Complete genome sequence of a novel dsRNA mycovirus isolated from the phytopathogenic fungus *Fusarium oxysporum* f. sp. *dianthi*.

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Abstract

A novel double-stranded RNA (dsRNA) mycovirus, designated *Fusarium oxysporum f. sp. dianthi mycovirus 1* (FodV1), was isolated from a strain of the phytopathogenic fungus *F. oxysporum* f. sp. *dianthi*. The FodV1 genome has four dsRNA segments designated, from the largest to the smallest one, dsRNA 1, 2 3, and 4. Each one of these segments contained a single open reading frame (ORF). DsRNA 1 (3555 bp) and dsRNA 3 (2794 bp) encoded a putative RNA-dependent RNA polymerase (RdRp) and a putative coat protein (CP), respectively. Whereas dsRNA 2 (2809 bp) and dsRNA 4 (2646 bp) ORFs encoded hypothetical proteins (named P2 and P4, respectively) with unknown functions. Analysis of its genomic structure, homology searches of the deducted amino acid sequences, and phylogenetic analysis all indicated that FodV1 is a new member of the *Chrysoviridae* family. This is the first report of the complete genomic characterization of a mycovirus identified in the plant pathogenic species *Fusarium oxysporum*.

Introduction

Fungal viruses (mycoviruses) are widespread throughout the major taxonomic groups of fungi, including plant pathogenic species. Mycoviruses with RNA genomes are classified in 13 families, of which 6 accommodate mycoviruses with single stranded RNA genomes, and the remaining 7 include those with double stranded RNA (dsRNA) genomes. Mycoviruses with dsRNA genomes are classified into seven major families depending on the number and size of dsRNA segments: *Totiviridae* (monopartite, 4.6-7 kbp)*, Endornaviridae* (monopartite, 14-17 kpb), *Partitiviridae* (2 segments, 1.4-2.3 kbp)*, Megabirnaviridae* (2 segments, 7-9 kbp), *Chrysoviridae* (4 segments, 2.4-3.6 kbp), *Quadriviridae* (4 segments, 3.7-4.9 kbp), and *Reoviridae* (11/12 segments, 0.7-5 kbp) [7, 8]. In recent years, the potential use of mycoviruses as biocontrol agents for their pathogenic host has increased the research interest in this topic [13, 14].

Fusarium oxysporum is the causal agent of vascular wilt in many different plant species, including crops of agronomic interest [1, 5]. Although a number of mycoviruses belonging to different taxonomic families have been identified in different species of the genus *Fusarium* (e. g. *F. poae*, *F. graminearum*, *F.solani*) [2], to date only one mycovirus (*Fusarium oxysporum chrysovirus 1*, FoCV1) has been identified in the species *Fusarium oxysporum*. FoCV1 was found in a strain of the forma specialis *melonis* and has been assigned to the family *Chrysoviridae*, genus *Chrysovirus* , but its complete genomic sequence has not been elucidated [16]. *Fusarium oxysporum* f. sp. *dianthi* (*Fod*) is the forma specialis that infects carnation (*Dianthus caryophillus*), causing the most devastating carnation disease worldwide [6]. While analyzing a wide *Fod* collection to determine genetic/pathogenic diversity [10], we detected the presence of dsRNA in one of the isolates (isolate *Fod* 116).

In this study, the four dsRNA segments purified from isolate *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi* were fully sequenced. Homology searches and phylogenetic analysis of the deducted amino acid (aa) sequences showed that these dsRNAs constitute the genome of a new *Chrysoviridae* family member, that has been designated *Fusarium oxysporum f. sp. dianthi mycovirus 1* (FodV1).

Provenance of the virus material

The identified mycovirus was found in strain *Fod* 116 of *Fusarium oxysporum* f. sp. *dianthi*, which was isolated in 2008 from a diseased carnation plant in the Cádiz province of Spain [10]. The dsRNA was purified by column chromatography using cellulose (Sigma-Aldrich) [21] from mycelium of the isolate grown for 7 days in potato dextrose broth (PDB). The purified dsRNA was analyzed by electrophoresis on 0.8% agarose gels (Fig. 1a). The results indicated that isolate *Fod* 116 contained 4 dsRNA extrachromosomal elements that were resistant to digestion with DNase I, RNAse H, Exonuclease III and Exonuclease λ, but susceptible of degradation when digested with RNase A (data not shown). To determine the complete sequence of the dsRNAs, a combination of techniques was applied. Single Primer Amplification Technique (SPAT) [3] and production and analysis of cDNA libraries [20] generated partial sequence data for each segment. Analysis of these partial sequences with the BLASTX algorithm in the NCBI database (http://blast.ncbi.nlm.nih.gov/) allowed arranging them in segments corresponding to segments in other mycovirus with high sequence similarities. Specific primers were then designed to fill in the gaps. See Supplementary Fig. 1 for a schematic representation of the strategy used and Supplementary Table 1 for primer sequences. Full sequences were assembled using the software Lasergene SeqMan™ Version 7.0.0 (DNASTAR®Inc., Madison, USA). For comparison of the conserved motifs and nucleotide sequences of 5'-UTRs and 3'-UTRs the software CLUSTALX Version 2.1 (Conway Institute UCD Dublin, Ireland) was used. Phylogenetic analyses were achieved using the MEGA 6.06 program [17]; evolutionary history was inferred using the Neighbor-Joining (NJ) method [15], and the evolutionary distances were computed using the JTT matrix-based method [12].

Sequence properties

A schematic representation of the genomic organization of FodV1 is shown in Fig 1b. We determined that dsRNA 1 is 3555-bp long (accession number: KP876629) and codes for a putative RNA dependent RNA polymerase (RdRp) of 1139aa; dsRNA 2 is 2809-bp long (accession number: KP876630), and codes for a putative protein (P2) of 878aa; dsRNA 3 segment is 2794-bp long (accession number: KP876631), and codes for a putative capsid protein (CP) of 852aa; and dsRNA 4 is 2646-bp long (accession number: KP876632) and encodes a putative protein (P4) of 830aa.

The deduced aa sequence of the protein potentially encoded by dsRNA 1 (theoretical molecular mass = 128.76 KDa) contained the conserved motifs characteristic of the RNA-dependent RNA polymerases (RdRps) of mycoviruses (Fig 1c). A homology search with BLASTP indicated that this protein was closely related to the RdRps of viruses in the family *Chrysoviridae* [9]. Specifically, the most closely related RdRps were those of Fusarium graminearum dsRNA mycovirus-2 (FgV2) [23] and Fusarium graminearum mycovirus-China 9 (FgV-ch9) [4], showing 63% and 62% aa sequence identity, respectively. In contrast, the sequence identity with the RdRp of the other chrysovirus reported in the same fungal species, Fusarium oxysporum chrysovirus 1 (FoCV1) [16], was only 26%. The deduced aa sequence of hypothetical protein (named P2) encoded by dsRNA 2 ORF (theoretical molecular mass = 95.2 kDa) showed similarity to hypothetical proteins found only in known and tentative members of the family *Chrysoviridae*. These included the deduced aa sequences of segment 2 of FgV-ch9 (48% aa sequence identity), segment 2 of FgV2 (49% aa sequence identity), segment 4 of Botryosphaeria dothidea chrysovirus 1 (BdCV1) (32% aa sequence identity) [22], segment 3 of Magnaporthe oryzae chrysovirus 1-A (MoCV-1A) (30% aa sequence identity) [18], and segment 3 of Magnaporthe oryzae chrysovirus 1-B (MoCV-1B) (29% aa sequence identity) [19]. All these hypothetical proteins have unknown functions. The BLASTP search of the putative protein encoded by dsRNA 3 ORF (theoretical molecular mass = 92.8 kDa) indicated that it probably corresponded to a coat protein. This BLASTP analysis identified significant similarity with putative coat proteins (CPs) from other tentative members of the family *Chrysoviridae*; the putative CPs encoded by segments 3 of FgV2 (52% aa sequence identity) and FgV-ch9 (51% aa sequence identity), by segment 2 of BdCV1 (25% aa sequence identity), by segments 4 of MoCV-1A and MoCV-1B (24% aa sequence identity), and the protein encoded by gene pL3 of Agaricus bisporus virus 1 (AbV1) (22% aa sequence identity) [11]. A BLASTP search of the putative protein encoded by dsRNA 4 ORF (named P4; theoretical molecular mass = 92 kDa) showed similarity only with two other proteins from mycoviruses, those encoded by segments 4 of FgV2 (42% aa sequence identity) and FgV-ch9 (40% aa sequence identity). The function of these hypothetical proteins is unknown.

All four dsRNA segments that comprised the FodV1 genome contained 5' and 3' untranslated regions (UTRs). The 5'-UTRs were 82 and 84 nt in length for dsRNAs 1 and 2, respectively, and 97 nt long for dsRNAs 3 and 4. The sequences of the four 5'-UTRs were ~62% identical and contained a 5'-terminal identical stretch of 14 nt (Fig. 1d). The 3'-UTRs were 53, 88, 138 and 56 nt long for dsRNAs 1, 2, 3 and 4, respectively. These 3'-UTR sequences were, apparently, slightly conserved (~15% identical), and contained a 3'-terminal identical stretch of 9 nt (Fig 1d).

To provide further evidence of the relationship of FodV1 with previously reported mycoviruses, we performed phylogenetic analyses using RdRp and CP aa sequences. The RdRp aa sequence of FodV1, along with other 40 RdRp aa sequences of mycoviruses of *Partitiviridae*, *Totiviridae* and *Chrysoviridae* families, were used to build a phylogenetic tree (Fig 2a). The generated tree distributed the previously described mycoviruses in 3 groups, which corresponded to each of the families analyzed. The RdRp of FodV1 was placed in the *Chrysoviridae* family branch, very close to the RdRps proteins of FgV-ch9 and FgV2. These *F. graminearum* mycoviruses have been recently designated as "chryso-like" viruses, which group in a divergent clade corresponding to tentative members of the *Chrysoviridae* family not

yet recognized as a distinct taxa by the ICTV [8] . Furthermore, the results assigned the RdRp of FoCV1 (from *F. oxysporum* f. sp. *melonis*) to a different clade (clade II) inside the *Chrysoviridae* family, providing further evidence that these two *Chrysoviridae* members identified in the same fungal species are not closely related. The CP aa sequence of FodV1, and other 14 CP aa sequences of chrysovirus, were used to build a second phylogenetic tree (Fig. 2b). The generated tree displayed a distribution of the mycoviruses into two different clades (I and II). The CP aa sequence of FodV1 grouped in the same clade with other "chryso-like" viruses (clade I), again presenting the highest similarity with FgV-ch9 and FgV2 CP aa sequences. As previously observed in the RdRp phylogenetic tree, the CP phylogenetic analysis distributed FodV1 (from *Fod*) and FoCV1 (from *Fom*) in different clades inside the *Chrysoviridae* family.
In summary, the banding profile, the genomic sequence and organization, and the high bootstrap values observed in the phylogenetic analyses, support the classification of the mycovirus isolated from *Fusarium oxysporum* f. sp. *dianthi* isolate *Fod* 116 as a novel unreported member of the *Chrysoviridae* family, closely related to other "chryso-like" viruses previously reported. As far as we know, this is the first report of the complete genomic characterization of a mycovirus identified in the plant pathogenic species *Fusarium oxysporum*.

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Figure Captions

Fig. 1 Characterization of Fusarium oxysporum f. sp. dianthi mycovirus 1 (FodV1). (a) dsRNA banding pattern of FodV1 analyzed by 0.8% agarose gel electrophoresis. Lane 1 corresponds to the molecular weight marker λ HindIII; lane 2 corresponds to a dsRNA purification of *Fusarium oxysporum* f. sp. *dianthi* isolate *Fod* 116. (b) Schematic representation of FodV1 genome. Rectangles in each segment represent the open reading frame (ORF); the amino acids length is shown on the right (inside the boxes), and the 5' and 3'-UTRs nucleotide length is indicated as well (arrows). (c) Alignment of amino acid sequences of the RdRp motifs of FodV1 and other mycoviral RdRps. Conserved amino acids, motifs 1-8, are shown by horizontal gray bars below the amino acid areas. Black shades indicate identical or similar amino acid residues in all RdRp sequences included; dark grey shades, indicates a conservation of residues below a 100% and above an 80%; and light grey, below an 80% and above a 60%. Numbers in brackets correspond to the number of amino acid residues separating the motifs. The abbreviations of the virus names are as follows: FodV1, Fusarium oxysporum f. sp. dianthi mycovirus 1; FgV-ch9, Fusarium graminearum mycovirus-China 9; FgV2, Fusarium graminearum dsRNA mycovirus-2; BdCV1, Botryosphaeria dothidea chrysovirus 1; MoCV1-A, Magnaporte oryzae chrysovirus 1-A; MoCV1-B, Magnaporte oryzae chrysovirus 1-B; TocV-2, Tolipocladium cylindrosporum virus 2; AsV1816, Aspergillus mycovirus 1816; ACD-CV, Amasya cherry disease associated chrysovirus; CCRS-CV, Cherry chlorotic rusty spot associated chrysovirus; FoCV1, Fusarium oxysporum chrysovirus 1; CnV-1, Chryphonectria nitschkei chrysovirus 1; PcV, Penicillium chrysogenum virus. (d) Nucleotide sequence alignment of the 5' and 3'-UTR of the four genomic segments of Fusarium oxysporum f. sp. dianthi mycovirus 1 (FodV1).

Fig. 2 Phylogenetic analyses of FodV1 and selected members of the families *Partitiviridae*, *Totiviridae* and *Chrysoviridae.* (a) Phylogenetic tree based on the RdRp amino acid sequences. (b) Phylogenetic tree based on the CP amino acid sequences. Multiple sequence alignments of the deduced amino acid sequences were performed using CLUSTALW with the "Gap Opening Penalty" in 3 and the "Gap Extension Penalty" in 1.8 of the Multiple Alignment parameters. Phylogenetic trees were constructed using the program MEGA 6.0, and generated by the NJ method with 1000 bootstrap replicates. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. Only bootstrap percentages over 50 are shown. Sequences of the RdRp and CP genes were obtained from the GenBank database for the following viruses (abbreviations and accession numbers are in parenthesis): Fusarium oxysporum f. sp. dianthi mycovirus 1 (FodV1; KP876629, KP876631), Agaricus bisporus virus 1 (AbV-1; CAA64144.1, BAA01612.1), Amasya cherry disease associated chrysovirus (ACD-CV; YP_001531163.1, YP_001531162.1), Aspergillus fumigatus chrysovirus (AfuCV; CAX48749.1, CAX48751.2), Aspergillus mycovirus 1816 (AsV1816; ABX79996.1, N/A), Aspergillus ochraceus virus 1 (AoV1; ABV30675.1, ABV30676.1), Atkinsonella hypoxylon virus (AhV; NP_604475.1, NP_604476.1), Botryosphaeria dothidea chrysovirus 1 (BdCV1; AGZ84312.1, AGZ84313.1), Botryotinia fuckeliana totivirus 1 (BfTV1; YP_001109580.1, YP_001109579.1), Ceratocystis resinifera virus 1 (CrV1; YP_001936016.1, YP_001936015.1), Cherry chlorotic rusty spot associated chrysovirus (CCRS-CV; CAH03664.1, CAH03665.1), Coniothyrium minitans mycovirus (CmV; YP_392467.1, YP_392466.1), Cryphonectria nitschkei chrysovirus 1 (CnV-1; ACT79255.1, ACT79251.1), Discula destructiva virus 1 (DdV-1; NP_116716.1, NP_116742.1), Discula destructiva virus 2 (DdV-2; NP_620301.1, NP_620302.1), Epichloe festucae virus 1 (EfV1; CAK02788.1, CAK02787.1), Fusarium graminearum dsRNA mycovirus-2 (FgV2; ADW08802.1, ADW08804.1), Fusarium graminearum mycovirus-China 9 (Fgv-ch9; ADU54123.1, ADU54125.1), Fusarium oxysporum chrysovirus 1 (FoCV1; ABQ53134.1, ABQ58816.1), Fusarium poae virus 1 (FUPO-1; NP_624349.1, NP_624348.2), Fusarium solani virus 1 (FsV-1; NP_624350.1, NP_624351.1), Gremmeniella abietina RNA virus L1 (GaRV-L1; AAK11656.1, NP_624332.2), Gremmeniella abietina RNA virus MS1 (GaRV-MS1; NP_659027.1, NP_659028.1), Helminthosporium victoriae 145S (Hv145SV; YP_052858.1, YP_052859.1), Helminthosporium victoriae 190SV (Hv190SV; NP_619670.2, NP_619669.2), Heterobasidion annosum partitivirus (HaV; AAL79540.1, N/A), Magnaporthe oryzae chrysovirus 1-A (MoCV1-A; YP_003858286.1, YP_003858288.1), Magnaporthe oryzae chrysovirus 1-B (MoCV1-B; YP_008914864.1, YP_008914862.1), Magnaporthe oryzae virus1 (MoV1; YP_122352.1, YP_122351.1), Magnaporthe oryzae virus2 (MoV2; YP_001649206.1,YP_001649205.1), Ophiostoma himal-ulmi partitivirus 1 (OPV1; CAJ31886.1, CAJ31887.1), Penicillium chrysogenum virus (PcV; YP_392482.1, YP_392483.1), Penicillium stoloniferum virus S (PsV-S; YP_052856.2, YP_052857.1), Rhizoctonia solani virus 717 (RsV-717; NP_620659.1, NP_620660.1), Saccharomyces cerevisiae virus L-A (L1) (Sc V-L-A; NP_620495.1, NP_620494.1), Saccharomyces cerevisiae virus L-BC (La) (ScV-L-BC; NP_042581.1, NP_042580.1), Sphaeropsis sapinea RNA virus 1 (SsRV1; NP_047558.1, NP_047557.1), Sphaeropsis sapinea RNA virus 2 (SsRV2; NP_047560.1, NP_047559.1), Tolipocladium cylindrosporum virus 2 (TocV-2; CBY84993.1, N/A), Ustilago maydis virus H1 (UmV-HI; NP_620728.1, NP_620728.1). *N/A = not available in the NCBI database.