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New cell lines from *Lymantria xylina* (Lepidoptera: Lymantriidae): Characterization and susceptibility to baculoviruses

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Abstract

Four new cell lines, designated as NTU-LY-1 to -4, respectively, were established from the pupal tissues of *Lymantria xylina* Swinhoe (Lepidoptera: Lymantriidae). These cell lines have been cultured approximately 80 passages during 2 years in TNM–FH medium supplemented with 8% fetal bovine serum, at a constant temperature of 28 °C. Each line consists of three major morphological types: round cells, spindle-shaped cells, and giant cells. The characterization of these cell lines showed that they are different from previously established lines derived from related Lepidopteran species. All new lines were susceptible to the *L. xylina* multiple nucleopolyhedrovirus (LyxyMNPV) and appeared to have a good potential for studying this virus.

Keywords: *Lymantria xylina*; Cell line; RAPD–PCR; Isozyme; Nucleopolyhedrovirus

1. Introduction

Since Grace (1962) successfully established long-term cultures of insect cells, over 500 continuous cell lines have been established from over 100 insect species (Lynn, 1999). Insect cell lines have become more important as a tool for the production of insect pathogenic viruses and recombinant proteins by the use of the baculovirus expression vector as well as in studies on physiology and developmental biology (Granados and McKenna, 1995; Hink et al., 1991; Maeda et al., 1985; Smith et al., 1983; Wu et al., 1989; Vaughn, 1981). Virtually, every cell line is a potential or actual source of material for molecular biologists to use in a wide range of studies.

The casuarina moth, *Lymantria xylina* Swinehoe (Lepidoptera: Lymantriidae), is a forest pest in Taiwan, Japan, India, and on the eastern coast of mainland China (Chao et al., 1996; Matsumura, 1933; Xiao, 1992). Due to the extension of its host plant range and the increasing agricultural loss, this moth now becomes a significant quarantine pest (Chang and Weng, 1985; Chao et al., 1996). An epizootic disease, nucleopolyhedrosis, of *L. xylina* larvae occurs from spring to early summer in Taiwan and mainland China, and the key pathogen was found to be *L. xylina* multiple nucleopolyhedrovirus (LyxyMNPV) (Cheng et al., 1987; Liang et al., 1986; Wu and Wang, 2005; Yu et al., 1997). This virus appears to effectively suppress *L. xylina* population in field trials (Cheng et al., 1987) and is being investigated as a possible addition to the integrated pest management (IPM) programs against this moth.

Production of LyxyMNPV involved the large-scale rearing of *L. xylina* larvae. This process is labor intensive and several challenges need to be met (e.g. the conditions for breaking embryonic diapause and the development of artificial diets) to obtain a reliable source of the host insect. *In vitro* production of LyxyMNPV in a highly susceptible cell line would be an alternative solution. Besides, this strategy possesses a potential advantage of screening and maintaining highly virulent LyxyMNPV strains. In the present study, we used pupal tissues of *L. xylina* to successfully establish four LyxyMNPV permissive cell lines. These new lines represent a great hope for extensive study of LyxyMNPV at the pathologic, cellular, and molecular levels.

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2. Materials and methods

2.1. Primary culture and subculture

Larvae of *L. xylina* were collected from the low-elevation mountain area located in central Taiwan (Mingjian Township), and reared with leaves of the Formosa sweet gum (*Liquidambar formosana*) at 25 °C. The larvae were allowed to go through the pupal stage. The 2–4-day-old pupae were collected and surface-sterilized with a 10% Clorox solution and 70% iodine alcohol. The internal tissues (without specific selection) from each pupa were picked with a fine forceps and a pipet, and placed in a 25 cm^2 flask with 2 ml TNM–FH medium (Hink and Strauss, 1976) containing 100 IU/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), 1.25 mg/ml amphotericin B (Sigma), and 16% heat-inactivated (56 °C for 30 min) fetal bovine serum (FBS, Hyclone). The primary cultures of pupal tissues were incubated at 28 °C. The first subculture of the primary cultures was performed after 1 month when the cells reach confluence. When subculturing, 2 ml of suspended cells were transferred to a new 25 cm^2 flask containing 4 ml of fresh media plus supplements. Thereafter, 3/4–4/5 volume of the medium was replaced at intervals of 14 days followed by 4–7 days intervals. After 25 passages, the cells were adapted to 8% FBS and were routine subcultured at 5 days intervals. Four cell lines from different primary cultures have been successfully established and are designated NTU-LY-1 to -4, respectively. The approximate passage numbers of the NTU-LY-1 to -4 cells for the following studies were 55, 61, 58, and 63, respectively.

2.2. Cell morphological observation

Microscopical images of cells from individual cell lines were captured with an Olympus IX-71 inverted phase-contrast microscope with a digital camera. Cell sizes were calculated according to a calibrated magnification factor. Average cell dimensions were determined from measurements of 30 cells.

2.3. Cell growth curves

Cells in log phase were seeded into 25 cm^2 flasks, about 1×10^6 cells each, and cultured with 8% FBS supplementation at 28 °C. Cell densities were determined by counting the cell numbers within a microscope reticule of which the area at a certain objective was known. The cell densities in five areas of each flask were determined at 24 h intervals. Cell population doubling time was calculated using the exponential formula described by Kuchler (1977).

2.4. Random amplified polymorphic DNA-polymerase chain reaction (RAPD–PCR) analysis

The extraction of total cell DNA from *L. xylina* larva and the six cell lines, NTU-LY-1 to -4, IPLB-LD-652Y (*L. dispar* cell line, Goodwin et al., 1978), and NTU-PN-HH (*Perina nuda* cell line, Wang et al., 1996) were carried out using a tissue and cell genomic DNA purification kit (GeneMark) according to the protocol provided by the manufacturer. Three 10 mer random primers (OPU-09: 5′CCA CAT CGG T; OPU-10: 5′ACC TCG GCA C and OPO-05: 5′CCC AGT CAC T) and PCR conditions used were modified from Wu et al. (2002). Each PCR consisted of 50 μl mixture containing 1× reaction buffer (with 2 mM $MgSO_4$), 200 μM dNTP, 2.5 U HiFi DNA polymerase (Yeastern Biotech), 1 μM of primer, and 50 ng cellular DNA templates. The PCR was performed under the following conditions: 2 min at 94 °C for the initial denaturation; 1 min at 94 °C, 1 min at 40 °C, and 2 min at 72 °C for the next 40 cycles; and 15 min at 72 °C for the final extension. The PCR products were analyzed by electrophoresis in a 2% agarose gel prestained with ethidium bromide and run at 100 V for 20 min in TAE buffer.

2.5. Isozyme analysis

Cell samples were prepared and run on gels modified from Wang et al. (1996). Briefly, the confluent cells of the five cell lines, NTU-LY-1 to -4, and IPLB-LD-652Y, were harvested from each individual 25 cm^2 flask. The cells were centrifuged at 70*g* for 10 min at 4 °C, resuspended in 500 μl of grinding buffer (0.125 M Tris–HCl, 46 mM citric acid, 10% sucrose, 1% Triton X-100, and 0.02 mM bromophenol blue), and lysed by three freeze/ thawing cycles in liquid nitrogen and a 37 °C water bath. The cell lysate was centrifuged at 8500*g* for 5 min. The supernatant liquid was collected and stored at −20 °C. For sample separation, 10–20 μl of lysate was loaded into each well of a 10% polyacrylamide gel, and electrophoresed at a constant current of 20 mA for 2 h. The gels were tested for the three isozymes, esterase, malate dehydrogenase (MDH), and lactate dehydrogenase (LDH) following the protocol of Harris and Hopkinson (1977).

2.6. Virus infection

The following viruses were used to test the viral susceptibility of NTU-LY-1 to -4 cells: AcMNPV (*Autographa californica* NPV) kindly supplied by Dr. M.J. Fraser of Notre Dame University; PnMNPV (*P. nuda* NPV) collected from infected NTU-PN-HH cells (Wang et al., 1996); LdMNPV-like virus initially isolated from an infected larva of *L. xylina* and *in vitro* propagated in IPLB-LD-652Y cells (Yu et al., 1997); and LyxyMNPV collected from an infected larva of *L. xylina* that was LyxyMNPV-positive for PCR-RFLP method described by Wu and Wang (2005).

These four viruses were used to examine the viral susceptibility of three other cell lines as a positive control for virus infectivity (Sf-9, NTU-PN-HH, and IPLB-LD-652Y). AcMNPV, PnMNPV, and LdMNPV-like virus were obtained from their permissive cell lines, Sf-9, NTU-PN-HH, and IPLB-LD-652Y, respectively. Semiconfluent cells