Virus Research 253 (2018) 62–67

Contents lists available at ScienceDirect

Virus Research

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

Genomic analysis of a cypovirus isolated from the eucalyptus brown looper, *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae)

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ARTICLE INFO

Keywords:
Forest protection
Biological control
Eucalyptus
Thyrinteina arnobia
Cypovirus

ABSTRACT

The eucalyptus brown looper, *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae), is the main lepidopteran defoliator of eucalyptus plantations in Brazil. Outbreaks of this insect pest are common in Brazil and can affect the productivity of planted forests severely. *T. arnobia* caterpillars from a laboratory colony with viral infection symptoms were analyzed by electron microscopy that revealed polyhedral occlusion bodies (OBs) with several icosahedral virus particles embedded. Analysis of its genetic material showed ten segments of dsRNA, which confirmed this virus as a possible member of the genus *Cypovirus*. Phylogenetic analysis of the whole genome sequence revealed its close relationship with other isolates of *Cypovirus 14* species and according to these results we proposed the name Thyrinteina arnobia cypovirus 14 (TharCPV-14) for this new virus isolate. Further research will be necessary in order to analyze the potential of this virus as a biopesticide.

1. Introduction

Wood production in countries located in tropical and subtropical regions is based on forest plantations. Brazil has a prominent position as a global leader in forest productivity. According to the Brazilian Industry of Trees (Portuguese acronym IBÁ, 'Indústria Brasileira de Árvores') in 2016, the export revenue from the Brazilian planted tree industry reached US$ 8.9 billion; mainly due to *Eucalyptus*, the most planted hardwood species worldwide (Rockwood et al., 2008). The eucalyptus plantations occupy approximately 73% of 7.84 million hectares of planted forests in Brazil. The growing areas of non-native forests cultivated as monocultures have created conditions to the establishment and reproduction of many species of insect-pests at high population densities, which can severely reduce tree development and economic yields (Hurley et al., 2016).

One of the most important insect-pests of eucalyptus forests in Brazil is the larval stage of *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae), the eucalyptus brown looper (Oliveira et al., 2005; Ribeiro et al., 2016). The control of this insect is carried out mainly by biopesticides and releases of natural enemies, such as parasitoids and predatory bugs (Barbosa et al., 2016; Zanuncio et al., 2014). However, severe outbreaks in Brazil have led to the prospection of alternative methods for its effective control (Jesus et al., 2015), including new biological agents.

Cypoviruses, also known as cytoplasmic polyhedrosis viruses (CPVs), are insect viruses with a genome segmented consisting of 10 to 12 dsRNA molecules in a non-enveloped and icosahedral capsid that belong to the family *Reoviridae* (Attoui et al., 2012). Remarkably, during virus infection CPVs produce in the cell cytoplasm occlusion bodies (OBs) that protect virions from environmental adversities. The main infection route is by ingestion of OBs present on contaminated food followed by the dissolution in the alkaline pH of the caterpillar

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https://doi.org/10.1016/j.virusres.2018.05.026
Received 22 March 2018; Received in revised form 24 May 2018; Accepted 30 May 2018
Available online 06 June 2018

midgut. The dissolution releases virions from OBs to infect the midgut epithelium cells (Attoui et al., 2012). In lethal infections, disease symptoms appear two or three days after the initial ingestion by the larva and include reduction of insect feeding and movement, alterations of tegument coloration, pasty excrements, and climbing up behavior to the plant top before death. After death, the OBs are released in the environment, contaminating the plant leaves, and favoring the horizontal transmission among susceptible hosts (Belloncik, 1989). Cypoviruses are considered less pathogenic than baculoviruses (another OB-producing insect virus with large double-stranded DNA genome) and have been shown to induce sublethal effects on some infected hosts such as lower weight of the infected inset, shorter adult lifespan, and development (for a review see Rothman and Myers, 1996). In this work, we characterized the first virus isolated from *T. arnobia,* which was shown to be an isolate belonging to the species *Cypovirus 14* and we tentatively named Thyrinteina arnobia cypovirus 14 (TharCPV-14). Moreover, we performed ultrastructural characterization, sequencing, phylogenetic analysis, and genome diversity description.

2. Materials and methods

2.1. Samples origin

The samples of *T. arnobia* dead caterpillars with symptoms of virus infection were collected from an insect colony at the Laboratory of Plant Resistance to Insects at São Paulo State University (USP) (Jaboticabal, Brazil). This population was obtained from a commercial eucalyptus plantation, being maintained healthy for several generations through insect feeding with leaves of *E. camaldulensis* under controlled conditions of temperature (25 °C ± 2), humidity (70%) and photoperiod (12 h). Caterpillars with symptoms of virus infection were collected before death and small drops of its regurgitation liquid were analyzed under a light microscope. One leaf of *E. camaldulensis* treated with these drops was offered to 5 apparently healthy 3rd instar *T. arnobia* caterpillars and mortality observed every 24 h.

2.2. Purification and ultrastructural analysis of OBs

Ten dead caterpillars of *T. arnobia* between the 5th to 6th instar were macerated in phosphate buffered saline (PBS: 137.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na2HPO4, 2.0 mM KH2PO4, pH 7.4) and the homogenate was filtered once through cheesecloth and centrifuged once at 12,000 × *g* for 10 min at 4 °C. The pellet was washed with PBS and centrifuged three times at the same conditions. The pellet was resuspended with distilled RNase-free water and placed onto a linear sucrose gradient (20, 40, 50, 60 and 80%) with distilled RNase-free water, and centrifuged at 140,000 × *g* for 75 min at 4 °C. A main white band containing OBs was collected, diluted 10 times in PBS and centrifuged again at 12,000 × *g* for 10 min at 4 °C. The pellet was collected, resuspended in 1 mL of distilled RNase-free water, counted in a hemocytometer, as previously described (O'Reilly et al., 1992) and maintained at −20 °C. For scanning electron microscopy (SEM), 100 µl (10%) of the OBs suspensions were incubated with 300 µl of acetone at 25 °C for 1 h. The suspensions were loaded onto two metallic stubs, dried overnight at 37 °C, coated with gold in a Sputter Coater (Balzers) for 3 min, and observed in a Scanning Electron Microscope Jeol JSM 840 A at 10 kV. For transmission electron microscopy (TEM), pellets of purified OBs were fixed in Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1 M, cacodylate buffer, pH 7.2) for 2 h, post-fixed in 1% osmium tetroxide in the same buffer for 1 h and then stained *en bloc* with 0.5% aqueous uranyl acetate, dehydrated in acetone, and embedded in Spurr's low viscosity embedding medium. The ultrathin sections (60–80 nm) were obtained with a Leica ultramicrotome equipped with a diamond knife, contrasted with 2% uranyl acetate and observed in a ZEISS TEM 109 at 80 kV.

2.3. SDS-PAGE analysis

10 µl of purified OBs (10^6) were mixed with the same volume of loading buffer (0.25 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue), heated for 5 min at 100 °C and subjected to electrophoresis in 12% SDS-PAGE gels using the Mini Protean Tetra Cell apparatus (BioRad) following the manufacturer's instructions. The gel was then photographed using an ImageQuant™ LAS 4000 (GE).

2.4. RNA isolation, genome sequencing, and analysis

RNA extraction was carried out using 200 µl (10^8/mL) of the purified OBs suspension using the TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. The RNA quality was checked by electrophoresis in an agarose gel (1%), stained with 0.01% ethidium bromide, and visualized under UV light and photographed in an AlphaImager® Mini (Alpha Innotech). The viral RNA genome segments were used to build cDNA libraries and sequenced using 2 × 100 bp read length on the HiSeq™ 2000 platform (Illumina, San Diego, CA, USA) at Macrogen Inc (Seoul, Republic of Korea). The resulting reads were trimmed and the genome *de novo* assembly was carried out using CLC Genome Workbench 6.5.2 (CLC bio, Denmark). The read mapping and genome annotation was performed using Geneious 7.1.8 (Kearse et al., 2012) and selecting the following parameters for Open Reading Frames (ORFs): AUG start codons (methionine), minimal overlapping of adjacent ORFs and at least 150 bp long. ORFs were annotated using BLASTx search against the NCBI non-redundant protein database. The

Table 1
Properties of the dsRNA segments of TharCPV-14. Segments with the respective GenBank accession numbers, segment sizes in nucleotides, including ORFs, protein's sizes in amino acids, putative function, nucleotide identity with other cypovirus 14 species.

Segment number	Accession number	Segment size (nt)	ORF posistion (nt)	Protein size (aa)	Putative Function	Nucleotide identity (%)		
						HearCPV/ TharCPV	LydiCPV/ TharCPV	HearCPV/ LydiCPV
S1	MF161423	4466	446–4363	1305	RdRp	84,5	83,6	97,5
S2	MF161424	4075	59–3970	1303	major capsid protein	84,3	84,3	97,7
S3	MF161425	3953	34–3834	1266	minor capsid protein	81,8	81,7	98,6
S4	MF161426	3358	46–3270	1074	mRNA 5′ capping synthesis	83,2	83,1	98,4
S5	MF161432	3119	58–3009	982	Unknown	65,2	65,0	97,9
S6	MF161427	1772	19–1671	550	RNA guanylyltransferase	84,0	83,9	99,3
S7	MF161428	1412	8–1279	423	NTPase activity	80,5	79,8	94,1
S8	MF161429	1332	43–1182	379	Unknown	81,3	81,2	99,0
S9	MF161430	1269	71–1024	317	Unknown	79,9	81,0	95,8
S10	MF161431	978	55–804	249	Occlusion body protein (POLH)	88,0	87,9	98,5