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Isolation and RNA1 nucleotide sequence determination of a new insect nodavirus from *Pieris rapae* larvae in Wuhan city, China☆

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Abstract

A new insect nodavirus is isolated from *Pieris rapae* larvae in Wuhan city, China and tentatively designated Wuhan nodavirus (WhNV). We here report the physicochemical characterization of WhNV and determine the nucleotide sequences of its larger segment of genome, RNA1. The results show that WhNV particles are isometric, non-enveloped, with a diameter of about 29 nm. The virus has a major capsid protein and a minor capsid protein with estimated molecular mass of 40 and 44 kDa, respectively. WhNV RNA1 is determined to be 3149 nt long, containing a 1014-amino-acid open reading frame (ORF) encoding protein A with a calculated molecular mass of 114,608 Da. The protein A shows 39 and 27% identity to its homologues in *Pariacoto virus* (PaV) and *Striped jack necrosis nervous virus* (SJNNV), respectively, but shows only 24% or less identity to its homologues in other insect Nodaviruses such as *Nodamura virus* (NoV), *Black beetle virus* (BBV), *Boolarra virus* (BoV) and *Flock house virus* (FHV). Predicted domains for six RNA-dependent RNA polymerase motifs and putative ORFs (protein B) are confirmed by sequence analysis of WhNV RNA1.

Keywords: Wuhan nodavirus; Nodavirus; Protein A; ssRNA

1. Introduction

The family *Nodaviridae* contains two genera, alphanodaviruses and betanodaviruses, which predominantly infect insects and fish, respectively. Nodaviruses are small, non-enveloped spherical viruses with $T=3$ icosahedral capsid which is composed of 180 copies of coat protein (Hosur et al., 1987). Its genome contains two single-strand positive-sense RNA molecules, RNA1 (3.1 kb) and RNA2 (1.4 kb), which are copackaged in the same particle (Newman and Brown, 1973; Longworth and Carey, 1976; Newman and Brown, 1977; Krishna and Schneemann, 1999). RNA1 encodes protein A, catalytic subunit of RNA-dependent RNA polymerase (RdRp) which replicates both genome segments and can replicate autonomously when transferred alone into appropriate cells (Gallagher et al., 1983), and RNA2 encodes coat precursor protein α which undergoes an autocatalytic mature cleavage into two viral capsid proteins β and γ, and the mature cleavage is required for viral infectivity and enhancement of stability (Schneemann et al., 1992). 5′-ends of the two RNAs are capped and 3′-ends, which are not polyadenylated (Schneemann et al., 1998; Ball and Johnson, 1999), are blocked by an undetermined covalent modification or unusual secondary structure (Guarino et al., 1984; Dasmahapatra et al., 1985; Ball, 1995).

In addition to RNA1 and RNA2, a subgenomic RNA3, which is not encapsidated into virions, is synthesized during RNA replication from the 3′ terminus of RNA1 and encodes one or two proteins (B1 and B2) in overlapping ORFs (Guarino et al., 1984; Dasmahapatra et al., 1985; Ball, 1995; Johnson et al., 2003). Proteins B1 and B2 are both identified to be dispensable for RNA replication (Ball, 1995). However, protein B2 encoded by FHV RNA3 has recently been demonstrated to be a RNA-silencing

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suppressor in animal and plant cells (Li et al., 2002) and NoV protein B2 can enhance viral RNA accumulation in both mammalian and insect cells (Johnson et al., 2004).

Although original hosts of alphanodaviruses are limited to insects (Hendry, 1991; Ball, 1994; Ball and Johnson, 1998), their RNAs can replicate in insect (Gallagher et al., 1983; Dasgupta et al., 2003), vertebrate (Ball et al., 1992), plant (Selling et al., 1990), and even yeast (Price et al., 1996, 2005) cells. Features that alphanodaviruses can replicate abundantly in a wide range of cells make them attractive experimental system for the studying viral structure, replication, assembly and evolution, and provide the potential applications of the nodavirus system for the development of long-term, high-level vectors expressing foreign genes (Dasgupta et al., 2003).

Pieris rapae is a worldwide-distributed polyphagous pest. In China, many members of *Cruciferae* especially cabbage and broccoli are affected by larval instars resulting in this species to be considered an agricultural pest of major importance.

Currently, a small icosahedral RNA virus is isolated from the dead larval population of *Pieris rapae* infected by granulosis virus (PrGV)-a baculovirus, in a cabbage field near Wuhan city, China. We have characterized the particle properties of this virus and determined the nucleotide sequences of the larger segment of genome. The results indicate that it is a new virus that should be classified within the family *Nodaviridae*. The virus is tentatively named Wuhan nodavirus (WhNV). WhNV is the first nodavirus isolated from an insect host in China.

2. Materials and methods

2.1. Infected larvae collection

Dead larvae of *Pieris rapae* infected by WhNV and PrGV were collected during an outbreak of PrGV from a cabbage field near Wuhan city, Hubei province, China and frozen at −20 °C immediately until use.

2.2. Virus purification

Frozen infected larvae were thawed three times and homogenized in TNE buffer (10 mM Tris–Cl, 1 mM EDTA, 50 mM NaCl; pH 7.5). After a low-speed centrifugation at 100,000 × *g* in a Biofuge stratos rotor for 30 min of the homogenate, the supernatant was precipitated at 120,000 × *g* for 2 h in a Beckman coulter Ti70 rotor. The virus pellets were resuspended in TNE buffer and then centrifuged through a 40% (W/V) sucrose cushion at 180,000 × *g* in a Beckman coulter Ti40 rotor for 3 h. Virus pellets were resuspended again, and then layered on a 10–40% (W/V) sucrose gradient prepared with the same buffer and centrifuged at 100,000 × *g* in a Beckman coulter SW40 rotor for 2.5 h. The band containing virus particles was collected, diluted with TNE buffer and pelleted at 120,000 × *g* for 2 h to concentrate virus. Virus pellet was resuspended again in the same buffer and kept at −20 °C until use.

2.3. Transmission electron microscopy

Purified virus was examined under a HitachiH-8100 transmission electron microscope.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The capsid proteins of WhNV were analyzed by SDS-PAGE. Purified virions were separated on 12% glycine SDS-PAGE gel (Laemmli, 1970). Protein bands were stained with Coomassie Brilliant Blue R-250. The molecular masses of the proteins were estimated by comparison with a wide range protein molecular mass marker (MBI) using a UA scanning system (UVP).

2.5. Nucleic acid extraction, cDNA synthesis and cloning

Nucleic acid was extracted from purified virions using TRIzol reagent (Invitrogen) following the manufacturer's instructions and digested with RNase A or DNase I (Promega).

The first strand of cDNA fragments complementary to WhNV RNA were synthesized using Thermoscript reverse transcriptase (Invitrogen) and random hexamer primer, as recommended by the manufacture. The cDNA was blunt-ended with T4 DNA polymerase (TaKaRa) and inserted into the *Sma* I site of PUC18 plasmid. The ligation mixtures were transformed into *Escherichia coli* Top10 cells by electroporation.

Subsequent clones were constructed by RT-PCR to cover missing sequences using specific primers based on the flanking sequences available from the above cDNA clones.

To determine the 5′ non-coding region (NCR) and 3′ NCR sequence, 5′ RACE (rapid amplification of cDNA ends) and 3′ RACE were performed (Iwamoto et al., 2001). For 5′ RACE, the first strand of cDNAs were polyadenylated with terminal deoxy-nucleotidyl transferase (TaKaRa). For 3′ RACE, viral RNA was polyadenylated with poly A polymerase (Invitrogen) in the presence of ATP. The amplified products were inserted into the PMD18-T vector (TaKaRa).

2.6. Nucleotide sequencing and sequence analysis

Clones were sequenced as double-strand DNA by dideoxynucleotide chain-termination method (Sanger et al., 1977) using an ABI Prism 377 DNA sequencer (Applied Biosystems) with universal sequencing and walking primers.

Sequence analyses were performed with the following programs: Bioedit program, CLUSTAL-W version 1.8 (Thompson et al., 1994), Blast (http://www.ncbi.nlm.nih.gov/gorf/). DNAMAN program (Lynnon corporation), Tmpred (Transmembrane domain (TMD) prediction) (www.ch.embnet.org/software/tmdred). Mfold (http://www.bioinfo.rpi.edu/applicattions/mfold).

The virus sequences and accession numbers used in this paper are as follows: FHV (X77156), BBV (X02396), BoV (AF329080), NoV (AF174533), PaV (AF171942) and SJNNV(AB025018).