Further Studies on the Biological Activity and Identification of Nucleopolyhedrovirus Isolated from *Spodoptera litura* in Japan

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Abstract

The Japanese nucleopolyhedrovirus isolate from *Spodoptera litura* (SpltNPV Satsuma) has been well studied as a prospective bio-pesticide. Low LC_{90} and LC_{99} values of about 10^5 OB/ml were recorded on second instars of larvae of *S. litura.* The estimated LT_{50} for every instar-concentration combination was between 4 and 9 days. Based on the restriction endonuclease profiles of viral DNA digested with four restriction enzymes, the genome size, estimated to be between 96 and 130 kbp was close to those reported for other SpltNPVs.

Key words: Biological control , nucleopolyhedrovirus , *Spodoptera litura*, virus characterization.

Introduction

The biology of the cotton leafworm (tobacco cutworm) *Spodoptera litura* (Fabricius) (Lepidoptera, Noctuidae) pest has been widely studied by several authors (KUMAR *et al.* 1992, KHARUB *et al.* 1993, PACHORI and GARVAV 1997, SINGH *et al.* 1989, OU-YANG and CHU 1990, RAO *et al.* 1989, CHU and YANG 1989). It has a life cycle of about 5 weeks (EPPO 1979).

S. litura is an international pest with a host range covering over 40 families of economically important plants. Among the main crop species attacked by *S. litura* are soybeans, cotton, rice and vegetables (EPPO 1979). The pest is found in North America, Oceania, Reunion, and Asia, including Japan, where the species undergoes four generations between May and October (NAKASUJI 1976). On most crops, damage arises from extensive feeding by larvae, leading to complete loss of foliage (EPPO 1979) and yield losses attain 50% (PATEL *et al.* 1971).

To date, utilization of broad-spectrum chemical insecticides is the main practical method of controlling the larval stages (caterpillars) of this insect. However, the threats of development of insecticide-resistance and concerns over safe-food supply and environmental contamination, as well as delay or suppression of field colonization of natural enemies necessitate the development of alternative interventions. Thus

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a safe alternative pest control method using its pathogens as a biological intervention was considered.

Several species of entomopathogenic bacteria, protozoa, fungi, and viruses were isolated and evaluated as possible biological control agents of the cotton leafworm and other cutworms. To date, more than 600 different insect species infected with baculoviruses have been reported in literature. Baculovirus are enveloped viruses (singly or multiply) that have double-stranded, super coiled and circular DNA genome ranging in size from 88 to 160 kbp (KOOL *et al.* 1995). They belong to the family *Baculoviridae* divided into two genera: the *Nucleopolyhedrovirus* (NPV) and the *Granulovirus* (GV). The NPVs are characterized by the occlusion of virions in a large occlusion body (OB) called polyhedra and range in size from 1 to 15 μm. They also exhibit peculiar characteristics in that they are primarily transmitted horizontally to their host via OBs that are released after the death of infected individuals.

Over the last decades, the specificity of baculoviruses has been reviewed by several researchers, with increasing interest on baculoviruses. The baculovirus genera *Nucleopolyhedrovirus* (NPV) has a specificity limited to one or a few related insect specices, but exceptionally *Autographa californica* NPV (AcMNPV) and *Anagrafa falcifera* NPV (HOSTETTER and PUTTLER 1991) have been found to infect a wide range of host (EVANS 1986). Thus before application as bio-pesticide, a range of studies are needed to evaluate the bio-safety of the use of the virus. Some information on characterization of a Japanese NPV isolate from *S. litura* (SpltNPV Satsuma) have been given by KOUASSI *et al.* (2009), but the available information still remains inadequate for a practical and safe use of the virus.

The present paper attempts to give a better overview of the evaluated virus, including its biochemical and biological properties. The results presented in this paper support the concluded apparent relatedness of SpltNPV S and *S. litura* NPVs, and the assessment of the virus as a good candidate for the biological control of *S. litura* moth outbreaks in future.

Materials and Methods

Insects

The larvae of *S. litura* used in the experiment were hatched from egg masses originally collected from a field in Kagoshima University during summer 1997. Insects were then reared until 2003 in laboratory together either with other larvae from egg masses collected on a number of occasions in the University, or with male adults from pheromone traps set in the University. At the beginning of the experiment in 2003, larvae were reared without crossing with any new population. The insects were reared continuously in the laboratory at ca. 25℃ on artificial diet (OKADA 1977), prepared by a modified process in which dried maize leaves were used instead of dried

pinto beans. To reduce stress-related mortality due to crowding, larvae were first reared in petri dishes (ϕ 90×20mm) from egg masses to third instar, and then transferred to plastic containers measuring 15×20×5cm where 60 larvae of the fourth and fifth instars were maintained.

Virus

The *S. litura* NPV Satsuma isolate (SpltNPV S) was obtained from diseased larvae collected from a filed at Kagoshima University in 1997 and viral stocks prepared by infecting *S. litura* larvae *per os* with virus-contaminated artificial diet. Occlusion bodies (OBs) were collected by grinding larval cadavers in 0.1 % sodium dodecyl sulfate (SDS) and filtering through four layers of muslin. The filtrates were purified by glycerol density gradient centrifugation (HUNTER-FUJITA *et al.* 1998), and then suspended in distilled water. OBs in the suspension were counted with a Thoma hemocytometer and stored at 4℃ until use.

DNA extraction and REN analysis

Purified OBs were incubated in 0.02 M NaOH for 10 min. to release virions, and centrifuged at 13,000 g for 30 min. Virions were disrupted by suspending the pellet in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 % SDS and 200 μg/ml proteinase K, and incubating the mixture at 55℃ for 3 h. DNA was extracted with TE-saturated phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. After washing with 70 % cold ethanol, the DNA was suspended in TE and stored at 4 ℃. Viral DNA was digested with *Eco*RI, *Bam*HI, *Pst*I, or *Hind*III (Takara Bio) for 4 h under conditions recommended by the supplier. Digested fragments were loaded together with λ-*Eco*T14 I/*Bgl*II (Takara Bio) digest, λ-*Hind*III (Takara Bio), and 100 bp DNA Ladder (Takara Bio) onto 0.5 to 1.5 % agarose gels (SAMBROOK and RUSSELL 2001). The gels were stained with ethidium bromide and photographed under UV light using a Toyobo FAS-III transilluminator. DNA fragment sizes were estimated by the graphical method (SOUTHERN 1979).

Bioassays

Bioassays were conducted in second-, third-, fourth- and fifth-instar *S. litura* larvae. Larvae just before molting were distinguished by head capsule slippage and transferred to other containers to ensure that test larvae were inoculated within 12 h of molting. Virus preparations were serially diluted from 10^9 to 10^2 OBs/ml, and 10 μl of four to eight virus concentrations spread on small pieces of artificial diet in glass test tubes. Newly molted larvae were allowed to feed individually on the contaminated diet for 48 h. A total of 25 larvae that had consumed the entire piece of diet were then transferred to virus-free diet in all the instar stages except in the second instar, where 30 larvae were used because of their weakness to manipulation. As controls, larvae were fed on diet without virus suspension. All treatments were incubated at 25 ℃ in a 14-h light and 10-h dark photoperiod. Mortality due to infection was recorded every day until pupation. The presence of OBs in tissue smears of dead larvae

was confirmed by phase contrast microscopy at ×400 magnification.

Statistical analysis

The concentrations resulting in 90 % (LC_{90}) and 99 % (LC_{99}) mortality of larvae at $\alpha = 0.05$ were estimated for SpltNPV S at each instar by probit analyses (FINNEY 1978), using the microcomputer program PriProbit 1.63 (SAKUMA 1998). The "All or nothing model (natural response rate = 0, natural immunity rate = 0)" was used.

Mean time to death and the median lethal time (LT_{50}) per treatment was calculated by the Kaplan-Meier product limit estimator, and the effect of virus concentration on the lethal time was determined by regression analysis using the JMP 5 software package (SAS-INSTITUTE 2002). Only insects that died from NPV infection were included in the analysis (FARRAR and RIDGWAY 1998).

Results

REN profiles

Electrophoresis profiles of SpltNPV S genome after digestion with *Bam*HI, *Hind*III, *Pst*I and *Eco*RI together with a diagram of these profiles obtained using 0.5%, 1% or 1.5% agarose gels for a better separation of the smallest and largest DNA fragments are presented in Fig. 1. Fragments were lettered in alphabetic order and the genome size of SpltNPV S estimated was between 96 and 130 kbp (Table 1).

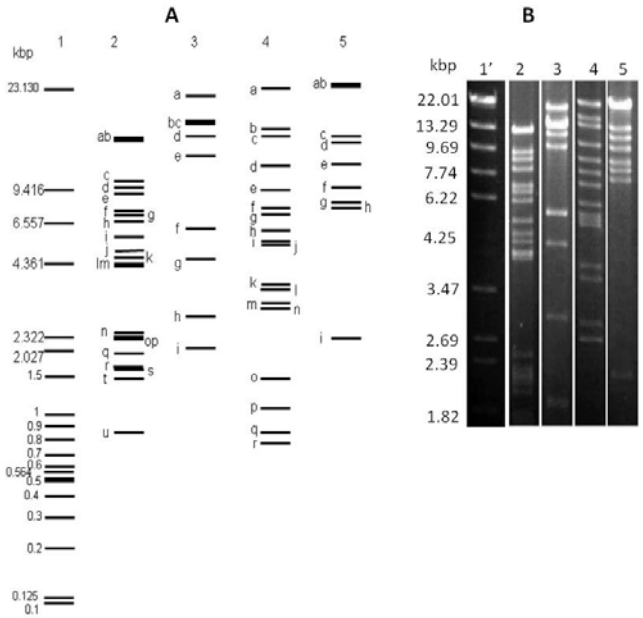

Fig. 1. Restriction enzyme profiles of SpltNPV S DNA digested with digested with *Eco*RI (lane 2), *Pst*I (lane 3), *Bam*HI (lane 4), and *Hind*III (lane 5) using 1% agarose gel (B), and their diagram using 0.5% , 1%, and 1.5% agarose gel (A). Markers (lane 1): λ-*Hind* III digest + 10 bp DNA Ladder, (lane 1'): λ-*Eco*T 14 I/*Bgl*II digest.

Table 1. Restriction endonuclease fragment sizes (in kilobase pairs) of MyseNPV G isolate DNA

fragment	*Eco*R I	*Pst* I	*Bam* HI	*Hind* III
a	14700	22000	23000	24500
b	14700	16500	17500	24500
c	10750	16500	14700	14700
d	10250	14000	11500	13000
e	9000	12000	9000	11500
f	8000	6000	8250	9500
g	7000	4800	7400	8750
h	6750	3100	6000	8000
i	5410	2000	5410	2300
j	5000		5200	
k	4800		4000	
l	4300		3600	
m	4300		3000	
n	2500		2700	
o	2300		1425	
p	2300		1025	
q	1900		850	
r	1675		775	
s	1600			
t	1500			
u	850			
Total (kb)	119585	96900	125335	116750

Infectivity of SpltNPV S against S. litura larvae

In Table 2 the LC_{90} and LC_{99} values are presented, showing the effectiveness of SpltNPV S against larvae of *S. litura*. At $\alpha = 0.05$ for LC_{90}, second and third instars of *S. litura* were significantly more susceptible to SpltNPV S than fourth and fifth instars. For LC_{99}, second instar was significantly more susceptible than third, fourth and fifth instars of *S. litura*. No mortality was observed in the control group for each instar.

Table 2. Probit analysis of dose mortality response of SpltNPV S against second, third, fourth, and fifth instars of *Spodoptera litura*

Larval instar	LC_{90} *(95% CL)	LC_{99}*(95% CL)	$\chi^{2\,a}$	df
Second	8.5×10^4 (4.6×10^4- 3.3×10^5)	3.7×10^5 (1.5×10^5- 2.1×10^6)	4.51	2
Third	8.4×10^5 (2.4×10^5- 3.8×10^6)	1.1×10^7 (2.6×10^6- 1.3×10^8)	3.60	4
Fourth	2.8×10^7 (1.2×10^7- 9.7×10^7)	2.4×10^8 (7.2×10^7- 1.8×10^9)	4.91	6
Fifth	3.5×10^7 (1.5×10^7- 1.3×10^8)	3.6×10^8 (1.0×10^8- 2.9×10^9)	7.74	6

*OB/ml

[a] Likelihood Ratio Chi-square for the homogeneity test. Values are not significant ($p > 0.05$)

Time-mortality relationship

Time-mortality relationship for second to fifth instars of *S. litura* infected with SpltNPV S at various concentrations is shown in Fig. 2. Lethal time was negatively correlated with applied concentration in second instar ($F_{1,74} = 43.08$, $P < 0.0001$), third instar ($F_{1,73} = 7.34$, $P = 0.008$), fourth instar ($F_{1,85} = 62.74$, $P < 0.000$); and fifth instar ($F_{1,85} = 21,92$, $P < 0.0001$). The estimated LT_{50} for every instar-concentration combination was between 4 and 9 days (Fig. 3).

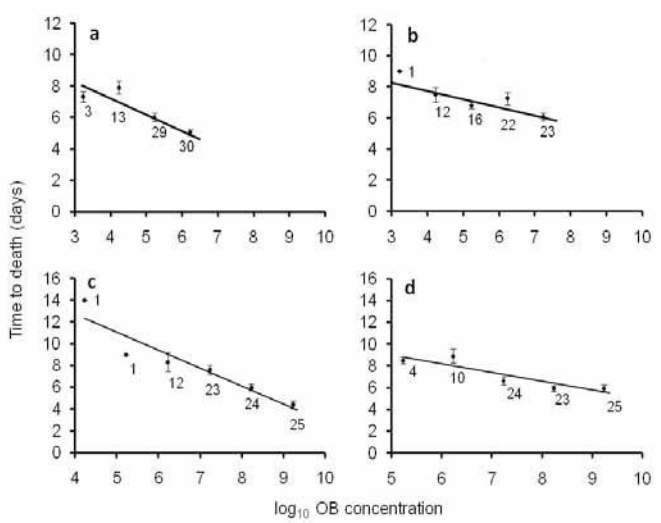

Fig. 2. Time to death (mean±SE) for second (a), third (b), fourth (c), and fifth (d) instars of *S. litura* infected with SpltNPV S. The sample sizes of second instar and third to fifth instar larvae were 30 and 25, respectively. Values next to the plots are the number of dead larvae in that treatment. The regression equation with SpltNPV S (solid line) was time to death = 8.17 - 1.16 x $\log_{10}$ OB concentration, time to death = 8.25 - 0.43 x $\log_{10}$ OB, time to death = 12.44 - 1.46 x $\log_{10}$ OB, time to death = 9.03 - 0.75 x $\log_{10}$ OB against second, third, fourth, and fifth instar, respectively.

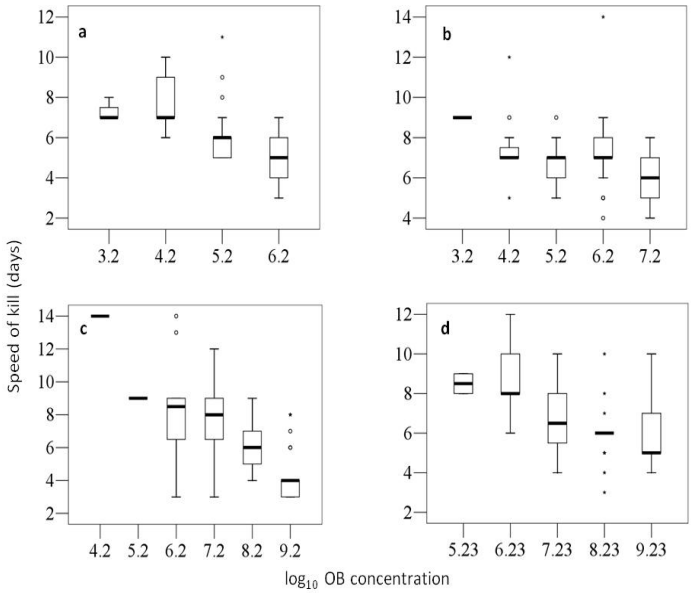

Fig. 3. Time to death for second (a), third (b), fourth (c), and fifth (d) instars of *S. litura* infected with various concentration of SpltNPV S. The horizontal line in each box indicates the median mortality time for each treatment. The upper and lower hinges of the box indicate the third quartile and the first quartile, respectively. The upper and lower ends of the vertical lines indicate the largest and smallest non-outliers, respectively; open circles and asterisks indicate extreme and mild outliers, respectively.

Discussion

Genetically, the isolate SpltNPV Satsuma has been shown by REN profiles analysis and PCR amplification to be close to other SpltNPVs. PANG *et al*. (2001) and WEI *et al*. (1999) reported that the genome size of SpltNPVs was 139,342bp and 136.6kp, respectively and the electrophoresis profiles of SpltNPV S genome after digestion with *Bam*HI, *Hin*dIII, *Pst*I and *Eco*RI in this study, that lead to an estimation of the genome size between 96 and 130 kbp supports the identification confirmed by KOUASSI *et al.* (2009) for SpltNPV S.

The increase of LC_{90} and LC_{99} value of SpltNPV S on *S. litura* larvae is attributed to a decrease of the susceptibility to the virus of *S. litura* with age. Generally, decrease of lepidopteran's susceptibility to NPVs with age is explained by the "dilution effect" related to increase in larval weight as development proceeds (BRIESE 1986). In earlier studies, PRASAD and RAMAKRISHNAN (1993) after conducing bioassay of SpltNPV against *S. litura* larvae reported a steady increase of LC_{50} values with larval age, but concluded that increase in LD_{50} or LC_{50} values with increase in age of larvae was not due to true resistance mechanism but due to dilution effect of viral inoculum (BRISE 1986) related to increase in larval weight. An increase in LD_{50} and LC_{50} values with increased larval age was also recorded in the bioassay study of NPV infecting *S. litura* (MONOBRULLAH and NAGATA 2000). However, MONOBRULLAH and NAGATA (2000) noted that old larvae were not infected through oral inoculation, but intrahaemocoelic injection of NPV into mature larvae resulted in death due to viral infection. Thus, they concluded that the gut appears to act as a barrier to infection in the late stage of larval development, leading to a kind of resistance. Furthermore, MONOBRULLAH and NAGATA (2000) reported that although there seemed to be a difference in slopes of regression lines between younger and older larvae, there was no statistical significant difference in slope values of the dosage mortality regression lines indicating similar changes in response to increasing virus dose for most of the larval instars. They also concluded that increase in LD_{50} values with increase in larval age was mainly due to the dilution effect (BRIESE 1986). In this study, although no statistical analysis of the slope has been done, in concordance with MONOBRULLAH and NAGATA (2000) and PRASAD and RAMAKRISHNAN (1993), we attribute the decrease of the susceptibility to SpltNPV of *S. litura* larvae with age to dilution effect, as well as the gut acting as a barrier as larvae approach pupation. However, an increased intrinsic heterogenecity in the test population or an increased variability in the bioassay can not be ruled out as factors affecting the variation in host susceptibility with age.

The results of bioassay of *S. litura* larvae with SpltNPV Satsuma isolate characterize the extremely high infectivity of the virus used in our study. KOUASSI *et al.* (2009) reported that SpltNPV S was highly pathogenic to second instar of *S. litura* with a LC_{50} value of 1.48×10^4 OB/ml. This value is not far from the LC_{90} and LC_{99} estimated to 8.5×10^4 OB/ml and 3.7×10^5, respectively. Even though the LC_{90} and

LC_{99} values for third-, fourth-, and fifth-instar *S. litura* were a little higher (between 10^5 OB/ml and 10^8 OB/ml), this study confirms that SpltNPV S isolate from Kagoshima was very active against *S. litura* larvae above all in earlier stages, and therefore can be use as a suitable bio insecticide to control the pest in this area.

The survival times for NPV-infected lepidopterans tend to decrease with increasing viral dose (VAN BEEK *et al.* 1988), and this pattern was found for every instar stage of *S. litura* infected with SpltNPV S. The short LT_{50} (4-9 days) for every instar-concentration combination supports the idea that practical application of the isolate SpltNPV S as a bio-insecticide to control *S. litura* presents wide opportunities.

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