Molecular characterization of the genome of a partitivirus from the basidiomycete Rhizoctonia solani

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The bisegmented genome of a double-stranded (ds) RNA virus from the fungus Rhizoctonia solani isolate Rhs 717 was characterized. The larger segment, dsRNA 1, is 2363 bases long whereas the smaller segment, dsRNA 2, has 2206 bases. The 5’ ends of the coding strands of dsRNA 1 and dsRNA 2 are highly conserved (100% identity over 47 bases), and contain inverted repeats capable of forming stable stem-loop structures. Analysis of the coding potential of each of the two segments showed that dsRNAs 1 and 2 could code for polypeptides of 730 aa (bases 86–2275; molecular mass 86 kDa) and 683 aa (bases 79–2130; molecular mass 76 kDa), respectively. The 86 kDa polypeptide has all the motifs of dsRNA RNA-dependent RNA polymerases (RDRP), and has significant homology with putative RDRPs of partitiviruses from Fusarium poae and Atkinsonella hypoxylon. The 76 kDa protein shows homology with the putative capsid proteins (CP) of the same viruses. Northern blot analysis revealed no subgenomic RNA species, consistent with the fact that the long open reading frames encoding the putative RDRP and CP cover the entire length of the respective dsRNAs.

Introduction

The fungus Rhizoctonia solani (telomorph Thanatephorus cucumeris) (Kühn) is a cosmopolitan, soil-borne plant pathogen causing consistent economic losses in a wide range of vegetable and field crops, ornamentals and tree species worldwide (Sneh et al., 1996). As is the case with many fungi, double-stranded (ds) RNA is ubiquitous in field isolates of this basidiomycete (Bharathan & Tavantzis, 1990). Recently, we provided strong indirect evidence suggesting that a 3–6 kb dsRNA (M2) is associated with suppression of virulence in R. solani (Jian et al., 1997). An open reading frame (ORF) of M2 encodes a putative polypeptide which is phylogenetically related to the penta-functional AROM protein of the shikimate pathway and the suppressor QUTR protein of the quinate pathway in fungi (Lakshman et al., 1998). In contrast, a 6–4 kb dsRNA (M1) is associated with increased virulence (Jian et al., 1997). M1 possesses six ORFs, one of which encodes a polypeptide of 1747 aa that has a significant sequence similarity, including six conserved helicase domains and an ATP/GTP binding motif, with a helicase gene of plant bromoviruses (Jian et al., 1998).

Although progress has been made, we have no knowledge of the genetic content of a large number of dsRNAs found in field isolates of R. solani (Zanzinger et al., 1984; Hyakumachi et al., 1985; Kousik et al., 1994). The high degree of genetic diversity among dsRNA elements found in natural populations of R. solani (Bharathan & Tavantzis, 1990, 1991) suggests that it is reasonable to assume that the genetic information carried by a dsRNA is more important in phenotype determination than the mere presence of any dsRNA in a R. solani isolate. We have previously described the physico-chemical properties of a dsRNA mycovirus from isolate Rhs 717, which belongs to anastomosis group (AG) 2 of R. solani. The virus consists of icosahedral virions, 33 nm in diameter, containing dsRNAs of 2–4 kb (dsRNA 1) and 2–2 kb (dsRNA 2), and an RNA-dependent RNA polymerase (RDRP) (Tavantzis & Bandy, 1988). We report here the complete sequence of both genomic segments of this virus. Genetic information on dsRNA 1 includes a putative RDRP, whereas that on dsRNA 2 includes a putative capsid protein (CP). Both segments have a high

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The GenBank accession numbers of the sequences reported in this paper are as follows: dsRNA 1, AF133290; dsRNA 2, AF133291.
degree of sequence similarity with partitiviruses from the ascomycetes *Atkinsonella hypoxylon* (Oh & Hillman, 1995) and *Fusarium poae* (Compel et al., 1999).

**Methods**

- **Fungal isolates.** The AG 2 isolate Rhs 717 is weakly virulent on cabbage and radish and moderately virulent on snapbean and maize. Fungal mycelium was grown, harvested and stored according to Tavantzis & Bandy (1988).
- **Virus purification.** Purification of the *R. solani* 717 virus, and subsequent isolation of the virion RNA, were carried out as previously described (Tavantzis & Bandy, 1988).
- **Cloning of viral RNA.** cDNA cloning was as described by Lakshman & Tavantzis (1994) using virion dsRNAs as templates.
- **Total RNA extraction.** Total RNA was extracted from mycelium according to Logemann et al., (1987), with minor modifications.
- **dsRNA extraction.** The procedure for extraction of dsRNA from fungal mycelium using CF-11 cellulose column chromatography was as described by Morris & Dodds (1979) with minor modifications (Lakshman & Tavantzis, 1994). Contaminating DNA and single-stranded RNA were removed as described previously (Hoch et al., 1985).
- **Northern blot hybridization analysis.** Total RNA was de-natured in formalin/formaldehyde and fractionated on 1.2% agarose gels containing 2.2 M formaldehyde (Sambrook et al., 1989). dsRNAs were denatured by immersing gels in 50 mM NaOH for 30 min at room temperature (Lakshman & Tavantzis, 1994). RNA was transferred to Hybond-N (Amersham) membranes by capillary transfer, and Northern blots were prehybridized, hybridized and washed as described by Lakshman et al. (1998).
- **Sequencing.** cDNA sequences were determined by the Sanger dideoxy method using cycle sequencing and an Applied Biosystems 373A automated DNA sequencer. Ambiguous sequences were confirmed by direct sequencing of the products of RT–PCR reactions, which where carried out using purified viral RNA as templates. The sequences of the extreme ends of both segments were determined by amplification using 5’ RACE (Gibco-BRL), followed by direct sequencing of the amplified products.
- **Sequence analysis.** DNA sequences were analysed using PC Gene (Intelligenetics), the GCG sequence analysis package (Genetics Computer Group) and the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple sequence alignment was performed with CLUSTAL W (Thompson et al., 1994), and the tree topology was obtained using the exhaustive search strategy in PAUP* (Swofford, 1998).

**Results**

**Expression and form of the viral genomic segments in vivo**

Northern blot hybridization analysis of total RNA from Rhs 717 was performed under both native and denaturing (formaldehyde) conditions using radiolabelled cDNA probes.
Fig. 2. Homology at the 5′ ends of the coding strands of the two genomic segments of the R. solani virus 717. There is 100% identity over a 47 base region of dsRNA 1 and a 41 base region of dsRNA 2 if a 6 base gap is allowed in dsRNA 2. The identity drops to 65% (26 out of 40 bases) over the next 40 bases which also includes the translation initiation sites. AUG codons are shown in bold letters and the CAA motifs (possibly involved in translational enhancement) are underlined. Identical positions are shown with colons (:) beneath the sequence. The double underlined bases are complementary and thus are capable of forming a stem–loop structure.

Table 1. Properties of the putative genes located on dsRNA 1 and dsRNA 2 of the Rhs 717 partitivirus

<table>
<thead>
<tr>
<th>Genome</th>
<th>ORF</th>
<th>Frame</th>
<th>Nucleotide coordinates</th>
<th>Amino acids</th>
<th>Mol. mass (kDa)</th>
<th>Putative function</th>
<th>Kozak sequence</th>
<th>Inverted repeats*</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsRNA1</td>
<td>1</td>
<td>2</td>
<td>86–2275</td>
<td>730</td>
<td>85.8</td>
<td>RDRP+</td>
<td>502–510</td>
<td>736–747</td>
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<tr>
<td>(2363 bases)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>dsRNA2</td>
<td>1</td>
<td>1</td>
<td>79–2130</td>
<td>683</td>
<td>76.4</td>
<td>CP</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>(2206 bases)</td>
<td></td>
<td></td>
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* Inverted repeats longer than 12 bases.
† The Rhs 717 partitivirus RDRP (dsRNA 1, ORF 1) contains the dsRNA RDPR motifs according to Bruenn (1993). The residue coordinates of these motifs are as follows: Motif I, 242-LVTGT-246; Motif II, 315-FLKSFPTMM-323; Motif III, 363-ARKPECCIMYG-373; Motif IV, 397-IDWSGYDQRL-406; Motif V, 479-GVPSGMLLTQFLDSFGNLY-LII-500; Motif VI, 519-FIMGDDNSIF-528; Motif VII, 569-IETLSYRC-576; Motif VIII, 584-DVEK-587.

specific to each dsRNA segment. Under native conditions, total RNA from Rhs 717 had a single, genome-size dsRNA species hybridizing to a cDNA probe specific for dsRNA 1 (Fig. 1, autoradiograph a of panels A and B) or dsRNA 2 (Fig. 1, autoradiograph b of panels A and B). Under denaturing conditions, in addition to the expected genome-size band, a weaker, slower-migrating band hybridized to a dsRNA 1- or a dsRNA 2-specific probe (Fig. 1, autoradiograph b of panels A and B). The slow-migrating RNA species was not detected in virion RNA samples (Fig. 2, panel B, autoradiograph c). To determine whether this slow, hybridizing RNA is a circular or concatemeric replicative form of the virus, we carried out inverse RT–PCR (Ochman et al., 1988), using primers which primed away from the centre of the genome, in an attempt to amplify regions involved in circularization or concatemerization. The primer coordinates for the inverse RT–PCR were as follows. dsRNA 1: 137 5′-GAGCTTAATCTCCTCTCTG 3′ and 2216 5′-TCTCATCTCTCTCAGAAGC 3′; and nested primers 87 5′-CTGTTCTGCTTCAAGAAGC 3′ and 2074 5′-ACTCTGAATCGAGATC 3′. The inverse RT–PCR reactions failed to amplify virus-specific RNA isolated from virions or mycelial tissue (total RNA) (data not shown). Exposure times longer than those shown in Fig. 1 revealed no subgenomic RNAs in any of the Northern blotting experiments.

Sequence and analysis

The complete sequence of each genomic segment of the Rhs 717 partitivirus has been deposited in GenBank. The 2.4 kb dsRNA, referred to as dsRNA 1, is 2363 bases long and has a GC content of 41%. The 2.2 kb dsRNA, referred to as dsRNA 2, is 2206 bases long and has a GC content of 46.3%. dsRNA 1 has GenBank accession no. AF133290 and dsRNA 2 has accession no. AF133291.

The 3′ end of the coding strand of dsRNA 1 has an ‘interrupted’ poly(A) tail. Thirty-three of the 47 3′ terminal bases are adenosines. This poly(A) region is broken up into four stretches of A’s, with the longest uninterrupted stretch being 14 bases long (data not shown).

Direct comparison of the sequences of the two dsRNAs of the Rhs 717 virus revealed a stretch of similarity between the 5′ ends of the coding strands. This region has 100% identity over 47 bases, with a 6 base gap introduced into the dsRNA 2 sequence, and 65% identity over the next 40 bases (Fig. 2). Within the 47 base identical region, there is a short inverted
Fig. 3. Alignment of putative RDRPs from *R. solani* virus 717 (RhsR1), *F. poae* virus 1 (FpV1R2) (Compel et al., 1999), *A. hypoxylon* 2H virus (Ah2HR1) (Oh & Hillman, 1995) and *F. solani* virus dsRNA M1 (FusoVM1) (Nogawa et al., 1996). Identical residues are black-boxed and similar residues are gray-boxed.
repeat sequence which could allow for the formation of a stem–loop structure having a 6 base stem and a 24 base loop in dsRNA 1, and a stem–loop structure consisting of a 6 base stem and a 19 base loop in dsRNA 2 (data not shown). BLAST searches (Altschul et al., 1990) were performed comparing the core region with the complete GenBank DNA sequence database, but no significant homologies with other sequences were observed.

A BLAST search of the GenBank DNA database using the nucleic acid sequence of dsRNA 1 revealed a phylogenetic relationship (p score 8.2 e−62) between this dsRNA and the first genomic segment of Atkinsonella hypoxylon 2H partitivirus (Oh & Hillman, 1995). A slightly higher homology (p score 4.4 e−130) was observed between dsRNA 1 of the Rhs 717 virus and dsRNA 2 of Fusarium poae virus 1 (Compel et al., 1999).

Analysis of the coding potential of each of the two dsRNAs revealed that dsRNA 1 could potentially code for a 730 aa protein (bases 79–2130) (Table 1). The estimated molecular masses of the two proteins are 85.8 and 76.4 kDa, respectively.

### Discussion

Isometric dsRNA mycoviruses are grouped into two families, the Totiviridae and the Partitiviridae (Murphy et al., 1995). Totiviruses have single genomic segments, the size of which varies between 4.6 and 6.7 kb, and a single CP of 73 to 88 kDa. Partitiviruses have two genomic segments ranging between 1.4 and 3.0 kb each, and a single CP of 42 to 73 kDa. The sizes (2.4 and 2.2 kb) of the two viral dsRNAs from R. solani strain 717 as well as the estimated molecular mass (76.4 kDa) of the putative CP encoded by ORF 2 of dsRNA 2 suggest that this is a member of the Partitiviridae.

An RNA moiety migrating slower than the respective genome-sized band was visible in autoradiograms from Northern blots of denaturing gels (Fig. 1), but inverse RT–PCR failed to show that dsRNA 1 or dsRNA 2 occur as covalent circles, head-to-tail, head-to-head or tail-to-tail concatemers in vivo (data not shown). In contrast, the M2 dsRNA from R. solani has been shown to have a circular form (Lakshman et al., 1998). The absence of subgenomic RNAs can be attributed to the fact that ORF 1 and ORF 2 span the entire coding capacity of dsRNA 1 and dsRNA 2, respectively.

In addition to the Rhf 717 virus, other partitiviruses have been reported to have interrupted poly(A) tails at their 3’ ends. The first dsRNA of A. hypoxylon 2H partitivirus has 13 A’s out of 21 bases whereas the second segment has 36 A’s out of 48 bases. The third segment of the 2H virus has an interrupted poly(U) tail (31 U’s out of 58 bases) (Oh & Hillman, 1995). dsRNA 2 of F. poae virus 1 also has an interrupted poly(U) tail (Compel et al., 1999). dsRNA 2 of the Rhs 717 virus has no poly(A)- or poly(U)-rich sequences, while neither dsRNA 1 nor dsRNA 2 has a eukaryotic polyadenylation signal (AAUAAA).

The 5’ ends of the coding strands of dsRNA 1 and dsRNA 2 are highly conserved and contain inverted repeats capable of forming stem–loop structures (data not shown). Several multipartite viruses have 5’ end sequences that are conserved among their genomic segments (Matthews, 1991; Anzola et al., 1987; Wickner, 1993; Oh & Hillman, 1995), and some satellite RNAs show sequence similarity with their helper viruses at the 5’ and/or 3’ ends (Simon, 1988). Conserved sequences at the 3’ ends of the L-A and L-BC dsRNAs of Saccharomyces cerevisiae are involved in replication (Ribas & Wickner, 1996), whereas small stem–loops are present in dsRNA replication/packaging recognition sequences (Wickner, 1993; Mindich et al., 1994; Schuppli et al., 1994; Ribas & Wickner, 1996).

Each of the genomic segments has four CAA repeats within the respective 5’-end conserved regions, but downstream of the 47 base core region (Fig. 2). CAA repeats, located in the 5’ untranslated region of the tobacco mosaic virus genomic RNA, have been associated with initiation and enhancement of translation (Zaccomer et al., 1995). It appears that the 5’-end conserved regions are involved in replication and packaging of the respective dsRNAs as well as translation of the RDRP and CP genes.

The deduced protein sequence encoded by ORF 1 of dsRNA 1 showed homology with the putative RDRP of F. poae virus 1 (Compel et al., 1999) (46% identities and 13% conservative substitutions over 681 aa) and the corresponding RDRP of dsRNA 1 of A. hypoxylon 2H partitivirus (39% identities, and 15% conservative substitutions over 641 aa) (Oh & Hillman, 1995). Homologies were much higher within the core motifs of these polymerases (Fig. 3). Homology (24% identities and 15% conservative substitutions over 357 aa) was also observed between ORF 1 of dsRNA 1 (aa 275–625) and
the putative RDRP core motif region (aa 149–468) of the Fusov virus of *F. solani* (Nogawa *et al.*, 1996). Sequence similarity between the RDRP ORF (dsRNA 1) of the Rhs 717 partitivirus and the RDRP of plant partitiviruses (*Crypoviridae*), such as beet cryptic virus 3 RNA 2, was lower (19.6% identities, 11.5% conservative substitutions over 480 aa) (data not shown) than that observed among fungal partitiviruses (Fig. 4). The phylogenetic tree of Fig. 4 shows that when only partitivirus RDRP amino acid sequences are compared, fungal and plant partitiviruses form distinct branches sharing a relatively low homology. Similar relationships were observed when other virus groups were included in the comparison (Hong *et al.*, 1998), although there was no grouping into fungal and plant partitiviruses.

The deduced protein sequence of ORF 1 of dsRNA 1 has all the motifs of RDRPs (Bruenn, 1993). Motif swap experiments between *Giardia lamblia* virus and *Saccharomyces cerevisiae* virus L1 (ScVL-A) indicated that motifs I–VI are critical for the maintenance of ScVL-A (Routhier & Bruenn, 1998). The similarities observed among the putative RDRP of the Rhs 717 virus dsRNA 1 and the respective RDRPs of *A. hypoxylon* 2H partitivirus and *F. poae* virus 1 (Fig. 3) are much higher than those that are generally found among homologous RDRPs of dsRNA viruses (Shapira *et al.*, 1991; Koonin & Dolja, 1993).

The 683 aa (76.4 kDa) putative polypeptide encoded by ORF 2 of dsRNA 2 exhibited a moderate degree of phylogeny (22% identities and 16% conservative substitutions over 583 aa) with the putative capsid proteins of *F. poae* virus 1 (Compel *et al.*, 1999) and *A. hypoxylon* 2H partitivirus (20% identities and 15% conservative substitutions over 632 aa) (data not shown) (Oh & Hillman, 1995). Tavantzis & Bandy (1988) showed that in vitro translation of the Rhs 717 virion dsRNA yielded two major polypeptides of 77 and 71 kDa, which were immunoprecipitated by antibodies raised against purified Rhs 717 virions. The deduced size (76.4 kDa) of the polypeptide encoded by ORF 2 (dsRNA 2) is very similar to that of the largest in vitro translation product. Thus, it appears that ORF 2 encodes the CP of the Rhs 717 partitivirus. This hypothesis is supported by the homology observed between this ORF and the putative CPs of both *A. hypoxylon* 2H virus (Oh & Hillman, 1995) and *F. poae* virus 1 (Compel *et al.*, 1999). The immunoprecipitation data (Tavantzis & Bandy, 1988) in conjunction with the sequence data suggest that the 71 kDa polypeptide is either an incomplete in vitro translation product or a result of proteolytic activity on the 77 kDa CP, a phenomenon observed with CPs of partially purified plant viruses (Tavantzis, 1980).

*R. solani* 717 partitivirus is closely related to *F. poae* virus 1 and the 2H partitivirus from *A. hypoxylon*. dsRNAs viruses evolve and diverge rapidly, and thus RDRPs are identified by a small set of weakly conserved motifs (Koonin, 1992; Bruenn, 1993; Koonin & Dolja, 1993). It is significant in this regard that the RDRP genes of three dsRNA viruses, one found in a basidiomycete (*R. solani*) and two occurring in ascomycetes (*F. poae* and *A. hypoxylon*), retain a relatively strong homology both at the nucleic acid (see Results) and the amino acid level (Fig. 3).

**References**


DNA form and is genetically related to the pentafunctional AROM protein of the shikimate pathway. Proceedings of the National Academy of Sciences. USA 95, 6425–6429.


Received 1 July 1999; Accepted 18 October 1999