VIROLOGY 252, 399–406 (1998)
ARTICLE NO. VY989480

Coinfection of a Fungal Pathogen by Two Distinct Double-Stranded RNA Viruses

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Received July 15, 1998; returned to author for revision August 4, 1998; accepted October 19, 1998

Unsegmented double-stranded (ds)RNA viruses belonging to the family Totiviridae persistently infect protozoa and fungi. In this study, two totiviruses were found to coinfect the filamentous fungus *Sphaeropsis sapinea*, a well known pathogen of pines. Isometric, virus-like particles ~35 nm in diameter were isolated from extracts of this fungus. The nucleotide sequences of the genomes of the two *S. sapinea* RNA viruses named SsRV1 and SsRV2 were established. The linear genomes of 5163 and 5202 bp, respectively, are identically organized with two large, overlapping ORFs. The 5′ located ORF1 probably encodes the coat protein, whereas the gene product of ORF2 shows the typical features of RNA-dependent RNA polymerases. The absence of a pseudoknot and a slippery site at the overlapping region between ORF1 and ORF2, as well as the shortness of that region, leads us to suggest that the translation of ORF2 of both viruses is internally initiated. The mode of translation and the genomic organization are similar to those of *Helminthosporium victoriae* 190S virus (Hv190SV; Huang, S., and Ghabrial, S. A. (1996). *Proc. Natl. Acad. Sci. USA* 93, 12541–12546). Hv190SV thus appears to be closely related to the SsRVs. Interestingly, based on amino acid sequence homology SsRV1 is more closely related to Hv190SV than to SsRV2.

INTRODUCTION

Biological control of plant pathogenic fungi is increasingly being viewed as a viable disease management strategy. One of the routes to achieve such biological control is through the exploitation of fungal viruses. A good example of this approach is found in studies of the hypovirus that mediates hypovirulence in the chestnut blight pathogen *Cryphonectria parasitica* (Chen *et al.*, 1994). After the accidental introduction of this devastating tree pathogen into Europe, the natural occurrence of a double-stranded (ds)RNA mycovirus has substantially reduced the impact of chestnut blight in Europe. In contrast, the American chestnut has virtually been eliminated by the disease (Nuss, 1992). Although most mycoviruses that have thus far been characterised are members of the family Totiviridae, the *C. parasitica* hypovirus resides in its own family, known as the Hypoviridae.

Totiviruses are isometric dsRNA viruses with a monopartite genome of 4.5–6 kb that are known to persistently infect fungi [e.g., *Saccharomyces cerevisiae* (Icho and Wickner, 1989; Park *et al.*, 1996) and *Helminthosporium victoriae* (Huang and Ghabrial, 1996)] as well as protozoa [e.g., *Leishmania braziliensis* (Stuart *et al.*, 1992) and *Giardia lamblia* (Wang *et al.*, 1993)]. The effect of their presence varies from cryptic (no symptoms) to a lytic reaction. The presence of satellite dsRNA genomes often explains the drastic differences in phenotype of these viruses. Satellite dsRNAs are not physically linked to the genome of the helper virus, but they require their gene products for replication and encapsidation. The best known example is the satellite dsRNA associated with the *S. cerevisiae* L-A virus (ScV-L-A), which encodes a protein toxin responsible for the killer phenomenon of certain yeast strains (Wickner, 1986).

Sphaeropsis sapinea is a well known pathogen of pines throughout the world. In South Africa, this fungus is responsible for extensive damage to pine plantations (Swart and Wingfield, 1991). Despite the fact that no sexual state has been discovered for it, *S. sapinea* isolates in South Africa are morphologically diverse and show different degrees of virulence (H. Smith and M. J. Wingfield, unpublished observations). The fungus is a latent pathogen that is able to infect *Pinus* spp. without causing symptoms (Smith *et al.*, 1996). After the onset of stress (e.g., hail damage or drought), *S. sapinea* usually invades trees, causing severe die-back. Other symptoms include root disease, stem canker, shoot blight, and blue stain (Swart and Wingfield, 1991).

Recently, the presence of dsRNA species in South African isolates of *S. sapinea* was reported for the first time. Around 10% of the isolates studied contained dsRNA (Steenkamp *et al.*, 1998). The apparent length of the dsRNA was estimated to be ~4.3 kb. In this preliminary study, pathogenicity tests revealed no obvious cor-

The sequences reported in this article have been deposited in the GenBank database: SsRV1 with accession no. AF038665 and SsRV2 with accession no. AF039080.

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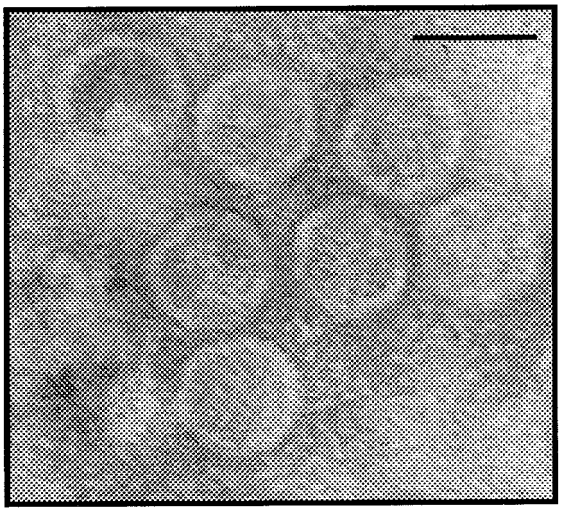

FIG. 1. Electron micrograph of isometric virus-like particles from *S. sapinea* isolate CMW4254. Particles were enriched by PEG precipitation followed by CsCl centrifugation. The sample was negatively stained with 2% uranyl acetate. Bar, 35 nm.

relation between the presence of the dsRNA species and hypovirulence in *S. sapinea*. However, the lack of an isogenic strain with and without infecting viruses could have resulted in the fact that no correlation was found between the presence of dsRNA and changes in the physiology of the pathogen. Even if the virus itself is not mediating hypovirulence in *S. sapinea*, the potential still exists for the introduction of a heterologous satellite dsRNA with a hypovirulent phenotype into the fungus, such that the virus might act as a helper virus.

Any means of reducing the impact of *S. sapinea* is of considerable interest to forestry in South Africa. We have therefore initiated a programme to characterise the mycoviruses in *S. sapinea* at a molecular level. In this study, we report for the first time the sequence-based identification of two distinct dsRNA viruses, which are members of the family Totiviridae coinfecting a single conidial isolate of a filamentous fungus. The complete sequence of both viruses and their close structural relationship to the *H. victoriae* 190S virus (Hv190SV) (Huang and Ghabrial, 1996) are presented.

RESULTS AND DISCUSSION

Detection of virus-like particles

A recent study has shown the presence of dsRNA genomes with an estimated size of 4–5 kb in 4 of 31 South African isolates of *S. sapinea* (Steenkamp *et al.*, 1998). To correlate these dsRNA species with viral genomes, a purification protocol for virus particles was applied to a cell extract from a dsRNA-containing *S. sapinea* isolate. Virus-like particles (VLPs) of ~35 nm in size and an isometric shape were identified by transmission electron microscopy (Fig. 1).

The properties of these VLPs are similar to those found for the viruses of the family Totiviridae that infect fungi and protozoa (Ghabrial, 1994; Castón *et al.*, 1997). To confirm the relationship between the observed VLPs and the isolated dsRNA fragments, nucleic acids were isolated directly from an aliquot of the VLP sample and separated by agarose gel electrophoresis. A band of the same size as the previously isolated dsRNA species from mycelium was observed (data not shown). These data support our view that these VLPs represent viruses with a dsRNA genome.

The name *S. sapinea* RNA virus (SsRV) has been given to this virus. To confirm a possible relationship of SsRV to viruses of the family Totiviridae (Ghabrial, 1994), it was necessary to clone and sequence the SsRV genome. The nature and relationship of a virus such as SsRV can be readily determined using sequence comparisons with other published genomes.

Synthesis and sequencing of cDNA from *S. sapinea* dsRNA

Due to the stability of dsRNA, initial attempts to denature the nucleic acid resulted in a high renaturation rate. Heat treatment at 98°C of the dsRNA resuspended in ddH_2O improved the yield of denatured RNA (Fig. 2). Single-stranded RNA produced by denaturation of the gel-purified dsRNA was used as template for the reverse transcription reaction.

Using mixed hexanucleotides as primers, random cDNA fragments were produced and subsequently cloned into pUC18, yielding clones with inserts of up to 1 kb cDNA. The inserts of ~30 cDNA clones produced in this manner were sequenced. The sequences could be overlapped partially. Interestingly, the preliminary sequence analysis revealed that the isolated dsRNA band might represent a mixture of two viral genomes both

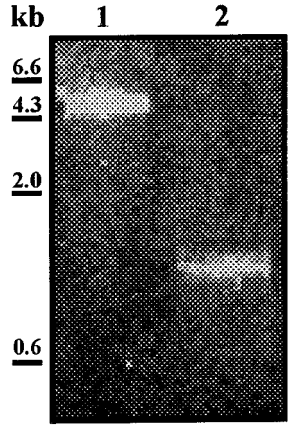

FIG. 2. Denaturation of gel-purified *S. sapinea* dsRNA. Electrophoreses of dsRNA in the native form (lane 1) and after heat treatment at 98°C (lane 2) on an 1% Agarose gel stained with SYBR Green II RNA stain. Marker values are derived from λ DNA restricted with *Hin*dIII.

belonging to the family Totiviridae. Based on the genome structure of Totiviruses, RT-PCRs with sequence-derived primers were used to complete the gaps in the sequence derived solely from the initial cDNA clones.

The 5′ RACE approach was applied to obtain the nucleotide sequences at the distal ends of the genomes (Frohman, 1994). The inserts of three to five clones per end were sequenced to verify the exact 5′ and 3′ termini of each virus. However, it was possible that some of the determined ends did not represent the most distal part of the genomes but were at least close to the ends.

The termini of the *Leishmania* RNA virus 1 (LRV1) genomes were found to be heterogeneous (Widmer, 1993). Thus it generally might be difficult to locate the exact termini of such viral genomes. Comparison of the different sequences further revealed that some variation occurs at identical positions of the same genome. This might be due to the error-prone activity of the RNA-dependent RNA polymerase (RDRP) (Domingo *et al.,* 1996; Holland *et al.*, 1982) producing a higher genome diversity. Thus the obtained nucleotide sequences might have represented only one species of a pool of nearly identical viral genomes.

The overall sequencing strategy established the full-length sequences of two, distinct totiviruses, which we have named SsRV1 and SsRV2. This confirmed our preliminary finding that the *S. sapinea* isolate used in this study is coinfected with two dsRNA viruses. This isolate was established from a single conidium, and there can be no doubt that the two viruses are infecting a single genotype. The notion that the fungal isolate represents a mixed, virus-infected fungal population, which might have led to the same observation, can be excluded with confidence.

Mixed infections by at least two different dsRNA viruses have previously been found in protozoa (e.g., in *G. lamblia*; Tai *et al.*, 1996) and fungi (e.g., in *H. victoriae*; Ghabrial, 1994; Sanderlin and Ghabrial, 1978). The only characterization at sequence level of two totiviruses coinfecting the same host was done in the case of the yeast *S. cerevisiae* with ScV-La and ScV-L-A (Park *et al.*, 1996). The totiviruses SsRV1 and SsRV2 in *S. sapinea* thus are the first viruses coinfecting a filamentous fungus to be characterized at a sequence level.

Genome organization and comparison of the two dsRNA viruses

The linear dsRNA genomes of SsRV1 and SsRV2 are 5163 and 5202 bp in length and have a GC content of 62% and 63%, respectively. Both genomes contain two large open reading frames (ORFs) that overlap by eight nucleotides (Fig. 3A). Two potential start codons for the ORF1 of SsRV1 (AUG) are found at positions 80 and 251 (Fig. 3B). Although the scanning model of ribosomes would favor initiation of translation at the first AUG codon of the RNA, the second AUG of the SsRV1 ORF1 appears to be in a slightly better context. The sequence UUU-CCGAUGG compares favorably with the optimal consensus sequence found in eukaryotic start codons of $GCC^{A}/_{G}CCAUGG$ (Kozak, 1991). The most proximal AUG codon in the SsRV2 ORF1 has a sequence of AGC-GCCAUGG. This also compares favorably with the consensus sequence and, therefore, most probably functions as the start codon of ORF1.

The short overlap of the end of ORF1 and the start of ORF2 was found in both SsRVs. This is similar to the structure found in the closely related Hv190SV (Huang and Ghabrial, 1996). In both SsRVs, the AUG (positions: SsRV1, 2574; SsRV2, 2658) of ORF2 is separated by two nucleotides from the downstream-located UAA stop codon of ORF1. In Hv190SV, the start and stop codons overlap directly (Fig. 3B). It is also important to note that there is a stop codon in the same reading frame, 8 and 12 codons upstream to the proposed start codon of ORF2 of SsRV1 and SsRV2, respectively. The presence of this stop codon severely restricts the area for any frameshift event. This region is important for the translation mechanism of ORF2 because eukaryotic ribosomes have problems with the translation of the second ORF of bicistronic mRNA (Kozak, 1991).

Totiviruses appear to translate ORF2 either by ribosomal frameshifting to produce a fusion protein of the coat and the RDRP or by internal initiation (Ghabrial, 1998). The ribosomal -1 or $+1$ frameshifting has been shown, for example, in ScV-L-A (Icho and Wickner, 1989) and *G. lamblia* virus (GLV) (Wang *et al.*, 1993). This involves a pseudoknot, which forces the ORF1 translating ribosome to pause over the slippery site and to resume the translation in the frame of ORF2. Both structures are located within the overlapping region of ORF1 and ORF2 or, in the case of the pseudoknot structure, adjacent to it. Neither a pseudoknot nor a slippery site was detected within or adjacent to the short overlap of ORF1 and ORF2 of both SsRVs. Therefore, translation by a frameshifting event is not probable. It is more likely that translation of ORF2 is internally initiated, as has been proposed for the Hv190SV (Huang and Ghabrial, 1996). In the latter case, no RDRP-coat fusion protein could be detected, which would indicate a ribosomal frameshifting event. However, a stem-and-loop structure can be predicted in the overlapping region of SsRV1 with the following inverted repeat sequence: 5′-GGCCC-GcC-AAUGAAuaagUUCAUUGaCaGGGCC-3′ (position 2565–2597; start and stop codons are underlined). Because such a structure is missing in SsRV2, a general function in the translation of SsRVs is unlikely.

The translation of ORF2 of SsRV1 and SsRV2 is probably terminated at the stop codon UAG at position 5088 and UAA at position 5133, respectively (Fig. 3B). The 3′ noncoding region of SsRV1 and SsRV2 consists of 73 and 67 nucleotides, respectively. For both SsRVs, as well

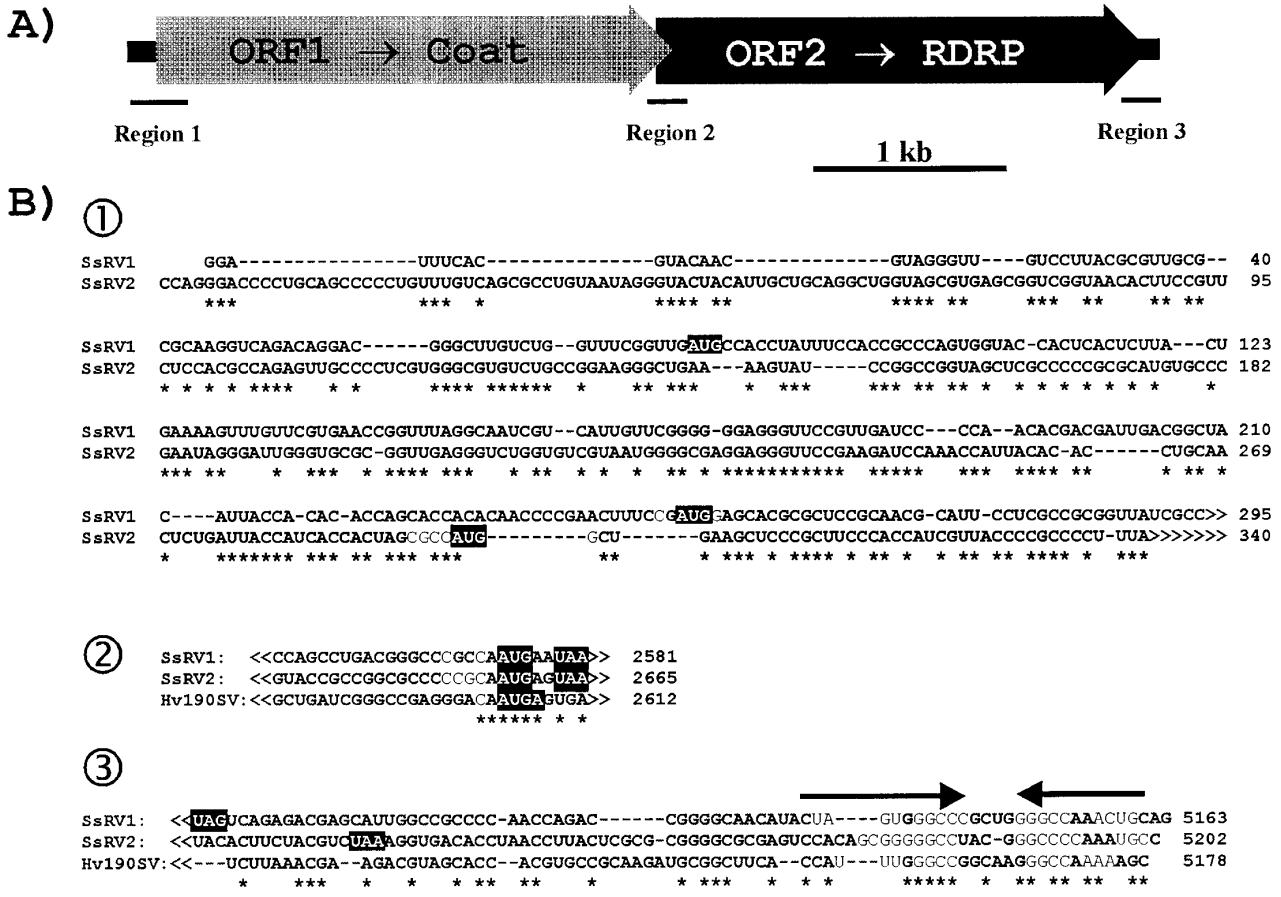

FIG. 3. Genomic details of SsRV1 and SsRV2. (A) Scheme of the genome of SsRV1 and SsRV2. The genomic organisations of both viruses are very similar. ORF1 and ORF2 are overlapped by eight nucleotides. ORF1 encodes a coat protein, whereas the gene product of ORF2 is an RNA-dependent RNA polymerase (RDRP). The RNA sequence of the three regions indicated in A are shown in more detail in B. (1) The alignment of the 5′ ends of the genomes of SsRV1 and SsRV2. The potential start codons are highlighted by black blocks with white letters. The gray letters around the AUG indicate optimal nucleotides for initiation of translation (Kozak, 1991). (2) The aligned, overlapping region of ORF1 and ORF2 of SsRV1, SsRV2, and Hv190SV (Huang and Ghabrial, 1996), respectively. The start codons (AUG) of ORF2, as well as the stop codons (UAA, UGA) of ORF1, are highlighted by black blocks with white letters. (3) The aligned 3′ ends of the genomes of SsRV1, SsRV2, and Hv190SV, respectively. The stop codons of the SsRVs are also highlighted. The Hv190SV stop codon is further upstream than the sequence presented. The sequence regions that form an inverted repeat are indicated by letters in gray with arrows above these sequences. Stars at the bottom of the alignment indicate identical bases at the same position in all sequences.

as for Hv190SV, a stem-and-loop structure can be predicted close to the 3′ end (Fig. 3B). Such a sequence at the 3′ terminus of ScV-L-A RNA is essential for its *in vitro* replication by the replicase of ScV-L-BC (Ribas and Wickner, 1996). This stem-and-loop structure might be one of the structures that the replicase has to recognize for the replication of the dsRNA.

ORF1 of SsRV1 encodes a coat protein of 833 or 776 amino acids with a predicted molecular mass of 88.7 or 82.2 kDa, depending on which of the two potential start codons is used for translation. The coat protein encoded by ORF1 of SsRV2 consists of 789 amino acids, with a predicted molecular mass of 82.9 kDa.

The coat and RDRP of Hv190SV (Huang and Ghabrial, 1996) showed the highest degree of homology to the gene products of SsRVs. The coat proteins of SsRV1 and SsRV2 have an identity of 38%. The Hv190SV coat protein shows an identity of 50% and 38% to the coat proteins of SsRV1 and SsRV2, respectively. The coat protein of the LRV1 (Stuart *et al.*, 1992) is only 22% identical to the coat proteins of SsRV1 and SsRV2. An alignment of the predicted amino acid sequences of the coat proteins of SsRV1, SsRV2, Hv190SV (Huang and Ghabrial, 1996), and LRV1 (Stuart *et al.,* 1992) is shown in Fig. 4A.

ORF2 of SsRV1 and SsRV2 encodes RDRPs of 838 and 825 amino acids with predicted molecular masses of 92.2 and 90.8 kDa, respectively. The alignment of RDRPs (Fig. 4B) of both SsRVs, Hv190SV (Huang and Ghabrial, 1996) and LRV1 (Stuart *et al.,* 1992), shows that all conserved motifs known in totiviral RDRPs are present (Bruenn, 1993). The RDRPs of the two SsRVs are 36% identical, whereas the SsRV1 polymerase is slightly more homologous to RDRP of Hv190SV, showing an identity of 40%. The SsRV2 RDRP has only a 36% identity

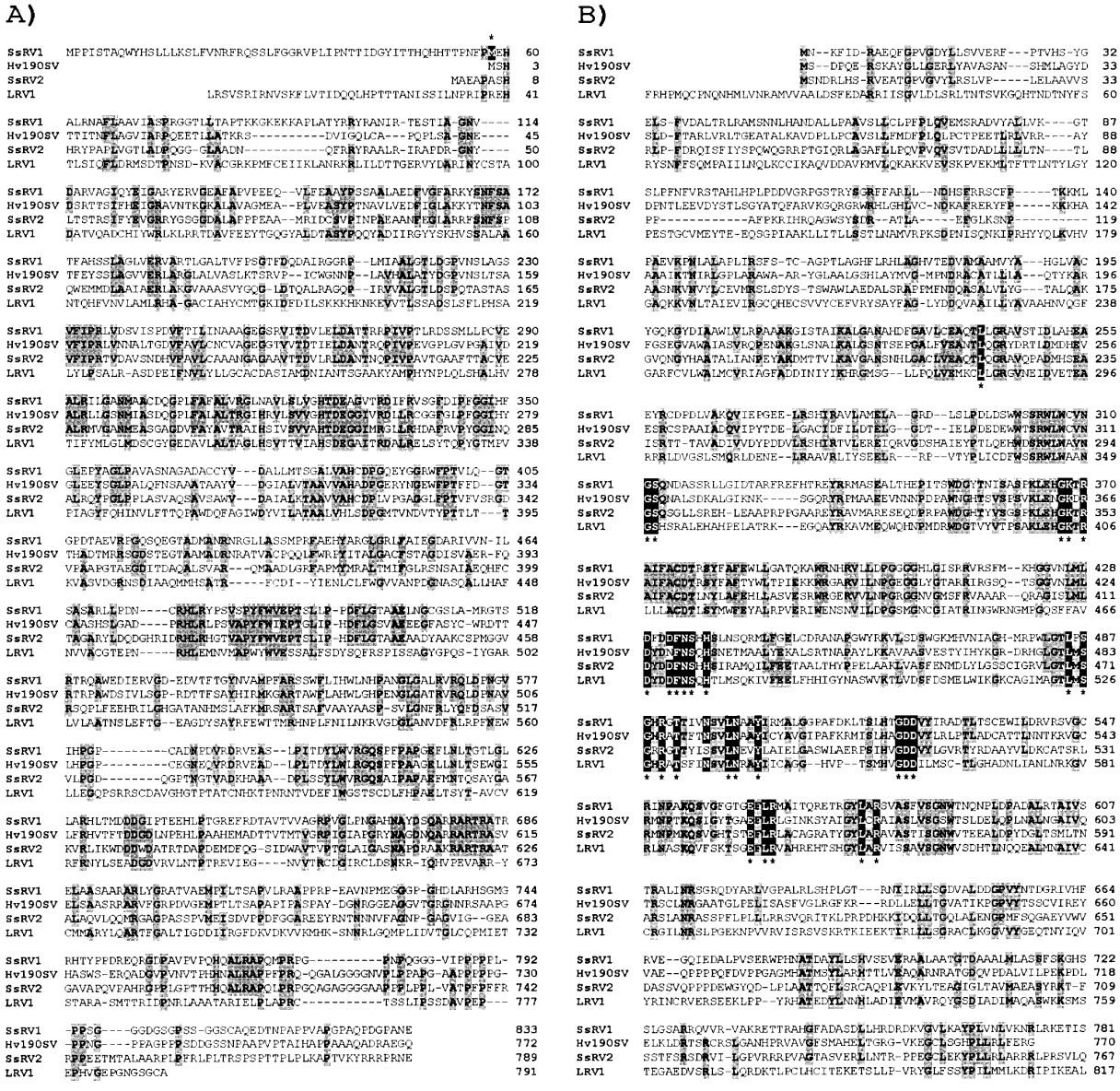

FIG. 4. Amino acid alignments of the coat proteins (A) and of RDRPs (B) of SsRV1, SsRV2, Hv190SV (Huang and Ghabrial, 1996), and LRV1 (Stuart *et al.*, 1992), respectively. Amino acids that are conserved at least in three sequences are marked by bold, highlighted letters. The highlighted methionine in the sequence of the SsRV1 coat protein may represent an alternative N-terminus of this protein. The highly conserved motifs in RDRPs of totiviruses (Bruenn, 1993) are highlighted by black blocks with white letters and indicated by asterisks.

to the Hv190SV polymerase. LRV1 RDRP has an even lower identity of 30% to both SsRVs RDRPs.

Sequence analyses show clearly that the Hv190SV, which also infects a filamentous fungus, is most closely related to both SsRVs. Furthermore, the organizations of the dsRNA genome as well as the gene products of all three viruses are similar.

Phylogenetic relationships of SsRV1 and SsRV2 to other totiviruses

The evolution of the dsRNA viruses in the Totiviridae is of particular interest because these viruses are persistent in fungal and protozoan cells. Bruenn (1993) postulated that an archetypical virus might have infected a single, ancient cell type before fungi and protozoans diverged.

Based on a multiple alignment of RDRPs encoded by SsRV1, SsRV2, Hv190SV (Huang and Ghabrial, 1996), LRV 1–1 (Stuart *et al.*, 1992), LRV 2–1 (Scheffter *et al.,* 1995), ScV-La (Park *et al.,* 1996), ScV-L-A (Icho and Wickner, 1989), GLV (Wang *et al.,* 1993), *Trichomonas vaginalis* virus (TVV) (Tai and Ip, 1995), and the unclassified La France isometric L1 dsRNA found in *Agaricus bisporus* (van der Lende *et al.*, 1996), a phylogenetic tree was

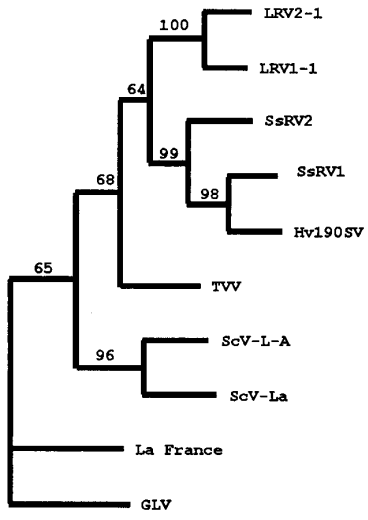

FIG. 5. Phylogenetic tree using PAUP of different Totiviruses based on an alignment of their respective RDRP amino acid sequence. For the calculation of the tree, RDRPs of SsRV1, SsRV2, Hv190SV (Huang and Ghabrial, 1996), LRV 1–1 (Stuart *et al.,* 1992), LRV 2–1 (Scheffter *et al.,* 1995), ScV-La (Park *et al.,* 1996), ScV-L-A (Icho and Wickner, 1989), GLV (Wang *et al.,* 1993), TVV (Tai and Ip, 1995), and the unclassified La France isometric L1 dsRNA (van der Lende *et al.,* 1996) were used. Bootstrap values are indicated at the branch points.

produced (Fig. 5). This tree shows that SsRVs group together with Hv190SV and that they are closely related to the LRVs, but it is arguable whether it would be appropriate to accommodate the SsRVs into the genus *Leishmaniavirus* (Ghabrial, 1994). The other two genera, *Totivirus* represented by ScVs and *Giardiavirus* represented by GLV, are clearly more distantly related. Both SsRVs and Hv190SV infecting filamentous fungi have a similar genomic organization and probably translate ORF2 by internal initiation, rather than by a frameshift event. Furthermore, all three viruses cluster together on a phylogenetic tree (Fig. 5). Therefore, the question might be raised as to whether they should not reside in a genus of their own. More sequence data from other totiviral genomes will be necessary to clarify this point.

The phylogenetic tree also confirms the hypothesis that SsRV1 is more closely related to Hv190SV than it is to the coinfecting SsRV2. This is a novel observation that implies that the *S. sapinea* isolate under investigation might have become infected on two separate occasions. This observation also suggests not only that these persistent viruses coevolved with their hosts from an infected ancestral cell type (Bruenn, 1993; Ghabrial, 1998) but also that noninfectious dsRNA viruses might be transmitted between different host species via a vector, such as insects. In this respect, it would be interesting to know how host specific these viruses are and to what extent host factors determine the replication of the viruses. A broad host specificity would be essential for a successful transmission.

Conclusions

Cloning, sequencing, and characterizing of the genome organization of the viruses infecting *S. sapinea* was the aim of this study. Coinfection by two distinct totiviruses was not expected, and the discovery of these two viruses was of particular interest. This is especially true because neither of these genomes appears to have any obvious effect on their host (Steenkamp *et al.*, 1998). The fact that mutation rate in RNA genomes is higher by several orders of magnitude than in DNA genomes (Domingo *et al.,* 1996) suggests that a virus should be able to adapt to environmental changes more easily than its host. It should, therefore, maintain its capacity to infect the host. So far, the only totivirus that has been described as infectious is GLV (Miller *et al.,* 1988). Alternatively, if the virus is persistent, then it would be reasonable to assume that its presence should benefit the host in some way. A study including a larger *S. sapinea* collection will be necessary to determine whether coinfection of the two viruses in this fungus is common.

Further investigation of satellite dsRNA of SsRV1 and SsRV2 will be worthwhile. In *S. cerevisiae,* it is the satellite dsRNA that encodes the toxin in the killer yeast system (Wickner, 1986). Similarly, in *H. victoriae,* it has been proposed that satellite dsRNA might be responsible for the lytic disease of the fungus that results in hypovirulence (Ghabrial, 1994). Four potential dsRNA species of ~2 kb copurify with SsRV1 and SsRV2 from the *S. sapinea* isolate used in this study (N. A. van der Merwe, O. Preisig, B. D. Wingfield, and M. J. Wingfield, unpublished data). Further investigations are necessary to elucidate the nature and function of these dsRNAs.

It was anticipated that characterization at the molecular level would enable us to better understand the function of these viruses in *S. sapinea*. The transfection of this fungus with SsRVs based on full-length cDNA clones will now be attempted. This will make it possible to characterize the individual viral effects in more detail. It might also be possible to use SsRVs as helper viruses for the replication of heterologous or specially designed satellite dsRNA. This could provide a viable approach to introducing phenotypic characteristics into the fungus that would be useful for biological control of the pathogen.

MATERIALS AND METHODS

Fungal isolate and culture conditions

The virus-infected *S. sapinea* isolate CMW4254 isolated from *Pinus roxburghii* Sarg. (Gauteng, South Africa; Steenkamp *et al.*, 1998) was derived from a single conidium extracted from one pycnidium. Pycnidia were induced to form by growing the fungus on water agar plates covered with a few sterile pine needles. The fungus was grown in 2% malt extract in Erlenmeyer

flasks under shaking conditions at 25°C. Mycelium was harvested in the stationary phase and subsequently freeze-dried before dsRNA extraction.

Purification and electron microscopy of virus-like particles

To isolate VLPs, freshly harvested mycelium was dried between filter paper and then ground in liquid nitrogen with a mortar and pestle. To the ground mycelium, 1.5× STE buffer (10× STE: 0.5 M Tris–HCl, 1 M NaCl, and 10 mM EDTA, pH 6.8) was added and mixed well. Cell debris was pelleted by centrifugation at 10,000 rpm in a Beckman JA20 rotor for 30 min at 4°C. PEG_{6000} was added to the supernatant to achieve a final concentration of 4%, and the mixture then was incubated for 2 hr at 4°C. The precipitated VLPs were pelleted at 15,000 rpm for 30 min. The pellet was resuspended in 2.5 ml of 1.5× STE buffer, layered onto a 2.5-ml CsCl solution (1 g/2.5 ml), and centrifuged in a Beckman 50Ti rotor at 42,000 rpm for 2 hr at 4°C. The resulting pellet was resuspended in 100 μl of water. A drop of this VLP suspension was placed on a Formvar/carbon-coated copper grid (Ted Pella Inc., Redding, CA). After a few minutes, the liquid was absorbed using a filter paper, and the virus particles were resuspended in a drop of 2% uranyl acetate for 2 min. After removal of the negative stain solution, the dried grid was examined for VLPs using a transmission electron microscope (Philips).

Extraction and purification of dsRNA

Freeze-dried, ground mycelium was resuspended in 2× STE with 1% SDS and incubated for 10 min at 60°C. The suspension then was incubated in the presence of 0.1 mg/ml Proteinase K (Boehringer-Mannheim) for 1 h at 37°C. An equal volume of phenol was subsequently added, and the mixture was shaken for 30 min. The aqueous phase was separated from the organic phase by centrifugation at 10,000 rpm in a Beckman JA20 rotor for 30 min at 4°C. The aqueous phase was extracted with an equal volume of chloroform and finally adjusted to 16% ethanol. To separate dsRNA from other nucleic acids, the suspension was applied to a CF11 cellulose (Whatman) column (Valverde *et al.*, 1990). The unbound nucleic acids were removed using a wash buffer of 2× STE containing 16% ethanol. The bound dsRNA was eluted using 2× STE without ethanol. The dsRNA then was precipitated with 0.6 volume of isopropanol. The dsRNA pellet was washed with 70% ethanol, dried, and resuspended in 2× STE. The dsRNA species was then further purified by agarose gel electrophoresis and isolated from excised gel pieces using an RNaid w/SPIN kit (BIO 101). The purified dsRNA was stored at −20°C.

Cloning of cDNA from viral genomes

DsRNA was denatured for 3–5 min at 98°C and immediately placed on ice. To monitor the efficiency of denaturation, an aliquot was loaded on an 1% agarose gel treated with diethyl-pyrocarbonate. The change in mobility of RNA could be visualized using SYBR Green II RNA stain (FMC).

Mixed hexanucleotides were used initially as primers to generate random cDNA fragments using AMV reverse transcriptase for the first-strand synthesis, and *Escherichia coli* DNA Polymerase I was used for the second-strand synthesis (cDNA synthesis kit; Boehringer-Mannheim). The cDNA fragments were cloned into the *Sma*I site of the pUC18 vector and transformed into *E. coli* strain DH5α. To determine gaps in the genomic sequences, sequence-derived 18- to 25-mer primers (DNAgency, Malvern, PA) were used in RT-PCR reactions with the Titan One Tube RT-PCR system (Boehringer-Mannheim). The cloning of the dsRNA ends was achieved by 5′ RACE approach (rapid amplification of cDNA ends) (Frohman, 1994) using a 5′/3′ RACE kit (Boehringer-Mannheim): cDNA was initially synthesized from dsRNA ends at 55°C for 60 min with AMV reverse transcriptase primed by sequence-derived, distal primers. A poly(A) tail was subsequently added to the cDNA by terminal transferase in the presence of 0.2 mM dATP. Then, both ends of each virus genome were PCR-amplified from the modified cDNA with a sequence-derived primer close to the end and an oligo(dT)-anchor primer applying the Expand High Fidelity PCR system (Boehringer-Mannheim). In some cases, a secondary PCR was necessary. Resulting fragments from either RT-PCR or RACE approaches were gel purified and, when necessary, cloned with the pGEM-T Easy Vector System II (Promega) before sequencing.

Sequencing and phylogenetic analyses

PCR and RT-PCR fragments, as well as plasmid inserts, were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The sequencing products were separated and analyzed with an ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

Sequence analyses were made using the computer program Sequence Navigator (Perkin-Elmer) and the programs Translate, SIM+LANVIEW, CLUSTALW, and WU-BLAST listed on the ExPASY home page (http://www.expasy.ch/www/). The GenBank/EMBL and the Swiss-Prot databases were used for homology searches. Secondary RNA structures were obtained with the program RNAdraw V1.1 (Mazura Multimedia, Stockholm, Sweden). Phylogenetic trees were calculated from CLUSTALW aligned sequences using the analysis program PAUP 3.1.1. (D. L. Swofford, Illinois Natural History Survey, Champaign, IL).

ACKNOWLEDGMENTS

We thank Pieter van Wyk (University of the Free State, Bloemfontein) for assistance with electron microscopy and Henk Huismans (University of Pretoria) for his initial assistance with cDNA synthesis. The Members of the Tree Pathology Co-operative Programme are thanked for providing the research facilities. Oliver Preisig was generously supported by postdoctoral bursaries from the Foundation for Research Development (South Africa) and the Swiss National Foundation for Scientific Research.

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