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Genetic and phenotypic comparisons of viral genotypes from two nucleopolyhedroviruses interacting with a common host species, *Spodoptera litura* (Lepidoptera: Noctuidae)

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ABSTRACT

Nucleopolyhedroviruses (NPVs) are known to be highly variable, both genetically and phenotypically, at several scales such as different geographic locations or a single host. A previous study using several geographic isolates indicated that two types of NPV, *Spodoptera littoralis* NPV (SpliNPV) and *S. litura* NPV (SpltNPV) types, were isolated from the common cutworm, *Spodoptera litura* (Fabricius), a polyphagous insect that causes serious damage to many forage crops and vegetables. That study also indicated that the SpliNPV type was widely distributed in Japan. Here, we investigated the genotypic and phenotypic variation of cloned NPVs that infect *S. litura*; such variation is an important resource for biological control agents, and may represent the genetic diversity of an NPV species. Eighteen genotypically distinct NPVs were cloned from four field-collected NPV isolates using an in vivo cloning technique. They were divided into two virus types according to the similarity of banding patterns of DNA fragments generated by restriction endonucleases, and Southern hybridization analysis. Partial polyhedrin gene sequences revealed that the two types corresponded to SpliNPV and SpltNPV. Bioassays seem to suggest that the SpliNPV virus type was, overall, more infectious and killed *S. litura* larvae faster, but yielded fewer viral occlusion bodies, than the SpltNPV type. These data provide a basis for explaining the distribution pattern of SpliNPV and SpltNPV types in *S. litura* populations in Japan.

1. Introduction

The common cutworm *Spodoptera litura* (Fabricius) is distributed in subtropical and temperate regions of Asia and Oceania, and is a serious insect pest of many forage crops and vegetables. Control of this pest has relied on chemicals. However, public concerns about environmental hazards resulting from the extensive use of chemicals have generated pressure for a considerable reduction in their use, and the development of resistance to many chemicals in *S. litura* has also been reported (Hirose, 1995; Takai, 1991). For these reasons, efforts have been made to develop alternative control methods for this pest (Honda, 2000; Oki et al., 2012; Shimoda and Honda, 2013).

Nucleopolyhedroviruses (NPVs) are promising candidates for controlling insect pests due to their minimal negative impact on non-target organisms and the environment, and indeed have already been extensively applied in horticulture, forestry and orchards (Fuxa, 1987; Hunter-Fujita et al., 1998; Shapiro et al., 2012). Further development and registration of NPVs may make pest control programs more powerful in a wide variety of situations.

Selection of viral strains for pest control is an important part of a microbial control program. Variation in genotypes within an NPV species has been observed at several ecological scales, including different geographical regions or countries (Gettig and McCarthy, 1982; Harrison et al., 2014; Laitinen et al., 1996; Lee and Miller, 1978; Maeda et al., 1990; Takatsuka et al., 2003), a single locality (Shapiro et al., 1991; Simón et al., 2008) and a single host cadaver (Cory et al., 2005; Garcia-Maruniak et al., 1996). Differences in biological traits have also been demonstrated among geographical isolates (Hatfield and Entwistle, 1988; Hughes et al., 1983; Shapiro et al., 1984), among genotypes from a single isolate (Lynn et al., 1993; Muñoz et al., 2000; Simón et al., 2008) and among genotypes from a single cadaver (Cory et al., 2005; Hodgson et al., 2001).

NPVs pathogenic for *S. litura* larvae have been isolated in several locations in Japan. Such wild isolates show heterogeneity regarding genotypes, and individual isolates can contain several viral

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genotypes (Maeda et al., 1990; Takatsuka et al., 2003) that may have different biological characteristics. Thus, these wild isolates are potential sources of viral strains to be selected as microbial control agents for controlling *S. litura*. Takatsuka et al. (2003) also indicated that the wild isolates contained two types of viruses, *S. littoralis* NPV (SpliNPV) and *S. litura* NPV (SpltNPV), with the former type distributed more widely in Japan. *Spodoptera littoralis* is distributed in Africa and Mediterranean Europe and does not exist in Japan. However, this insect is the sibling species of *S. litura*. Therefore, it is reasonable to assume that both SpliNPV and SpltNPV can be isolated from both insect species. Cherry and Summers (1985) reported that two types of NPV, SlNPV-B (corresponding to SpliNPV) and SlNPV-A (corresponding to SpltNPV) were isolated from *S. littoralis*, with equal frequency. The wider distribution of SpliNPV than SpltNPV in Japan may be in part explained by a difference in virus fitness in *S. litura* populations.

Maeda et al. (1990) cloned many genotypes from several wild isolates by an in vitro technique, but phenotypic characterization was only performed in vitro regarding their multiplication in cell culture. Takatsuka et al. (2003) compared virus infectivity and survival time of infected larvae, using wild isolates themselves, which were derived from pooled samples of numerous infected larvae in a single field at each of several localities. However, no comparison in vivo has been made of the phenotypic characteristics of virus genotypes between SpliNPV and SpltNPV.

Here, we address this issue by hypothesizing that (1) SpliNPV has fitness advantages over SpltNPV in Japan, but (2) there is a mechanism to allow the coexistence of SpliNPV and SpltNPV. We isolated genotypes from four wild isolates of NPV from *S. litura* in Japan and examined their phenotypic characteristics such as infectivity, speed of kill (the time required to kill the insect) and yield of occlusion bodies (OBs). How these laboratory-evaluated parameters translate into virus fitness in the field, or field performance of the viruses, will require further research; nevertheless, our observations provide basic information for the estimation of virus fitness.

2. Materials and methods

2.1. Insects

S. litura were originally collected from a soya bean field in the Tokyo University of Agriculture and Technology Field Science Center, Tokyo, Japan in 2000 and continuously reared in our laboratory. Larvae were kept in plastic cages (30 × 22 × 6 cm) at 25 °C with a 16-h photoperiod until pupation, and were reared on an artificial diet (Insecta: Nosan Corporation, Yokohama, Japan). Individual adults were transferred to paper bags (8 × 15 × 20 cm) with a 10% crude sugar solution for feeding. Nucleopolyhedrosis has never been observed in this insect culture. In addition, we found no evidence for the endogenous presence of either SpliNPV or SpltNPV: a PCR procedure (see Section 2.7) using polyhedrin gene-specific primers and larval genomic DNA as templates did not generate any product.

2.2. Viruses

NPV isolates were obtained from diseased *S. litura* larvae at four locations in Japan (the Ogasawara Islands, Mishima, Fukuyama and Kagoshima) (Fig. 1). The isolates from Mishima (Mi), Fukuyama (Fu) and Kagoshima (Ka) were provided by Ministry of Agriculture, Forestry & Fisheries GenBank. Dr. M. Okada (Japan Plant Protection Association) kindly provided the Ogasawara isolate (Og). These isolates are pooled samples of many infected larvae from a single agricultural field in each of the four locations. Autographa californica multiple NPV strain C6 (AcMNPV-C6) (Ayres et al., 1994) was kindly provided by Dr. S.G. Kamita (University of California, Davis). OBs were purified by homogenization of cadavers and density gradient centrifugation (Okuno et al., 2003), suspended in 0.05 M sodium phosphate buffer (pH 7.7), and then counted with a Thoma hemocytometer. Five counts per hemocytometer and three subsamples per suspension were measured to reduce counting and dilution errors. The suspensions were stored at 4 °C until use.

2.3. End-point cloning of genotypes in vivo

Genotypes were cloned from the above four isolates. The in vivo cloning technique described by Smith and Crook (1988) was modified. Larvae beginning to molt out of third instar, as determined by head capsule slippage, were transferred into plastic cages (8 × 15 × 20 cm) without food. The newly molted insects were collected after 20–24 h and each was allowed to imbibe a 1-μl droplet of OB suspension (10^3 OBs/ml) containing 1% Tinopal UNPA-GX (Fluorescent Brightener: Sigma-Aldrich, St. Louis, USA), 10% sucrose and 1% blue food coloring (Kyoritsu Food Co., Tokyo, Japan). Each larva should therefore theoretically ingest an average of one polyhedron. Previous studies showed that there was no difference in infectivity among the isolates and that, for Og, a dose of one OB/insect killed 10–20% of fourth-instar larvae in the presence of 1% Tinopal UNPA-GX (Okuno et al., 2003; Takatsuka et al., 2003). Therefore, each isolate was expected to kill 10–20% of the insects. Larvae that ingested the entire droplet were immediately transferred to 30-ml cups containing fresh artificial diet and were incubated at 25 °C with a 16-h photoperiod. More than 200 larvae were used for each isolate. OBs from each dead larva were used for a further round of cloning with the same procedure, except that 50 larvae were allowed to drink the OB suspensions. DNA extracted from OBs obtained from each larva was examined by restriction endonuclease (REN) analysis. This process was repeated until DNA profiles indicated homogeneity in the genotypes.

2.4. Isolation of viral DNA

Dead larvae containing OBs were individually transferred to microcentrifuge tubes and homogenized in 1 ml of sterile distilled water (SDW). OBs were purified by centrifugation and density gradient centrifugation using Percoll solution (GE Healthcare UK, Buckinghamshire, England) containing 0.25 M sucrose, and resuspended in 220 μl SDW. A 20-μl aliquot was kept aside at 4 °C for a further round of infections, while the rest was used for extraction and REN analysis of viral DNA. Virions were released from OBs using alkali buffer, pelleted by centrifugation at 20,400*g* for 10 min, and incubated in TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer with a final concentration of 200 μg/ml proteinase K and 1% (w/v) sodium dodecyl sulfate (SDS) at 55 °C for 3 h. DNA was purified using Protein Precipitation Solution (QIAGEN, Tokyo) and dialysis against 0.01× TE buffer.

2.5. REN analysis of viral DNA

Aliquots of 1 μg of viral DNA were digested with 10 U of EcoRI (Takara Bio, Shiga, Japan) at 37 °C for 4 h. Digested fragments were separated in 0.7% agarose gels using Marker3 (HindIII-digested and EcoRI-digested λ-DNA mixture) (NIPPON GENE, Tokyo, Japan) as a molecular size marker. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

2.6. Southern blot hybridization analysis of viral DNA

Viral DNA was blotted onto a Nytran N membrane (Schleicher & Schuell BioScience, Dassel, Germany) with transfer solution (3 M