Mycoviruses infecting the endophytic and entomopathogenic fungus *Tolypocladium cylindrosporum*

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Abstract

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A mixed virus infection in a strain of the endophytic and entomopathogenic fungus *Tolypocladium cylindrosporum* was deduced from a study of the transmission to conidia of several double-stranded RNA (dsRNA) elements. The transmission rates of each dsRNA were different, and monosporic isolates harbouring different combinations of the original set of six dsRNAs were obtained. A 5196 bp dsRNA element was sequenced and represents the genome of *Tolypocladium cylindrosporum* virus 1 (TcV1), a new member of the genus *Victorivirus* in the *Totiviridae* family. This virus was transmitted to 81.4% of the conidia; in contrast, four dsRNAs of 3.1-3.7 kbp were transmitted only to 4.7% of the monosporic isolates obtained from the infected parental strain. These four dsRNAs did not show segregation during transmission, one of them was sequenced and encoded an RdRp, suggesting that the four molecules might represent the whole genome of a multipartite chrysovirus. A third possible virus with a genome of approximately 4.2 kbp was transmitted to 79.1% of the monosporic isolates produced by the infected strain. Ribavirin was used to cure *T. cylindrosporum* from viruses, and TcV1 was sensitive to this drug. All monosporic cultures derived from the infected strain treated with 80 and 100 µM concentrations of the drug were free of TcV1.

Keywords: mycovirus, dsRNA, ribavirin, transmission, victorivirus

The ascomycete *Tolypocladium cylindrosporum* (Fam. *Ophiocordycipitaceae*) was first reported as a soil-borne species, and later as a pathogen of several species of insects, including mosquito genera like *Anopheles* and *Aedes* (Gams, 1971; Lam et al., 1988). The fungus is also pathogenic to crustaceans and arachnids like the ticks *Ornithodoros erraticus* and *O. moubata* (Herrero et al., 2011). In addition, this fungus has been isolated as an endophyte from leaves of some grasses (Sánchez Márquez et al., 2010). Other entomopathogenic fungi like *Beauveria bassiana*, *Lecanicillium lecanii*, or *Metarhizium anisopliae* have also been reported as endophytes

and soil inhabitants, and have been tested as biological control agents for invertebrate plant pests (Vega et al., 2008).

The presence of dsRNA molecules of viral origin has been reported in endophytic strains of *T. cylindrosporum*, and other species of entomopathogenic fungi such as *B. bassiana*, *M. anisopliae* and *Paecilomyces* spp. (Herrero et al., 2009; Inglis and Valadares-Inglis, 1997; Melzer and Bidochka, 1998). However, in none of these cases were these viruses classified.

Mycoviral infections can be very persistent and difficult to eliminate from their hosts (Martins et al., 1999; Romo et al., 2007). Different attempts to cure fungi from viruses have been reported, e.g., cycloheximide treatments, single conidium subculture, hyphal tip transfer, incubation at low or high temperatures (Carroll and Wickner, 1995; Romo et al., 2007; Souza-Azevedo et al., 2000), but they were not always successful. These difficulties to cure fungi from viral infections, plus the lack of simple methods for the artificial inoculation of mycoviruses, have greatly hampered progress in exploring mycovirus-host interactions (Ghabrial and Suzuki, 2009).

The purpose of the present work was to identify some of the mycoviruses associated to *T. cylindrosporum*, to study their transmission to asexual spores, and to test if they can be cured using the antiviral ribavirin, in order to obtain virus free isolates that could be used in further studies concerning to the effects of mycoviruses in this interesting fungal species.

Strain 11 of *T. cylindrosporum*, isolated as an endophyte from asymptomatic leaves of the grass *Holcus lanatus* in natural grasslands of Salamanca (Spain) (Sánchez Márquez et al., 2010), harbours six dsRNA elements of approximately 5.1, 4.2, 3.7, 3.4, 3.2, and 3.1 kbp, and it is pathogenic to the ticks *Ornithodoros erraticus* and *O. moubata* (Herrero et al., 2009). These dsRNA elements will be referred according to their size, dsRNA1 being the largest, and dsRNA6 the smallest (Fig. 1). To determine if all these dsRNA elements were transmitted to conidia, monosporic isolates obtained from strain 11 were analyzed. Strain 11 was grown in potato dextrose broth (PDB) under shaking (110 rpm) for 12 days at 24 °C. After this period the culture was filtered through sterile gauze and centrifuged for 5 min at 800 x g. The pellet was resuspended in water and different dilutions of the suspension were added to water agar plates. To obtain each monosporic isolate, single germinated conidia were collected under the microscope and plated in potato dextrose agar (PDA) plates. In parallel, strain 11 was cultured as above in two different curing media consisting of PDB containing 80 or 100 µM of ribavirin. Ribavirin is a nucleoside analog that induces mutations in RNA viral genomes, and its range of biologically active concentrations is 10 to 100 µM (Parker, 2005). This drug has been used in therapy against human RNA viruses such as hepatitis virus C or herpes virus, and against plant potyviruses (Mahmoud et al., 2009; Parker, 2005). However, it was not effective against a fungal virus infecting *Chalara elegans* (Park et al.,

2006). Monosporic isolates from each ribavirin treatment were obtained as explained before. For mycovirus detection all the monosporic isolates were cultured for three weeks over cellophane disks layered on top of PDA plates, after this period the mycelium was harvested, and dsRNA was extracted by CF-11 cellulose chromatography (Morris and Dodds, 1979). Isolates apparently cured of infection by all mycoviruses were analyzed two additional times.

To check which dsRNA elements were encapsidated in protein particles, twenty grams of mycelium were used to obtain partially purified virus preparations, as well as sucrose gradient purified preparations (Jiang and Ghabrial, 2004). Partially purified virus preparations from strain 11 were treated to disrupt virions by incubation for 20 min at 60 °C in the presence of 0.1% SDS, followed by extraction with 1 volume of phenol:chlorophorm (1:1). The aqueous phase was ethanol precipitated, and the presence of dsRNA was checked by electrophoresis. The six dsRNAs infecting *T. cylindrosporum* were recovered from the aqueous phase obtained after the disruption treatment. This result suggested that all six dsRNA elements are encapsidated in protein particles, naked dsRNA would not sediment at the high speed used for particle purification (Zabalgogezcoa et al., 1998).

Different dsRNA elements showed different rates of transmission to mitotic spores. Among the 43 monosporic isolates obtained from strain 11, dsRNA1 was present in 81.4% of the isolates, and dsRNA2 was transmitted to 79.1% of the monosporic isolates, alone or together with dsRNA1. dsRNAs 3, 4, 5, and 6 showed the same rate of transmission, they were present only in 4.7% of the isolates, and always accompanied by dsRNA1 and dsRNA2. These results suggest that the six dsRNA elements are the product of a multiple virus infection. Isolates infected only by dsRNA1 or by dsRNA2 were obtained. However, dsRNAs 3, 4, 5, and 6 did not segregate in conidial progeny. Therefore, dsRNA1 could be the genome of a virus, dsRNA2 the genome of another virus, and the four remaining dsRNAs could represent the multipartite genome of a third virus. In all reported cases of naturally occurring mixed virus infections in fungi (i.e. *Fusarium graminearum*, *Gremmeniella abietina*, *Epichloë festucae*, *Heterobasidion annosum*) all the viruses infecting a strain were transmitted together to the conidial progeny (Chu et al., 2004; Ihrmark et al., 2002; Romo et al., 2007; Tuomivirta and Hantula, 2005). In contrast, in *T. cylindrosporum* different rates of transmission occurred for each virus, with values ranging from 4.7% to 81.4%, depending on the virus. To our knowledge this is the first report of differential transmission rates among viruses in naturally occurring mixed infections.

In the absence of ribavirin 7.0% of the isolates were free of dsRNA, with 80 µM ribavirin (47 monosporic isolates analyzed) 44.7% of the monosporic isolates were free of all six dsRNAs, and the rest only harboured dsRNA2. With the 100 µM ribavirin treatment (48 isolates analyzed)

20.8% of the isolates were completely cured, and the remainder harboured only dsRNA2. The effectiveness of ribavirin seemed to be different for each dsRNA element. All the monosporic isolates were free of dsRNA1 at both concentrations, in the absence of ribavirin; the rate of transmission of dsRNA1 to conidia was 81%. In contrast, dsRNA2 was not as sensitive, and its percentage of transmission to conidiospores ranged from 55.5% to 79.2% for isolates treated with 80 and 100 μ M ribavirin, respectively. The natural transmission of dsRNA2 to asexual spores was 79.1%, almost the same observed with 100 μ M ribavirin. The natural transmission of dsRNAs 3, 4, 5 and 6 to conidia was very low (4.7%); and it is difficult to evaluate the effect of the drug in the elimination of these dsRNAs.

dsRNA purified from strain 11 was used as a template for the synthesis of a cDNA library. About 2 μ g of dsRNA dissolved in water were denatured at 95 °C for 10 min in the presence of 10 μ g of a degenerate primer (Totioligo: 5'-TTGAA(A/G)TC(A/G)TC(A/G)TA(A/G)TC(G/C)A(A/G)CA-3'). The design of this primer was based on the alignment of motif IV of the RNA-dependent RNA polymerase (RdRp) sequences of seven members of the *Totiviridae* family (Supplemental material Table 1). The heat-denatured mixture was cooled and cDNA was synthesized using the Universal RiboClone cDNA Synthesis System (Promega) and cloned in pGEM-T vector (Promega). *Escherichia coli* strain JM109 (Promega) was transformed and screened for recombinant plasmids. Thirty five different cDNA clones were sequenced and used to assemble three contigs. Northern blot hybridization of dsRNA extracts from strain 11 with probes complementary to each contig, showed that two contigs were complementary to dsRNA1, and the other one to dsRNA3 (Supplemental material Fig 1.). The gap between the two contigs complementary to dsRNA1 was completed by reverse transcription and PCR using primers complementary to sequences flanking the gap was developed, repeating this experiment three times. Four identical clones of the 5' end and five of the 3' end of dsRNA1 were sequenced from two independent RLM-RACE experiments (Coutts and Livieratos, 2003). To obtain a complete sequence of dsRNA3, four identical cDNA clones of the 5' end and three of the 3' end from two independent RLM-RACE experiments of each terminus were sequenced.

The complete sequence of dsRNA1 had 5196 bp, and contained two open reading frames (ORFs) (Fig. 2A). ORF1 consists of 2277 bp and encodes a hypothetical 758 amino acid protein (79.9 kDa); ORF2 is 2523 bp long and encodes an 840 amino acid protein (91.2 kDa). Both ORFs are in the same reading frame, the UAA stop codon from ORF1 is directly followed by the AUG start codon of ORF2. No other possible ORFs longer than 350 nucleotides were found in any strand. The complete genome has a GC content of 61%. The 5' untranslated region (UTR) has 326 bp and a GAAAT sequence in its terminus, similar to the GAAAA motif present in the genomes of *Ustilago*

maydis Virus (UmV) and the *Saccharomyces cerevisiae* viruses ScV-LA and ScV-L BC (Fujimura and Wickner, 1988; Kang et al., 2001). The 3' UTR has a length of 70 bp. The amino acid sequence deduced from ORF1 of dsRNA1 showed highest similarity to those of the capsid proteins (CP) of viruses of the family *Totiviridae*, particularly to that of *Botryotinia fuckeliana* virus 1 (BfV1; 61.5%). The C-terminus of this putative CP has an Ala/Gly/Pro-rich region, which occurs in mycoviruses of the *Victorivirus* genus (Ghabrial and Nibert, 2008). A sucrose gradient purified virus preparation of strain 11-1L, obtained from the transmission experiments and harbouring only dsRNA1 was made according to Jiang and Ghabrial (2004). Isometric virus like particles of approximately 50 nm of diameter were observed transmission electron microscopy (Fig. 2C). Part of this virus preparation was analyzed by SDS-PAGE (Laemmli, 1970), and Coomassie blue staining of the gel showed a major polypeptide with an apparent molecular mass of 79.70 kDa (Fig. 2D). This size is very similar to that expected from the hypothetical protein of 758 amino acids encoded by ORF1 (79.87 kDa). This supports that ORF1 encodes the CP of the virus whose genome is dsRNA1. The deduced amino acid sequence of ORF2 from dsRNA1 resembled those of RdRps of the *Totiviridae*, particularly that of BfV1 (49% identity). The eight conserved motifs of the sequences of RdRps of dsRNA viruses of simple eukaryotes (Bruenn, 1993) were present in this amino acid sequence. The size of dsRNA1 and its genes indicated that this molecule could constitute the genome of a virus belonging to the *Totiviridae* family. We nominated this new virus *Tolypocladium cylindrosporum* virus 1 (TcV1), and its complete genome sequence has been deposited in the EMBL nucleotide sequence database with accession number FR750562.

dsRNA3 had a length of 3486 bp, and contained a 3399 bp ORF that encodes a 1132 amino acid protein (127.7 kDa) (Fig. 2B). Its 45 bp 5' UTR had some sequences similar to those described in the 5' termini of some chrysovirus. For instance, a GAUAAA sequence in position 12 (Ghabrial, 2010), and an AAAAAA sequence in position 4 (Jamal et al., 2010). The 3' UTR is 42 bp long. The amino acid sequence of the unique ORF present in dsRNA3 exhibited the highest identity to the RdRp of *Magnaporthe oryzae* chrysovirus 1 (50% identity) (Urayama, et al., 2010), and also resembled other chrysovirus replicases. The eight conserved motifs of RdRps of dsRNA viruses (Bruenn, 1993) were present in the amino acid sequence. The sequence of dsRNA3 has been deposited in the EMBL nucleotide database with accession number FR750563.

No clones were obtained from dsRNA2, but its transmission was independent of other dsRNAs and it could constitute the complete genome of another virus that was tentatively named TcV3.

Phylogenetic analyses based on alignments of the amino acid sequence of the CP and RdRp of selected members of the *Totiviridae* and *Chrysoviridae* (Supplemental material Table 1) and of

TcV1 (dsRNA1) and dsRNA3 (Fig. 3) were made. MEGA software (Kumar et al., 2004) was used to estimate genetic distances with the Poisson correction model, and to make neighbour-joining trees. As expected, TcV1 was included in a clade within the genus *Victorivirus* (Fam. *Totiviridae*). In addition, the alignment of the 3' UTR sequences of eleven members of the *Victorivirus* genus and TcV1 (dsRNA1), revealed a region of 18 bp that is conserved in most members of the genus, except for *Helicobasidium mompa* totivirus 1-17 (HmV-17) and *Coniothyrium minitans* RNA virus (CmRV) (Supplemental material Fig. 2). Several victorivirus genomes, including TcV1, end with an AUGC 3' motif. This motif and a preceding stem loop, also predicted in TcV1, have an important role in the replication of *Saccharomyces cerevisiae* totivirus ScV-LA (Wickner, 1996). TcV1 differs from other victoriviruses in the fact that its two ORFs are in the same reading frame and do not overlap, as occurs with most members of the genus. In TcV1 the UAA stop codon of ORF1 is immediately followed by the AUG start codon of ORF2. A study performed with a mutant of the mycovirus CHV1-EP713 proved that the non existence of an overlap between ORF1 and ORF2 does not affect the translation of ORF2 (Guo et al., 2009). Therefore, TcV1 may also follow a coupled termination reinitiation mechanism of translation, which is typical of victoriviruses (Ghabrial and Nibert, 2008).

The phylogenetic analysis also confirmed that dsRNA3 is closer to the *Chrysoviridae* family than to the *Totiviridae* (Fig. 3). Since chrysoviruses have tetrapartite genomes of 2.4-3.6 kbp (Ghabrial et al., 2005), the fact that dsRNA3 encodes a putative chrysovirus RdRp supports the hypothesis of dsRNAs 3, 4, 5, and 6 being the multipartite genome of a virus, and in particular, of a member of the *Chrysoviridae*. We have named this virus *Tolypocladium cylindrosporum* virus 2 (TcV2).

In conclusion, this study presents the first reported sequences for mycoviruses infecting an entomopathogenic fungus, and adds a new member to the genus *Victorivirus*, TcV1. Curing and transmission experiments suggested that strain 11 of *T. cylindrosporum* was infected by three different viruses: TcV1 (dsRNA1), TcV2 (dsRNAs 3, 4, 5, 6) a hypothetical member of the *Chrysoviridae* family, and TcV3 (dsRNA2) an unknown virus with a genome or a replicative form of approximately 4.2 kbp. In contrast to other reports of virus transmission in mixed infections, the *T. cylindrosporum* viruses showed different rates of transmission to conidia, and as a result viral combinations different from those of the parental strains occurred in conidial progeny. In addition, this is the first report of the successful use of ribavirin to cure fungal viruses. However, the drug was efficient curing strains of TcV1, but not against TcV2 or TcV3. Therefore, ribavirin could be useful to cure some particular fungal viruses, and to obtain isogenic virus free strains, useful to

study the effects of viruses in their fungal hosts, something which is largely unknown, in spite of the ubiquity of viruses in the fungal kingdom.

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Figures

Fig. 1. (A) Electrophoretic banding patterns of the dsRNA elements present in strains 11 of *T. cylindrosporum*. Lane M contains λ -HindIII size marker; numbers on the left and right indicate kbp. (B) Enlarged picture of the larger band following extended electrophoresis.

Fig. 2. (A) Genome organization of *Tolypocladium cylindrosporum* virus 1 (TcV1). The 5196 kbp genome contains two ORFs; ORF1 encodes a putative CP and ORF2 a putative RdRp. (B) Organization of dsRNA 3, a 3486 bp element which encodes a putative RdRp. (C) Isometric virus-like particles observed by TEM from a purified virus preparation from *T. cylindrosporum* 11-1L isolate harbouring only dsRNA1. Bar = 25 nm. (D) SDS-PAGE analysis of purified TcV1 particles. Lane M, molecular weight marker (kDa on left); lane 1, SDS-treated TcV1 virions. The structural proteins were visualized by coomassie blue staining. The 79.7 kDa protein purified by electrophoresis (CP) has a molecular weight similar to the one estimated for the hypothetical protein coded by ORF1 (79.87 kDa).

Fig. 3. (A) Phylogenetic tree of viruses of the family *Totiviridae* based on CP amino acid sequences. (B) Phylogenetic tree based on RdRp amino acid sequences of members of the *Totiviridae* and *Chrysoviridae* families. The unrooted trees were based on the neighbour-joining method. Numbers at nodes represent bootstrap values as percentages estimated by 1000 replicates. The accession numbers of sequences used in the analyses are given in the supplemental material.

Supplemental material

Supplemental Table 1. Members of the families *Chrysoviridae* and *Totiviridae* used for phylogenetic analysis. Species used in the alignment for the design of the Totioligo primer are in bold type.

Virus	Abbreviation	EMBL accession no.
<i>Amasya</i> cherry disease associated chrysovirus	ACDACV	AJ781397
<i>Agaricus bisporus</i> virus 1	AbV 1	CAA64144
<i>Armigeres subalbatus</i> totivirus	AsTV	EU715328
<i>Aspergillus fumigatus</i> chrysovirus	AfuCV	FN178512
<i>Aspergillus</i> mycovirus 1816	AV-1816	EU289896
<i>Botryotinia fuckeliana</i> totivirus 1	BfV1	CAM33265.1
<i>Chalara elegans</i> RNA virus 1	CeRV1	AY561500
<i>Coniothyrium minitans</i> RNA virus	CmRV	AAO14999.1
<i>Cryphonectria nitschkei</i> chrysovirus 1	CnV-1	ACT79255
<i>Drosophila melanogaster</i> totivirus	DTV	GQ342961
<i>Epichloë festucae</i> virus 1	EfV1	CAK02788.1
<i>Fusarium oxysporum</i> chrysovirus 1	FoCV-1	EF152346
<i>Giardia lamblia</i> virus	GLV	L13218
<i>Gremmeniella abietina</i> RNA virus L1	GaRV-L1	AF337175
<i>Gremmeniella abietina</i> RNA virus-L2	GaRV-L2	AY615210
<i>Helicobasidium mompa</i> totivirus 1-17	HmV-17	BAC81754.1
<i>Helminthosporium victoriae</i> -145S virus	Hv-145S	AF297176
<i>Helminthosporium victoriae</i> virus 190S	Hv-190SV	AAB94791.2
<i>Infectious myonecrosis</i> virus	IMNV	EF061744
<i>Leishmania</i> RNA virus 1-1	LRV1-1	M92355
<i>Leishmania</i> RNA virus 2-1	LRV2-1	U32108
<i>Leishmania</i> RNA virus 1-4	LRV1-4	U01899
<i>Magnaporthe oryzae</i> virus 2	MoV 2	BAF98178.1
<i>Magnaporthe oryzae</i> chrysovirus 1	MoCV1	BAJ15133
<i>Omono River</i> virus-AK4	OMRV-AK4	AB555544
<i>Omono River</i> virus-Y61	OMRV-Y61	AB555545
<i>Penicillium</i> chrysogenum virus	PcV	AF296439
<i>Saccharomyces cerevisiae</i> virus-LA	ScV-L-A	J04692
<i>Saccharomyces cerevisiae</i> virus-L-BC	ScV-L-BC	U01060
<i>Sphaeropsis sapinea</i> RNA virus 1	SsRV-1	AAD11601.1
<i>Sphaeropsis sapinea</i> RNA virus 2	SsRV-2	AAD11603.1
<i>Tolypocladium cylindrosporum</i> virus 1	TcV 1	FR750562
<i>Tolypocladium cylindrosporum</i> virus 2	TcV 2	FR750563
<i>Trichomonas vaginalis</i> virus 2	TVV	AF127178
<i>Ustilago maydis</i> virus H1	UmV-H1	U01059
<i>Verticillium chrysogenum</i> virus	VcV	ADG21213

Supplemental Fig. 2. Alignment of the 3' UTR sequences of eleven members of the *Victorivirus* genus and TcV1. A conserved sequence (blue letters) among members of this genus is indicated.

Supplemental Fig. 1. Three contigs were obtained from assembling 35 cDNA clones obtained using dsRNA from strain 11 as template. (A) Northern blot hybridization using as a probe a clone belonging to the contig 1. (B). Northern hybridization using as probes clones from contigs 2 (B1) and contig 3 (B2). The hybridizations show that contig 1 belongs to dsRNA 3 while contigs 2 and 3 are part of dsRNA 1.