Journal of Invertebrate Pathology 109 (2012) 127–133

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

# Genome sequence of SeIV-1, a novel virus from the *Iflaviridae* family infective to *Spodoptera exigua*

Anabel Millán-Leiva, Agata K. Jakubowska, Juan Ferré, Salvador Herrero *

*Department of Genetics, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Spain*

ARTICLE INFO

*Article history:*
Received 26 August 2011
Accepted 17 October 2011
Available online 21 October 2011

*Keywords:*
*Spodoptera exigua*
Iflavirus
SeMNPV
RNA virus
Covert infection
Picorna-like virus

ABSTRACT

Analysis of the transcriptome of *Spodoptera exigua* larvae revealed the presence of several ESTs with homology to virus of the order *Picornavirales* and with the highest similarity to *Infectious flacherie virus* (*Iflaviridae*) that infects *Bombyx mori* larvae. *Iflaviridae* is a recently defined family of insect-infecting viruses that consist of positive single strand RNA genomes translated into a single polyprotein of around 3000 amino acids long. Using the sequence information derived from the obtained ESTs, we have completed the genomic sequence of this virus. The novel *S. exigua iflavirus* (SeIV-1) has a genome of 10.3 kb and codes for a 3222 aa polyprotein. Expression analysis has revealed the presence of the virus in all tissues tested and insect stages, being more abundant in the midgut of the larvae. High infectivity of this virus against *S. exigua* has been demonstrated after observing the presence of this virus in different colonies that were reared in the same chamber with the virus-infected colony, despite no evidence of pathological effects. Further study of viral covert infections of SeIV-1 could lead to a better understanding of its pathological effect as well as any possible interaction with other microbial pathogens used for the control of this pest.



## 1. Introduction

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is a worldwide pest that causes significant loses to agricultural and ornamental plant industries. To date, *S. exigua* has developed field resistance to a wide range of insecticides such as organophosphates, carbamates and pyrethroids, among others (Moulton et al., 2002). The development of resistance to chemical insecticides together with the environmental problems associated to their use have triggered the development and use of different types of microbial pathogens for the control of this pest. Currently two main types of entomopathogens are being used for its control, those based on the bacterium *Bacillus thuringiensis* and those based on the *S. exigua* nucleopolyhedrovirus (SeMNPV) (Fisher et al., 1999). Although field resistance of *S. exigua* to these pathogens has not been described yet, development of strategies for a long term sustainability of these pathogens as well as the finding of novel pathogens active against this pest is desirable.

In a recent study on the transcriptome of *S. exigua*, several Expressed Sequence Tags (ESTs) were obtained with high homology to the *Infectious flacherie virus* (IFV) (GenBank acc.: AB000906), which infects *Bombyx mori* larvae (Isawa et al., 1998). IFV belongs to the recently assigned viral family *Iflaviridae* (order *Picornaviridales*) (van Oers, 2008). Iflaviruses are insect-infecting viruses that form non-enveloped, icosahedral particles. These particles carry a single-stranded RNA genome of positive polarity that encodes one large polyprotein, which is post-translationally processed into viral proteins essential for its replication and transmission (van Oers, 2010). Among the genomic features, the 5′RNA terminus is attached to a small viral protein (VPg), followed by the 5′UTR. The 5′UTR includes an internal ribosome entry site (IRES) structure needed for the cap-independent translation (Borman et al., 1997; Lu et al., 2006). The 3′UTR is followed by a poly(A) tail encoded by the viral genome (van Oers, 2010).

Iflaviruses infect exclusively arthropods and, to date, around 15 species belonging to this family have been described (van Oers, 2010). So far, species of this family have only been found infecting insects from the orders Lepidoptera, Hymenoptera, Heteroptera, Diptera, and Orthoptera, although their presence in other insect orders cannot be discarded. Some of these iflaviruses have been identified as responsible for great economic losses in the sector of silk production (Aizawa and Kuruta, 1964) and apiculture (Bradbear, 1988; Ribiere et al., 2010). Despite these examples of virulence, many of the iflaviruses produce covert or asymptomatic infections

*Abbreviations:* IFV, *infectious* flacherie virus; IRES, internal ribosome entry site; EST, Expressed Sequence Tags; UTR, untranslated region; RACE, rapid amplification of cDNA ends; qPCR, real time quantitative RT-PCR; RdRp, RNA-dependent RNA polymerase.

* Corresponding author. Fax: +34 96 354 30 29.
*E-mail address:* sherrero@uv.es (S. Herrero).

0022-2011/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2011.10.009

that only under certain conditions, and for reasons that are not yet understood, can produce fatal infections (de Miranda and Genersch, 2010).

This work investigated if ESTs in the *S. exigua* transcriptome with homology to iflaviruses were revealing the presence of a novel Iflavirus and if so, to determine its complete genomic sequence and infectivity against *S. exigua* larvae.

## 2. Materials and methods

### 2.1. Insects

Three different laboratory colonies of *S. exigua* originally derived from different geographic locations worldwide were employed. The ALM colony was established from successive collections from southern Spain (Hernandez-Martinez et al., 2010a) and had been reared in the laboratory for more than 5 years. The FRA colony was kindly supplied by López-Ferber, INRA (St Christol les Alés, France) and had been reared in the laboratory for more than 10 years (Hernandez-Martinez et al., 2008). The Xen-R colony was derived from insects collected from cotton fields in Prattville, AL. (USA) and was later selected for resistance to *B. thuringiensis* (Hernandez-Martinez et al., 2010b). All three colonies were reared simultaneously in the same rearing chamber on artificial diet and at 25 ± 3 °C with 70 ± 5% RH and a photoperiod of 16/8 h (light/dark).

### 2.2. RNA isolation and genome sequencing

Total RNA was obtained from whole larvae as well as from different larval tissues. In all cases, RNA was isolated using RNAzol (MRC Inc., Cincinnati, OH) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using oligo-dT primers and SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA).

After alignment of the *S. exigua* ESTs with the IFV genome sequence, PCR primers were designed in order to complete the hypothetical gaps existing between ESTs (Table S1). Fragments were amplified from the cDNA pool used to obtain the primary EST sequences and derived from the three different *S. exigua* colonies using High Fidelity Polymerase (PrimeStar Polymerase, Takara, Shiga, Japan). Amplified fragments were cloned into pGemT-easy (Promega, Madison, WI) and sequenced. At least two clones were sequenced for each PCR fragment. In case of nucleotide discrepancy additional clones were sequenced for that region and a consensus sequence was assembled based on the more abundant nucleotide.

Specific primers (Table S1), distributed along the whole genome were designed in order to generate overlapping PCR fragments of the entire genome. These primers were used in order to obtain the full genome sequences of the viruses present in each of the three colonies employed in this study. Viruses derived from each *S. exigua* colony were individually re-sequenced. At least two clones from independent PCR reactions were sequenced for each genome region and virus. In case of nucleotide variation more clones were sequenced for the variable fragment and a consensus sequence assembled based on the highest nucleotide abundance. Viral genome sequences obtained in each colony were assembled using the Seqman program from the DNAstar software package (DNASTAR, Madison, WI).

For each virus the 5′ and 3′ ends were assessed by rapid amplification of cDNA ends (RACEs) methodology. RACE-cDNAs were synthesized using a SMART-RACE kit (Clontech, Saint-Germain-en-Laye, France), and 5′ and 3′ fragments were amplified using two sets of specific primers (Table S1). The amplified fragments were purified and cloned into the pGemT-easy vector for subsequent sequencing. At least three clones were sequenced for each PCR amplicon and each cDNA end.

### 2.3. Sequence comparison and phylogenetic analysis

Predicted amino acid sequences were aligned using ClustalX (Thompson et al., 1997) and visualized in GeneDoc (Nicholas et al., 1997). The phylogenetic analysis was conducted on amino acid sequences comprising the RNA-dependent RNA polymerase (RdRp) domain. Homolog proteins used in the analysis were obtained after blast-search against the NCBI database (proteins and ESTs) using the SeIV-1 RdRp domain as query. Only those hits with homology score less than 1e-20 were employed. Redundant sequences (identity higher than 95%) were omitted in our analysis for the sake of clarity. The RdRp domain from the *Foot-and-mouth disease virus* was employed as an out-group. *Spodoptera littoralis* EST derived from EST database (GenBank acc.: FQ021401) has been included due to its high similarity to presently described iflavirus. Phylogenetic analyses were performed using the neighbor-joining method with 100 bootstraps, using the ClustalX (Thompson et al., 1997) and MEGA 3.1 (Kumar et al., 2004) programs. Distances were corrected for multiple substitutions according to the method of Kimura (Kimura, 1980).

### 2.4. Tissue distribution and viral infectivity

The presence and abundance of viral sequences in various larval tissues was estimated by real-time quantitative RT-PCR (qPCR). For that purpose, last instar larvae were dissected and the midgut, fat body and hemocytes were collected. For each sample, a pool of at least five larvae was used and experiments were performed in duplicate. Total RNA was isolated and cDNA synthesized as described above. qPCR was carried out in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). All reactions were performed using Power SYBR green PCR master mix (Applied Biosystems) in a total reaction volume of 25 μl. Five-microliter cDNA templates (1:10 diluted) were added to each reaction mix. Forward and reverse primers, designed using Primer Express software (Applied Biosystems, Foster City, CA), were added to a final concentration of 0.3 nM. Reactions were performed in triplicate. Ct values for the SeIV-1 were normalized across the samples using the *ATP synthase* gene as internal control (Herrero et al., 2007). *ATP synthase* gene was previously tested to have similar expression levels in the different tissues.

SeIV-1 infectivity was determined in virus-free insects from the Xen-R colony. For that purpose, we used a duplicate of the Xen-R colony that had been reared by Prof. Shelton at Cornell University (New York, USA). Eggs from the Cornell Xen-R colony were obtained and larvae that emerged from those eggs were considered as the initial generation (F0). These insects were reared during several generations on the same chamber and conditions that the infected colonies. For each generation, second instar larvae (five larvae per sample) were collected and stored for further analysis. Presence of SeIV-1 in the different generations was determined by RT-PCR using specific primers (Table S1). As an internal control, RT-PCR was also performed using primers for the *ATP synthase* gene.

## 3. Results

### 3.1. Genome sequence of SeIV-1

Using the sequence information from the ESTs with homology to IFV, different primers were designed to amplify by PCR the genomic regions that remained unsequenced. Additionally, RACE-PCR