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Characterization of a novel member of genus *Iflavirus* in *Helicoverpa armigera*

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ABSTRACT

The cotton bollworm, *Helicoverpa armigera*, is one of the most important agricultural pests of many economic crops worldwide. Herein, we found a novel single-strand RNA virus by RNA-Seq and Polymerase Chain Reaction (PCR) method in *H. armigera* named *Helicoverpa armigera iflavirus* (HaIV), which possessed a genome with 10,017 nucleotides in length and contained a single large open reading frame (ORF) encoding a putative polyprotein of 3021 amino acids with a predicted molecular mass of 344.16 kDa and a theoretical isoelectric point (pI) of 6.45. The deduced amino acid sequence showed highest similarity (61.0%) with the protein of *Lymantria dispar Iflavirus 1*. Phylogenetic analysis with putative RdRp amino acid sequences indicated that the virus clustered with members of the genus *Iflavirus*. The virus was mainly distributed in the fat body of its host and was found to be capable of both horizontal and vertical transmission. The efficiency of perorally horizontal transmission was dose dependent (100% infection rate with a viral dose of 10^8 copies/μl) while vertical transmission efficiency was found to be relatively low (<28.57%). These results suggest that we have found a novel member of genus *Iflavirus* in *H. armigera*.

1. Introduction

Insects are susceptible to a variety of pathogens, which can result in chronic or lethal infections (Burden et al., 2003). Historically, viruses have been isolated and subsequently studied after the observation of overt disease symptoms in the host. However, there are also covert infections found within hosts displaying no obvious signs of disease, which could remain undetected (Burden et al., 2003; Kemp et al., 2011; Murillo et al., 2011). These covert persistent viral infections, such as baculoviruses, are almost ubiquitous in many lepidopteran insect species and their discovery is driving further research into the dynamics and behavior of covert infections and their role in the ecology of host populations, especially those of economic and agricultural importance (Graham et al., 2015).

The cotton bollworm, *Helicoverpa armigera*, is one of the most important agricultural pests of cotton and other important economic crops worldwide. The adult moth is highly migratory, and populations have been reported in Australia, Asia, Africa, Europe (Feng et al., 2007; Wu et al., 1997) and most recently from South America (Tay et al., 2013). Since the introduction of Bt-cotton into China in the 1990s, the *H. armigera* population has declined dramatically. However, several studies have reported that Bt-resistance has evolved in the field (Gunning et al., 2005; Zhang et al., 2012). Thus, other forms of biological pest control, including the use of host-specific viral pesticides, derived from baculovirus (Allaway and Payne, 1984; Chen et al., 2001; Fuxa, 2004; Sun et al., 2002, 2004), small RNA viruses (Christian et al., 2005) and densoviruses (El-Far et al., 2012), has attracted more attention from researchers. Moreover, high-through-put analytical methods such as metagenomics and RNA sequencing provide sensitive and effective methods for the discovery of novel viruses and asymptomatic disease agents that may be useful as biological control products (Diatchenko et al., 1996; Ge et al., 2012; Marguerat and Bähler, 2009; Mokili et al., 2012; Radford et al., 2012; Roossinck et al., 2015), or conversely, may negatively or positively impact upon the biopesticide products being used. For example, the recent discovery of a novel densovirus (HaDV2) from healthy migratory cotton bollworms revealed that HaDV2 infection significantly increased host resistance to the host-specific baculovirus HaNPV

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and to the generalist biopesticide Bt toxin (Xu et al., 2014).The development of molecular tools and next generation sequencing technology paves the way for a greater understanding as to how we may manipulate the host-pathogen system, with the aim of reducing pest outbreaks and economic crop damage.

In this study, a novel virus infecting *H. armigera* named Helicoverpa armigera iflavirus (HaIV), was discovered by RNA-Seq. Alignment and phylogenetic analysis revealed that the virus showed a high sequence identity with members of the *Iflavirus*, which is the only genus within the family *Iflaviridae.* Members of this genus possess a single positive-strand RNA genome and share several common features, including: (1) non-enveloped icosahedral particles measuring 30 nm in diameter; (2) genome translation into a polyprotein; (3) the viral coat proteins containing three jelly-roll domains; (4) a three-domain containing a superfamily III helicase, a (cysteine) proteinase with a chymotrypsin-like fold and an RNA-dependent RNA polymerase (RdRp) (Le Gall et al., 2008). The genome of *Iflavirus* is monocistronic with one single large open reading frame (ORF) encoding a single large polyprotein. To date, only nine species of iflaviruses have been recognized by The International Committee on Taxonomy of Viruses (ICTV), including *deformed wing virus* (Lanzi et al., 2006), *Ectropis obliqua virus* (Wang et al., 2004), *Infectious flacherie virus* (Isawa et al., 1998), *Lygus lineolaris virus 1* (Perera et al., 2012), *Nilaparvata lugens honeydew virus 1* (Murakami et al., 2013), *Perina nuda virus* (Wu et al., 2002), *Sacbrood virus* (Ghosh et al., 1999), *Slow bee paralysis virus* (de Miranda et al., 2010), and *Varroa destructor virus 1* (Ongus et al., 2004), although other iflaviruses have been reported (Silva et al., 2015; Suzuki et al., 2015). Herein, we report the nucleotide sequence, genome organization, phylogeny, transmission and tissue distribution of HaIV.

2. Materials and methods

2.1. Insect culture

A laboratory colony of *H. armigera* was originally captured in 2005 from Langfang (Hebei province, China). *H. armigera* larvae were reared on an artificial diet (Liang et al., 2008) and adult moths were cultured with a 10% sugar and 2% vitamin mix (Liang et al., 1999) at 25 ± 1 °C with a 14:10, light: dark photoperiod.

2.2. Transcriptome analysis and annotation

For transcriptome analysis, Illumina RNA-sequencing was conducted by Novogene (Beijing, China). Four 5th instar larvae (one day post-ecdysis) were individually collected and total RNA isolated using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA samples were dissolved in RNase-free water and used to construct the cDNA library of *H. armigera* with suitable fragments (about 200 bp). = Paired-end transcriptome sequencing was subsequently performed using an Illumina HiSeq™ 2000. Adaptor sequences and low-quality reads were trimmed and clean reads were used for *de novo* assembly using Trinity (Grabherr et al., 2011). The assembled contigs were annotated using BLASTx and BLASTn against the NCBI non-redundant nucleic acid database (NT) and the NCBI non-redundant protein database (NR), using a cut-off E-value of 10^{-5}.

2.3. Cloning the iflavirus genome of H. armigera

Total RNA was extracted from individual adult moths reared in laboratory using the TRIzol reagent kit as described above. Single-stranded cDNA was synthesized using the FastQuant RT Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Based on the assembled sequence from RNA-seq, nine pairs of primers were subsequently designed (Table S1). The genome of the iflavirus isolated from *H. armigera* was amplified and sequenced using cDNA as template, using the following PCR program: 4 min at 94 °C; 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C for 40 cycles. The PCR product was purified, inserted into the pEASY-T cloning vector (TransGen, Beijing, China), and sequenced.

2.4. Sequence and phylogenetic analysis

The open reading frame (ORF) of the viral genome was predicted using ORF finder at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequences encoding non-structural proteins were compared with members of the order *Picornavirales* using Clustal W (Thompson et al., 1994). The complete nucleotide sequence of the virus described in this study was submitted to GenBank under accession number KX228231. The deduced RdRp amino acid sequence of the new virus, together with that of members of the family *Iflaviridae,* was used in phylogenetic analysis. *Acyrthosiphon pisum virus* and *Spodoptera exigua virus AKJ-2014* were used as outgroup. The phylogenetic trees were constructed using the maximum likelihood method with a bootstrap of 1000 replicates in MEGA6 (Tamura et al., 2013). Gaps were regarded as a complete deletion unless specifically noted.

2.5. Virus detection and quantification

A partial sequence exhibiting high similarities with known iflaviruses was identified from the RNA-seq data. For the detection of the novel virus, a pair of specific primers, VPF1/VPR1 (Table S1) were designed to amplify a PCR product of 593 bp, according to the assembled sequence of the virus in *H. armigera*. The PCR program used was as follows: 30 s at 94 °C, 30 s at 57 °C, and 30 s at 72 °C for 40 cycles. To assess the detection threshold of the virus assay, a 10-fold dilution series of cDNA (3.37×10^9 to 3.37×10^1 - copies/μl) containing the virus was made and tested using the VPF1/VPR1 primers. 10 μl of each PCR product was analyzed by agarose gel electrophoresis.

Copy numbers of the novel virus were quantified using a standard curve by an absolute quantification qPCR method (Wong and Medrano, 2005). For quantification, the primers and probes of the virus (VPF/VPR, Table S1) were designed. A fragment was amplified using the primers and cloned into the pEASY-T Cloning Vector (TransGen, Beijing, China) and sequenced. The PCR program was as follows: 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C for 40 cycles. The plasmid was used for the construction of the standard curve. Virus quantification was conducted with TaqMan in 20 μl reaction agent, which comprised 1 μl of template DNA, 2 × SuperReal PreMix (Probe, Tiangen, Beijing, China), 50 × ROX Reference Dye, 0.2 mM of each primer and 0.4 mM probe. The thermal cycling conditions on a 7500 Real-time PCR System (Applied Biosystems) were as follows: 40 cycles of 95 °C for 3 s, 60 °C for 30 s. The standard curve equation of $y = -0.9990x + 41.6662$ (y = the logarithm of plasmid copy number to base 2, x = Ct value, $R^2 = 0.9998$) was used to calculate the copy number of the virus (Fig. S1).

2.6. Transmission of the virus

Filtered liquid containing an unpurified form of the iflavirus was prepared (Xu et al., 2014). RNA was isolated and the concentration of the virus was quantified. A NONINF strain was established from a single breeding pair that was not infected with the iflavirus, according to the method described by Xu et al. (2014). An infected line (INF strain) of *H. armigera* was established by orally infecting NONINF strain larvae with the filtered liquid.