against beet armyworm larvae.

MATERIALS AND METHODS

Virus

SeMNPV (P) was isolated in 1993 from laboratory reared *S. exigua* larvae and stored at –20°C in a suspension. The virus was freshly amplified in second instar larvae of *S. exigua* reared in the laboratory in the year 2000. Larvae were infected individually with diet discs contaminated with 10 μl of virus suspension in a concentration of 10^5 occlusion bodies (OBs) per ml. MbMNPV (French isolate, 30–85) was obtained from the National Institute for Agricultural Research, INRA (La Miniere, France).

Molecular analysis

For genetic analysis polyhedral occlusion bodies (OBs) were purified from dead larvae as described by Muńoz et al. (1997). Occluded virions were released by incubation of OBs in 0.1 M Na_2CO_3 for 10–15 min. at 37°C. DNA was purified from these virions according to the method described by Reed et al. (2003). The DNA was dialyzed against 0.1 × TE (1 mM Tris-HCl, 0.1 mM EDTA pH 8.0) after phenol: chloroform extraction. For restriction enzymes analysis 1 μg of DNA was digested for 3.5 h at 37°C with *Eco*RI and the fragments were separated by size in 0.7% agarose gels containing 40 mM Tris-acetate, 1 mM EDTA [pH 8.0] at 15 mA for 18 h and analyzed under UV light.

In order to determine the taxonomic status of SeMNPV (P) segment of three conserved baculovirus genes, polyhedrin, *lef-8* and *pif-2*, were PCR-amplified, sequenced and analyzed phylogenetically. Purified DNA from SeMNPV and MbMNPV isolates was used as a template for PCR. The degenerate primer set for *polh* gene was previously described by Moraes and Maruniak (1997), and for the

lef-8 and *pif-2* genes by Herniou et al. (2004) and successfully used recently to identify another baculovirus from Poland, *Leucoma salicis* MNPV (Jakubowska et al., 2005a). PCR products were cloned into pGEM-T easy plasmids (Promega) and the inserts were analyzed by automated sequencing (BaseClear, The Netherlands).

The BLAST program (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI) was used for nucleotide and predicted amino acid sequences homology searches. Polyhedrin, *lef-8* and *pif-2* gene sequences from other baculoviruses were obtained from GenBank and used for phylogenetic analysis. The latter was performed using PAUP 4.0 (Swofford 1998). The trees were bootstrapped using maximum parsimony method with 1000 replicates. The analysis of *lef-8* and *pif-2* sequences was combined, as those genes were found to be congruent in phylogenetic analyses (Herniou et al. 2004); the polyhedrin gene was analyzed separately. All three genes were previously found adequate for phylogenetic analyses and moreover their sequences are available for a large number of baculoviruses. The dendrograms were drawn using Tree View (Page 1996).

Bioassays

The biological activity of three NPV isolates, SeMNPV (P), SeMNPV (Spod-X^R) and MbMNPV (French isolate, 30–85) was compared in bioassays. Third instar *S. exigua* larvae were fed 10 μl of virus suspension applied on small piece of artificial diet (Poitout and Bues 1970). Each larva was reared separately in 12-wells NUNC plates. After consuming the whole piece of contaminated diet larvae were fed fresh diet and reared until death or pupation. Mortality was recorded daily. Deceased larvae were collected and kept in –20°C for further analysis.

LD_{50} values were calculated by the probit method according to Finney (Lipa and Śliżyński 1973).

RESULTS

Gene sequencing and phylogenetic analysis

In order to determine the taxonomic status of SeMNPV-P three conserved baculovirus genes were amplified, sequenced and analyzed phylogenetically. In the PCR amplification we obtained a 499-nt fragment of the polyhedrin gene, a 644-nt fragment of the *lef-8* gene and 342-nt fragment of the *pif-2* gene. BLAST homology searches for *polh* gene revealed the highest (98%) homology with polyhedrin of *M. brassicae* (Mb) MNPV at the nucleotide level. Translated BLAST gave the result of 98% amino acid sequence identity and similarity. For *lef-8* and *pif-2* genes the first BLAST match was *M. configurata* (Maco) NPV isolate B (Li et al. 2002) with nucleotide similarity of 98 and 99%, respectively. Amino acid sequences were 99% identical and 100% similar in case of the *lef-8* gene and 98% identical and 100% similar in case of the *pif-2* gene. Homology searches showed considerably lower values when SeMNPV(P) genes sequences were compared with SeMNPV sequence available in GenBank (IJkel et al. 1999).

Phylogenetic analysis of the polyhedrin gene placed SeMNPV(P) in group II NPVs, but distantly from SeMNPV (Spod-X^R). High bootstrap values supported the placement of SeMNPV (P) together with MbMNPV and MacoNPVs (Fig. 1A). Since

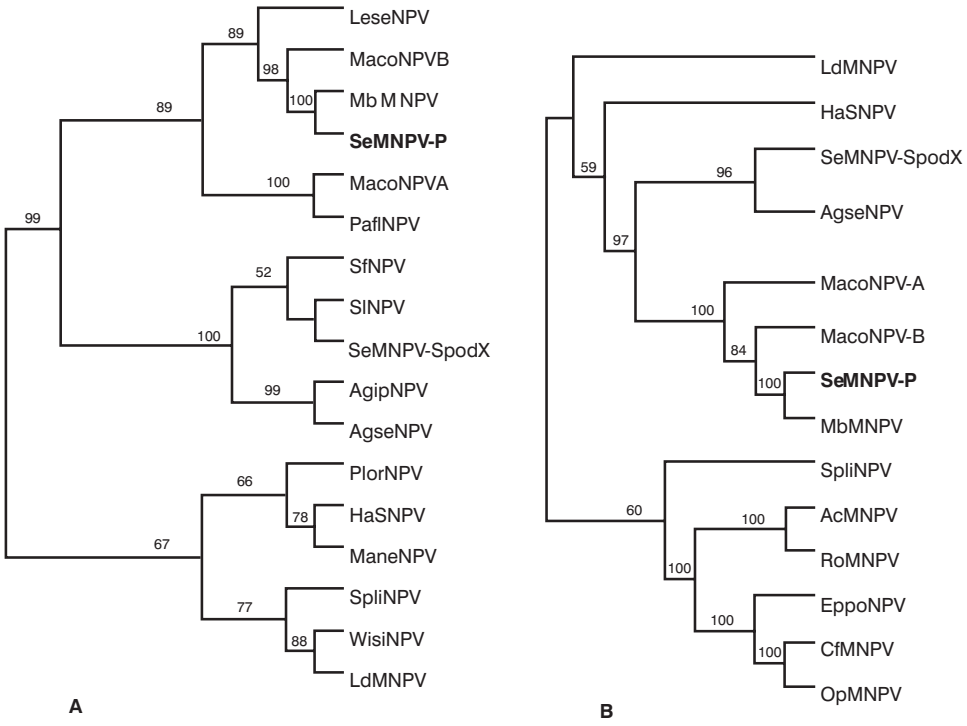

Fig. 1. Baculovirus phylogenies based on three conserved genes sequences: A. *polh*, B. *lef*-8 and *pif*-2

GenBank accession numbers: AcMNPV – NC001623, CfMNPV – NC004323, EppoNPV – NC003083, HearSNPV – G4 – NC002654, LdMNPV – NC001973, MacoNPV-A 90/2 – NC003529, MacoNPV-B – NC004117, OpMNPV – NC001875, SeMNPV – AF169823, SpltNPV – AY552474, AgipNPV – *polh* AY136484, AgseNPV-P – *polh* AY971675, *lef-8* AY971676, *pif-2* AY971677, LeseNPV – *polh* U30302, ManeNPV – *polh* AY127899, MbMNPV – *polh* iM20927, lef-*8*, *pif-2* – this study, PaflNPV – *polh* D0437, PlorNPV – *polh* AF019882, SfNPV – *polh* J04333, SpliNPV – *polh* AY600451, WisiNPV – *polh* AF016916. In bold – fully sequenced baculoviruses

the polyhedrin analysis revealed a close relationship of SeMNPV(P) with *Mamestra spp*. NPVs we have amplified, cloned and sequenced additionally the MbMNPV (French isolate, 30–85, Institute of Plant Protection collection) *lef-8* and *pif-2* genes. MacoNPV virus sequences are available in GenBank (NCBI). The combined *lef-8/*

Table 1. Median lethal doses, LD_{50}, of three nucleopolyhedroviruses for third instar larvae of *Spodoptera exigua*

Virus	Virus concentration (OBs/larvae)	Number of insects tested	LD_{50} values (OBs/larvae)	Confidence interval (95%)	Regression equation y=bx+a
SeMNPV(P)	$1 \times 10^1 - 1 \times 10^6$	432	5.4×10^2	$1.2 \times 10^2 - 1.1 \times 10^3$	y = 4.4x + 1.2
SeMNPV(Spod-X^R)	$0.5 \times 10^1 - 0.5 \times 10^3$	432	1.4×10^1	$0.8 \times 10^1 - 1.2 \times 10^1$	y = 1.1x + 1.5
MbMNPV	$1 \times 10^1 - 1 \times 10^6$	216	2.1×10^3	$5.3 \times 10^2 - 0.7 \times 10^4$	y = 0.9x + 1.9

pif-2 analysis confirmed placing SeMNPV (P) together with MbMNPV and MacoNPV spp. in the same clade, supported by high bootstrap values (Fig. 1B).

Restriction analysis

The restriction analysis with *Eco*RI was performed for SeMNPV (P), SeMNPV (Spod-X^R) and MbMNPV (30–85). The restriction profile of SeMNPV(P) was almost identical to that of MbMNPV(30–85), but very different from SeMNPV (Spod-X^R) (Fig. 2). This result supports the contention that SeMNPV (P) is closely related to MbMNPV and most likely a variant of the latter virus species.

Bioassays

Bioassays were performed in singlo in order to estimate the biological activity of SeMNPV (P), SeMNPV (Spod-X^R) and MbMNPV against *S. exigua* larvae. LD_{50} values against third instar larvae were 5.4×10^2, 1.4×10^1 and 2.1×10^3 OBs, respectively (Table 1). In terms of activity SeMNPV(P) differed significantly from SeMNPV (Spod-X^R). Although the LD_{50} values for SeMNPV(P) and MbMNPV seem different, they did not so at the 95% confidence interval. The latter may be due to the large variation in the assay with MbMNPV and requires repetition of the assays.

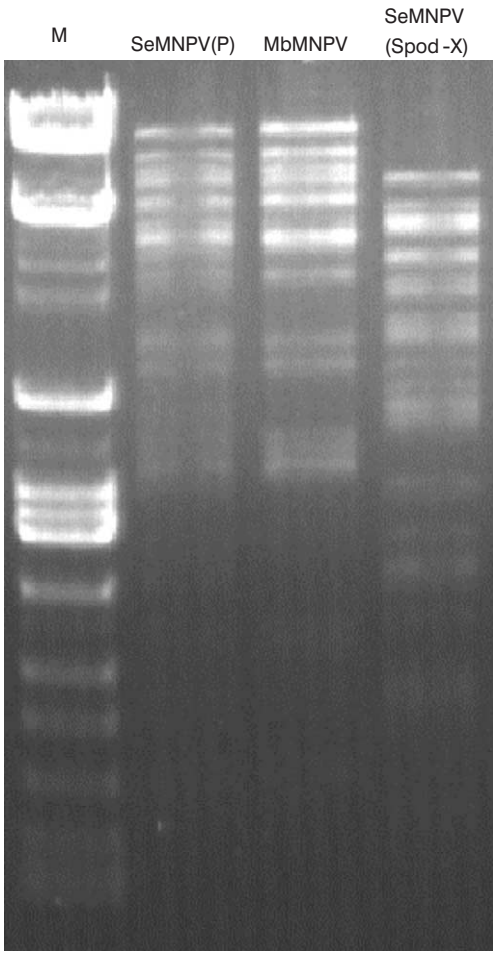

Fig. 2. *Eco*RI digestion profiles of SeMNPV(P), MbMNPV and SeMNPV(Spod-X); M – Lambda digested with *Bam*HI/*Eco*RI/*Hin*dIII

DISCUSSION

The aim of these studies was to characterize a multicapsid nucleopolyhedrovirus pathogenic to the beet armyworm *S. exigua* and isolated from *S. exigua* rearings in Poznan. Phylogenetic analysis showed that SeMNPV(P) belongs to the group II NPVs, but is distantly related to SeMNPV (IJkel et al. 1999). Both *polh* and combined *lef-8/pif-2* analyses placed SeMNPV (P) together with MbMNPV. Homology searches revealed high nucleotide and amino acid sequence similarities of SeMNPV (P) genes with MbMNPV and MacoNPVs and considerably lower with SeMNPV.

These findings were confirmed by *Eco*RI restriction profiles, which were nearly identical for SeMNPV(P) and MbMNPV.

Baculoviruses are identified according to the insect from which they were isolated. Thus we named our isolate SeMNPV (P). The biological activity of this isolate was lower than SeMNPV (Spod-X^R) but comparable with MbMNPV. Molecular analysis revealed that SeMNPV (P) is most likely a genotypic variant of MbMNPV. MbMNPV infectivity against *S. exigua* has already been reported by Smits and Vlak (1988) and earlier by Allaway and Payne (1984), but it is interesting to note that one of the MbMNPV isolates in the former study was isolated initially from *S. exigua* and probably the result of cross-infection. This outcome further underscores the utility of molecular analysis for baculovirus strain identification.

It is interesting to note that the infectivity of SeMNPV(P) against *S. exigua* larvae was slightly higher than of MbMNPV (Table 1). This may be the result of adaptation of the MbMNPV to the new host (*S. exigua*) by selection of a particular genotype or a genotype mixture that performs better in the alternate host. Multiple passage of a mixed isolate can change the genotypic structure and biological activity of the virus (Kolodny-Hirsch and van Beek 1997; Tompkins et al. 1988). This may have been the case of MbMNPV when passaged in a laboratory culture of *S. exigua* in our study. Cross-infections are very common in insect populations, specially those occupying similar ecological niches. Lower virus specificity which means broader host range increases its potential as biocontrol agent and also gives the possibility to produce the virus in an alternative insect, which may decrease the production costs.

Searching for new virus isolates to control pest insect species more effectively is still an important issue in biological control. The current study shows the relevance of molecular analysis for baculovirus strain identification to explain biological phenomena. In addition, such analysis may not only save time and money spent on bioassays and field work, but is also important for baculovirus registration and commercial use.

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POLISH SUMMARY

CHARAKTERYSTYKA WIRUSA WYIZOLOWANEGO Z LABOLATORYJNEJ HODOWLI ŚWIATŁOŁÓWKI NAZIEMNICY *SPODOPTERA EXIGUA* (HBN.) W POLSCE

Scharakteryzowano pod względem molekularnym i biologicznym wirusa poliedrozy jądrowej (NPV) SeMNPV(P) pochodzącego z gąsienic światłołówki naziemnicy *Spodoptera exigua* (polska hodowla laboratoryjna) podobnego morfologicznie do aktywnego wirusowego składnika biopreparatu Spod-X^{R}, SeMNPV(Spod-X^{R}). Analiza filogenetyczna polskiego izolatu wirusa oparta na trzech genach bakulowirusa *polh*, *lef-8* i *pif-2* wykazała niewielką homologię z wirusem SeMNPV(Spod-X^{R}) natomiast wysoką z wirusami *Mamestra brassicae* MbMNPV oraz *Mamestra configurata* MacoMNPV. Homologia ta została potwierdzona przez restrykcyjną analizę genomowego DNA. Wyniki testów biologicznych wykazały, że najbardziej aktywnym dla gąsienic *S. exigua* był wirus SeMNPV pochodzący z biopreparatu Spod-X^{R}, natomiast aktywność wirusów SeMNPV-P i MbMNPV była porównywalnie niższa.