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Molecular Cloning of Restriction Endonuclease Fragments of DNA Isolated from Nuclear Polyhedrosis Virus of Heliothis armigera

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ABSTRACT

Restriction fragments of *Heliothis armigera* nuclear polyhedrosis virus (HaNPV) DNA double digested with EcoRI and BamHI were cloned in *E. coli* DH 5α F' by using the Bluescript plasmid as a vector. Inserted DNAs were estimated to be 1.5 and 2.0 kb in size. DNA probe was constructed from DNA fragment of 1.5 kb labeled with digoxigenin, the nonradioactive DNA labelling. The probe hybridized well with DNAs isolated from positive colonies but not with the Bluescript plasmid. Dot blot hybridization data indicated that this digoxigenin labelling DNA probe can detect the viral DNA in the infected larva two days after inoculation. This probe can also be used to detect viral DNA isolated from a single NPV infected *H. armigera* larva.

INTRODUCTION

Nuclear polyhedrosis virus (NPV) is considered a potential bioinsecticide for the control of several lepidopterous insects. Among these pests, the cotton bollworm, *Heliothis armigera* is the most destructive to crop plants due to its resistance to a wide range of chemical insecticides.

The NPV belongs to sub-group A in the family Baculoviridae of insect viruses. This virus has an envelop, rod-shaped nucleocapsid, and a large, covalently closed, circular double stranded DNA (Summers, 1977; Tinsley and Harrap, 1978). The molecular weight of the viral genome was estimated from 50 to 100 million daltons (Bilimoria, 1986). Physical mapping of restriction endonuclease fragments of some viruses in this group has been constructed (Summers and Smith, 1979; Vlak, 1980; Loh *et. al.*, 1981; and Knell and Summers, 1984).

In Thailand an isolate of *H. armigera* NPV (HaNPV) was isolated from infected cotton bollworm from the field and now being developed as bioinsecticide (Attathom *et. al.*, 1988). Preliminary characterization of HaNPV was done by determining the specific restriction endonuclease cleavage pattern and its molecular weight of DNA was estimated to be 65 X 10^6 daltons (Attathom *et. al.*, 1988). Molecular characterization of HaNPV is needed to better understand the genome organization and its functions for further improvement of HaNPV as bioinsecticide.

In this study we report the cloning of the restriction endonuclease fragments of HaNPV genome and the construction of DNA probe for NPV diagnosis in order to study the distribution and transmission of this virus in nature. This is part of the efforts to understand the molecular biology and ecology of the Thai NPV isolate.

MATERIALS AND METHODS

Virus purification and viral DNA isolation :

Nuclear polyhedrosis virus of the cotton bollworm, *H. armigera* was isolated from diseased larvae collected from the cotton field at Utong district, Supanburi province, Thailand. The virus was propagated in host larvae which were mass reared on artificial diet. Virus purification and viral DNA isola-

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tion were followed the method described by Attathom *et. al.*, (1988). Briefly virus particles were extracted by dissolving the polyhedra in 0.1 M Na_2CO_3, pH 11.2 for 20 min. After removing undissolved polyhedra and debris by low speed centrifugation, virus particles were pelleted by centrifugation at 36,000 rpm for 2 hr. Viral DNA was isolated from purified virus by phenol:chloroform extraction, precipitated by ethanol and resuspended in TE buffer for further analysis.

Construction of recombinant plasmids :

Bluescript (Stratagene) and HaNPV DNA were each double digested to completion with EcoRI and BamHI. Enzyme treated Bluescript was separated by 0.7% agarose gel electrophoresis (Perbal, 1988), and eluted from the gel by using Geneclean Kit (BIO 101, La Jolla, California, USA). Restriction fragments of viral DNA were collected by phenol-chloroform : isoamyl alcohol (24:1) extraction and ethanol precipitation. Restriction fragments of HaNPV-DNA and Bluescript were then ligated at 14°C for 16 hr. Ligation reaction mixture consisted of 10 µl of 100 ng digested NPV-DnA, 5 µl of 100 ng digested Bluescript, 1 µl of T4 DNA ligase (Biolabs Inc.USA.), 2 µl of 10X ligation buffer, and 2 µl of 100 mM ATP.

Cloning of NPV-DNA fragments :

Recombinant plasmids were used to transform *E. coli* DH 5αF' by mixing 10 µl of ligated vector and 100 µl of competent cell suspension. The mixture was maintained on ice for 30 , incubated at 42°C for 50 sec and immediately chilled for 5 min. After supplementation with 900 µl of growth medium, the cells were incubated for 1 hr at 37°C and then plated on selective medium. Positive colonies were selected and recombinant plasmids were purified as outlined by Maniatis *et. al.* (1982). DNA inserts were analyzed by the EcoRI and BamHI double-digestion of purified plasmids followed by agarose gel electrophoresis.

Probe preparation :

A fragment of EcoRI and BamHI double digested HaNPV-DNA was used to construct the probe. Eluted DNA fragment was labeled with digoxigenin-11-dUTP, the nonradioactive DNA labelling (Boehringer, Mannehein GmbH, Biochemica, W. Germany). The reaction mixture of 20 µl was incubated for 16 hr at 37°C and stopped with 2 µl of 0.2 M EDTA, pH 8.0. Probe was precipitated with 2.5 µl of 4 M LiCl and 75 µl prechilled ethanol and kept overnight at -20°C. After centrifugation, the digoxigenin-labeled probe was vacuum dried and resuspended in 50 µl TE buffer, pH 8.0.

Sample preparation :

To prepare DNA samples for hybridization tests, each healthy and NPV infected *H. armigera* larva was homogenized in 1 ml of ddH_2O and 1 ml of 2X lysis buffer (0.02M tris, pH 7.5, 0.3 M NaCl and 4% SDS), centrifuged at 14000 rpm for 5 min and supernatant collected. DNA was precipitated from supernatant by cold absolute ethanol, vacuum dried and dissolved in ddH_2O for hybridization.

Dot blot hybridization :

DNA samples were dotted on nitrocellulose membrane. After prehybridization at 68°C for 1 hr in sealed plastic bag, they were hybridized overnight at 68°C in shaker bath with hybridized solution containing 5 µl/ml of denatured DNA probe. Detection of target DNA with digoxigenin labeled probe was performed according to the standard experimental procedure (Boehringer Mannheim GmbH Biochemica, W. Germany).

RESULTS

Cloning of NPV-DNA fragments :

Screening of over two thousand colonies, 23 colonies were shown positive to the ampicillin resistant test. Plasmids isolated from colony number 3, 5, 9, 12, 15, 18 and 20 revealed distinct bands in agarose gel electrophoresis with the size ranging from 3.0-3.5 kb (Fig. 1). These bands migrated slower than the 2.9 kb Bluescript plasmid indicating that they contained inserts.

Analysis of NPV-DNA inserts :

Double digestion of isolated plasmids with EcoRI and BamHI restriction endonucleases showed inserted DNA fragments of similar size (Fig. 2). Colony number 3, 9 and 18 contained inserted DNA with the size of 1.3-1.5 kb. Digestion product of colony number 5, 12 and 20 showed the DNAs with the size of 2.0 kb. The DNA fragment of 1.3 kb from colony number 3 was used for probe construction.

DNA probe and dot blot hybridization :

Digoxigenin labelling DNA probe constructed from 1.3 kb inserted DNA from colony number 3 reacted positively with all plasmids isolated from colony number 3, 5, 9, 15, 18, and 20. No reaction was observed when the probe was hybridized with non-insert Bluescript (Fig. 3). It is suggested that these

DNA inserts may derive from the same region of the HaNPV genome.

When the probe was dot blot hybridized with DNAs isolated from healthy and NPV infected *H. armigera*, positive reactions were observed only with diseased samples (Fig. 4). Viral DNA isolated from 1, 2 and 3 infected larvae gave the same result when hybridized with the probe. The intensity of color development showed the same level of hybridization independent from the amount of DNA being loaded per dot. This result indicated that DNA isolated from a single NPV-infected larva was sufficient for dot-blot hybridization with the 1.3 kb fragment NPV-DNA probe.

This study also demonstrated that the concentration of NPV DNA increased in infected larvae after inoculation (Fig. 5). When NPV-DNA was isolated from *H. armigera* larvae 1, 2, 3, 4, 5 and 6 days after inoculation and hybridized with the probe, the color development was observed with the increasing intensity respectively. The result indicated that NPV-DNA was first detected in the second day after inoculation. Viral DNA concentration increased up to the sixth day after inoculation. By that time, most of infected larvae died because of the viral infection.

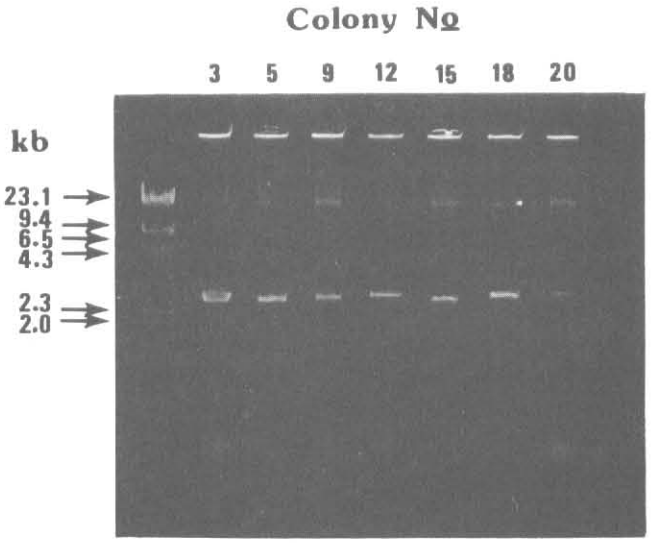

Figure 1 Agarose gel electrophoresis of recombinant Bluescript plasmids contained HaNPV DNA EcoRI and BamHI double-digested fragments. The plasmids were isolated from 7 selected colonies of ampicillin resistant *E. coli* DH 5αF'. The Hind III fragments of lambda DNA were used as standards for size determination.

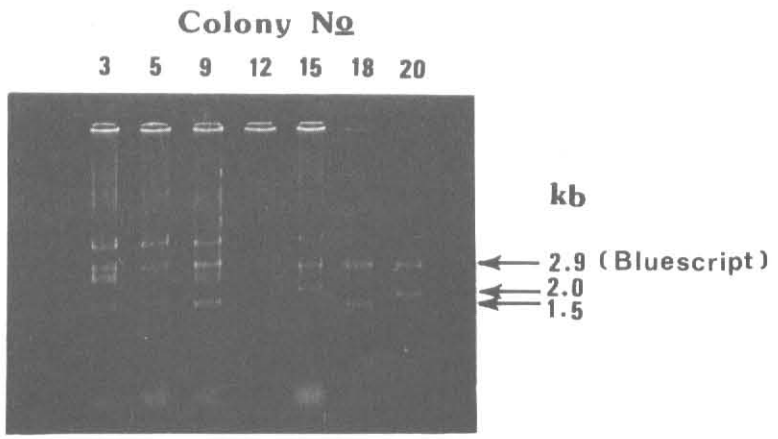

Figure 2 Agarose gel electrophoresis of recombinant Bluescript plasmids after double digestion with EcoRI and BamHI restriction endonucleases. The plasmids were isolated from 7 selected colonies of ampicillin resistant *E. coli* DH 5αF'.

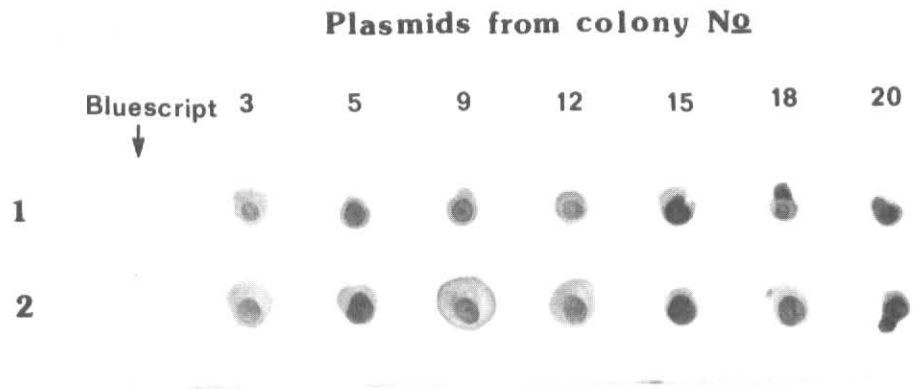

Figure 3 Dot blot hybridization of plasmids isolated from ampicillin resistant colonies to digoxigenin labelling NPV cDNA probe. DNA loaded per dot was 5 ng (row 1) and 10 ng (row 2) respectively. Non-insert Bluescript showed negative reaction to the probe.

DISCUSSION

Restriction endonuclease pattern is proven to be useful for the analysis of DNA rest on the fact that restriction enzymes cleave DNA at specific nucleotide sequences. Its usefulness for identification of strains and genomic variants of NPV had been well recognized (Cochran *et. al.*, 1982 and Brown *et. al.*, 1985). Our previous work has shown the difference of restriction endonuclease pattern of HaNPV-DNA after digested with EcoRI, HindIII, PstI, SacII, HpaI and ScaI (Attathom *et. al.*, 1988). However, the application of this technique for strain differentiation of NPV found in Thailand is very limitted. This is due to the fact that there is no rapid and reliable method to detect virus in the field so that more strains can be collected and studied. Molecular cloning of restriction endonuclease fragments of NPV-DNA will prove to be of great value for the search and the development of more effective strains of NPV in Thailand. We have selected the ECoRI and BamHI fragments because of its constant 1.3-2.0 kb products suitable for cloning. Although this work is at the beginning stage of constructing genome mapping and fullength sequence analysis, the probe derived from cloned DNA fragment has high application potential. With the probe and the digoxigenin labelling technique, NPV can be detected even in a single infected larva. It is hoped that

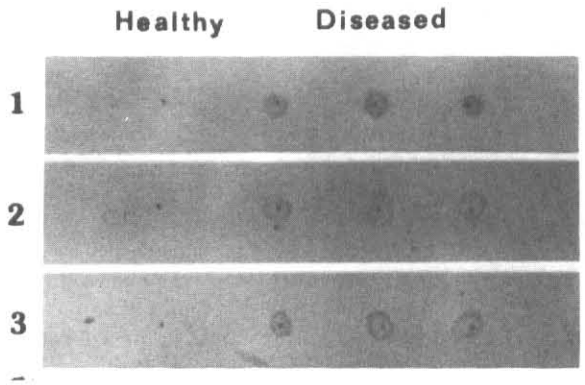

Figure 4 Dot blot hybridization of DNA isloated from healthy and NPV-infected (diseased) *Heliothis armigera* larvae to digoxigenin labelling NPV cDNA probe. NPV-DNA was isolated from 1, 2 and 3 larvae as indicated in row 1, 2 and 3 respectively.

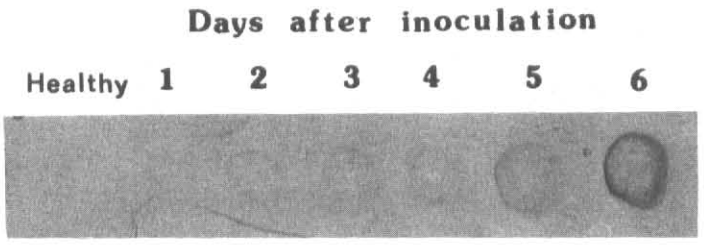

Figure 5 Dot blot hybridization of DNA isolated from healthy and NPV infected *Heliothis armigera* larvae to digoxigenin labelling NPV cDNA probe

more natural occurring strains of NPV will be detected by this method and then the selection and development of the effective strain of NPV for the control of *H. armigera* will be accomplished.

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LITERATURE CITED

Attathom, T., S. Chaeychomsri, S. Chaichuchot, S. Attathom and P. Chiemsombat. 1988. Characterization of the nuclear polyhedrosis virus of the cotton bollworm, *Heliothis armigera*. Kasetsart J. (Nat. Sci. Suppl.) 22:14-23.

Bilimoria, S. L. 1986. Taxonomy and identification of baculoviruses. *In* The Biology of Baculoviruses. Vol. 1. Biological Properties and Molecular Biology. Granados R. R. and Federici B. A. Eds., CRC Press. Inc. Boca Raton, Florida. p. 38-54.

Brown, S.E., J.E. Maruniak and D. L. Knudson. 1985. Baculovirus (MNPV) genomic variants : Characterization of *Spodoptera exempta* MNPV DNAs and comparison with other *Autographa californica* MNPV DNAs. J. Gen. Virol. 66:2431-2441.

Cochran, M. A., E. B. Carstens, B. T. Eaton and P. Faulkner. 1982. Molecular cloning and physical mapping of restriction endonuclease fragments of *Autographa californica* nuclear polyhedrosis virus DNA. J. Virol. 41:940-946.

Kneel, J. D. and M. D. Summers. 1984. A physical map for the *Heliothis zea* SNPV genome. J. Gen. Virol. 65:445-450.

Loh, L. C., J. J. Hamm and E. S. Huang. 1981. *Spodoptera frugipera* nuclear polyhedrosis virus genome:Physical maps for restriction endonucleases BamHI and Hind III. J. Virol. 38(3) : 922-931.

Maniatis, T., E. F. Fritsch, and J. Sambrooks. 1982. Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor. New York. 545p.

Perbal, B. 1988. A practical guide to molecular cloning .2nd ed. A Wiley-interscience Publication, John Wiley & Sons. New York. 811p.

Summers, M. D. 1977. Baculoviruses (Baculoviridae). *In* the Atlas of Plant and Animal Viruses. Maramorosch, K., Ed., Academic Press, New York. p.3-27.

Summers, M. D. and G. E. Smith. 1979. Restriction maps of five *Autographa californica* MNPV variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonucleases SmaI, KpnI, BamHI, SacI, XhoI, and EcoRI. J. Virol. 30:828-838.

Tinsley, T. W. and K. A. Harap. 1977. Viruses of invertebrates. *In* Comprehensive Virology, Vol. 12, Fraenkel-Conrat, H. and Wagner, R. R., Eds., Plenum Publishing Corp. New York. p.1-144.

Vlak, J. M. 1980. Mapping of BamHI and SmaI DNA restriction sites on the genome of the nuclear polyhedrosis virus of the alfalfa Looper, *Autographa californica*. J. Invertebr. Pathol. 36:409-414.