

A novel RNA mycovirus in a hypovirulent isolate of the plant pathogen *Diaporthe ambigua*

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Hypovirulent isolates of the fruit tree fungal pathogen *Diaporthe ambigua* have previously been shown to harbour a double-stranded (ds)RNA genetic element of about 4 kb. In this study, we established the complete cDNA sequence of this dsRNA, which represents a replicative form of a positive-strand RNA virus that we have named *D. ambigua RNA virus* (DaRV). The nucleotide sequence of the genome is 4113 bp and has a GC content of 53%. Two large ORFs are present in the same reading frame. They are most probably translated by readthrough of a UAG stop codon in the central part of the genome. The longest possible translation product (p125) has a predicted molecular mass of about 125 kDa. A significant homology can be found to the non-structural proteins of carmoviruses of the positive-strand RNA virus family *Tombusviridae*. These proteins also include the conserved RNA-dependent RNA polymerase (RDRP) domain. In contrast to the genome organization of these plant viruses, no ORF is present at the 3' end of the DaRV genome that encodes a coat protein. Therefore, it is proposed that DaRV is not encapsidated but that it occurs as RNA–RDRP complexes and/or that it might be associated with cell membranes. Interestingly, six putative transmembrane helices are predicted in the N-terminal part of p56 (translation product of the first ORF, N-terminal part of p125), which might direct and anchor the viral complex to membranes. DaRV is a mycovirus with a unique genome organization and has a distant relationship to the plant virus family *Tombusviridae*.

Introduction

It is common for fungi to be persistently infected by viruses that are not known to be infectious as purified virions (Ghabrial, 1998). The presence of mycoviruses in fungal mycelia is often detected by electron microscopy as virus-like particles in thin sections or by the isolation of distinct species of nucleic acid from the fungal mycelia. In most cases they are double-stranded (ds) RNA. In recent years, an increasing number of mycoviruses infecting plant pathogenic fungi have been classified based on the nucleic acid sequence of their genomes (Huang & Ghabrial, 1996; Preisig *et al.*, 1998; Hong *et al.*, 1999).

Various mycoviruses have been shown to mediate reduced virulence (hypovirulence) in their plant pathogenic hosts. A well-studied example is the hypovirus of the chestnut blight

fungus, *Cryphonectria parasitica* (Shapira *et al.*, 1991; Nuss, 1992). The American chestnut (*Castanea dentata*) has virtually been eliminated from the landscape by *C. parasitica*, which was introduced into North America early in the 20th century (Anagnostakis & Waggoner, 1981). In Europe, the presence of a hypovirulence-mediating dsRNA virus in the introduced population of *C. parasitica* reduced the devastating impact of this chestnut pathogen (Heiniger & Rigling, 1994). The horizontal transmission of mycoviruses depends on hyphal fusion (anastomosis) between two compatible isolates (Ghabrial, 1998). In Europe, a much smaller number of vegetative compatibility (VC) groups are found in the population of *C. parasitica* and this has facilitated spread of the hypovirus (Liu & Milgroom, 1996).

Besides the hypovirulence-mediating hypovirus infecting *C. parasitica*, other mycoviruses conferring hypovirulence have been reported. These viruses include different mitoviruses infecting the mitochondria of *Ophiostoma novo-ulmi* (Hong *et al.*, 1999), the totivirus Hv190SV of *Helminthosporium victoriae* (Huang & Ghabrial, 1996) as well as the unclassified dsRNA elements in *Leucostoma persoonii* (Hammar *et al.*, 1989). In other

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cases, such as the totiviruses infecting *Sphaeropsis sapinea*, no significant effect of the virus on the host could be observed (Preisig *et al.*, 1998; Steenkamp *et al.*, 1998). In plant pathology, the interest in mycoviruses derives from their potential to actively apply their hypovirulence-mediating effect in the biological control of pathogens.

Ascomycetes belonging to the genus *Diaporthe* are important plant pathogens of numerous agronomic and tree crops worldwide. In South Africa, the filamentous fungus *Diaporthe ambigua* causes cankers on apple, pear and plum trees and their rootstocks. This disease can lead to the slow death of mature trees, while affected nursery rootstocks usually die rapidly. The pathogen thus has an immense effect on the productivity of orchards of pome and stone fruit trees (Smit *et al.*, 1996a, 1998).

Isolates of *D. ambigua* have been observed to differ in virulence and morphology. The hypovirulence of isolates is coincidental with the presence of a single dsRNA species of about 4 kb in the fungal mycelia (Smit *et al.*, 1996b). Fungal isolates containing these dsRNA elements are not only hypovirulent, but also show hypovirulence-associated traits. These traits included reduced phenol oxidase activity, reduced gallic acid oxidation, diminished oxalate accumulation and suppressed production of ascospores (Smit *et al.*, 1996b). The dsRNA was transmissible between dsRNA-containing and dsRNA-free isolates of *D. ambigua* of the same VC group through anastomosis. Through this approach, previously virulent, dsRNA-free isolates were converted to hypovirulence. This result supported the idea that the presence of dsRNA mediates hypovirulence in *D. ambigua* (Smit *et al.*, 1996b). However, only transformation or transfection of dsRNA-free fungal isolates with cDNA constructs or RNA from the genetic RNA element might present conclusive evidence for this effect. The putative hypovirulence-mediating dsRNA element in *D. ambigua* could then be an ideal agent to be developed for biological control of this serious canker pathogen of pome and stone fruit trees (Smit *et al.*, 1998).

For application of the *D. ambigua* dsRNA element in biological control, the dsRNA must be characterized at a molecular level. In this study, we confirm the viral origin of the dsRNA based on its complete cDNA sequence. Furthermore, this sequence analysis shows that *D. ambigua* is infected by a novel RNA mycovirus related to the plant virus family *Tombusviridae*.

Methods

■ **Fungal isolates and culture conditions.** The dsRNA-containing *D. ambigua* isolate CMW3407 (RR19) was collected from an apple tree and the dsRNA-free isolate CMW5588 (99/815) was collected from a peach tree, both in the Western Cape, South Africa (W. A. Smit, unpublished). They were grown in ca. 2% potato dextrose broth in Erlenmeyer flasks at 20 °C without shaking. The mycelia were harvested after 2 weeks and then lyophilized for dsRNA isolation.

■ **Extraction and purification of dsRNA.** Freeze-dried mycelium

was ground and resuspended in 2 × STE (0.1 M Tris-HCl, 0.2 M NaCl and 2 mM EDTA, pH 6.8) with 1% SDS. The sample was incubated for 10 min at 60 °C and subsequently mixed with an equal volume of phenol. The mixture was shaken for 30 min and then centrifuged at 10000 r.p.m. in a Beckman JA25.50 rotor for 30 min at 4 °C. The aqueous phase was extracted with an equal volume of chloroform and separated by another centrifugation step. The aqueous phase was finally adjusted to 16% ethanol and centrifuged at 5000 r.p.m. for 5 min to pellet the precipitated genomic DNA. The supernatant was applied to a CF11 cellulose (Whatman) column (Valverde *et al.*, 1990) prepared in a syringe without the plunger. The column was washed with 2 × STE with 16% ethanol to separate the bound dsRNA from other nucleic acids. The dsRNA was eluted in 2 × STE and subsequently precipitated with 0.6 vols isopropanol. The dsRNA pellet was washed with 70% ethanol. The dried sample was then resuspended in DEPC-treated ddH₂O and separated by agarose gel electrophoresis. The dsRNA band was excised from the gel. An RNaid w/SPIN kit (BIO101) was used to isolate the dsRNA from the gel pieces. The purified dsRNA was stored in ddH₂O at -20 °C.

■ **Production of cDNA from viral dsRNA.** The cDNA synthesis was performed following the method of Gubler & Hoffmann (1983) using a cDNA synthesis kit (Roche Molecular Biochemicals). Mixed hexanucleotides were initially used to prime the first-strand cDNA synthesis of heat-denatured dsRNA (for 10 min at 99 °C) with AMV reverse transcriptase. The second strand was synthesized with *E. coli* DNA polymerase I from nicks introduced by RNase H in the RNA strand of the RNA-cDNA hybrid. The random cDNA products were then digested with *Sau3A*I. The restriction digest was purified through a column of a High Pure PCR Product Purification kit (Roche Molecular Biochemicals) to remove products with a length under 100 bp. Products were then cloned into the *Bam*HI site of pGEM-3Zf(+) vector and transformed into *E. coli* JM109 (Promega).

Sequence-derived 19- to 23-mer primers (MWG-Biotech) were used for RT-PCR experiments (Titan One Tube RT-PCR System; Roche Molecular Biochemicals) to amplify parts of the genome which were not cloned by the initial random cDNA synthesis. When necessary, the RT-PCR products were cloned in the pGEM-T easy vector (Promega) prior to sequencing. The distal ends of the dsRNA were amplified by the 5' RACE (rapid amplification of cDNA ends) approach (Frohman, 1994; Preisig *et al.*, 1998) using a 5'/3' RACE kit (Roche Molecular Biochemicals) and sequence-derived, nested primers. Primer pair Oli64 (5' GTCGCATCTCACAGCCGAGCGC 3') and Oli80 (5' CTCACCAGCCTCCAACCG 3') was used to amplify the conserved coding region of the RDRP in RT-PCRs.

■ **Construction of a full-length cDNA of viral dsRNA.** The construction of a full-length cDNA clone of the viral dsRNA was based on two large overlapping partial RT-PCR products (Fig. 1A). Both products were cloned in the pGEM T-Easy vector (Promega). The 2.8 kb 5' product was amplified using primer pair DaRV-5' (5' GGGAAAT-TTGTGAGATTATCGCC 3') and Oli65 (5' AACCTCGAGCACAGCGCAACG 3', including a *Xho*I restriction site) and cloned as pDV1. The 1.5 kb 3' product was amplified using primer pair Oli64 and DaRV-3' (5' GGGCCACAGGATCCGGAGAAC 3') and cloned as pDV2. The orientations of the clones were selected so that the 5' ends of viral cDNA were on the site of T7 promoter of the pGEM T-Easy vector. Plasmid pDV1 was linearized using the restriction enzymes *Xho*I and *Nsi*I. While *Xho*I has one restriction site within the viral cDNA, *Nsi*I cuts once in the multiple cloning site of pGEM T-Easy Vector. The 1.4 kb *Xho*I-*Nsi*I fragment of pDV2 was excised from an agarose gel and then ligated into the linearized pDV1. The authenticity of the resulting, full-length cDNA clone pDV3 was confirmed by sequencing.

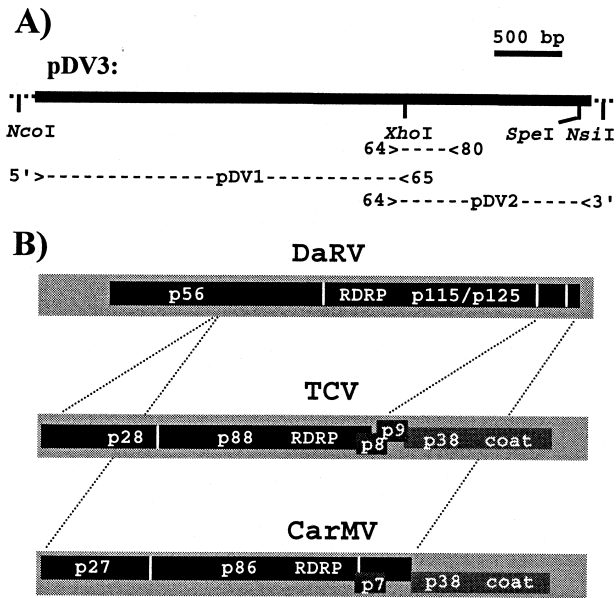


Fig. 1. (A) Construction scheme for the full-length cDNA clone pDV3 of DaRV. The thick line indicates the viral cDNA. The dotted lines at its ends indicate the vector's multiple cloning site. The location of the primers used for the construction of pDV3 (DaRV-5'/Oli65 and Oli64/DaRV-3') as well as for the amplification of the coding region for the conserved region of the RDRP (Oli64/Oli80) are shown. The restriction sites of *XhoI/NsiI* and *NcoI/SpeI* were used for the construction or for the linearization, respectively, of pDV3 for *in vitro* transcription. (B) Comparative scheme of genome organization of DaRV, TCV (Carrington *et al.*, 1989) and CarMV (Guilley *et al.*, 1985). All genomes are about 4 kb in size. In the scheme, ORFs are displayed as darker inlet blocks. The locations of potential readthrough stop codons (UAG) are indicated by white vertical bars. The names of the potentially translated proteins are derived from their molecular masses taking the potential readthrough into account. The location of the RDRP domain is indicated as well as the ORF encoding the coat protein in TCV and CarMV. Homologous genome regions of all three viruses are restricted by dotted lines.

■ **Sequence analyses.** Sequencing reactions of the PCR, RT-PCR and of the cloned cDNA products were performed with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). The sequenced products were separated and analysed with an ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

Sequences were analysed using the computer program Sequence Navigator (Perkin-Elmer) and the programs Translate, SIM/PRSS, PSORT, CLUSTALW and WU-BLAST listed at the ExPASy home page (<http://www.expasy.ch/tools/>). The GenBank/EMBL and SWISS-PROT/TrEMBL databases were used for homology searches.

■ **Northern blot hybridization.** Total nucleic acids from 1 mg of mycelia of isolate CMW3407 and CMW5588 were separated in a 1% agarose gel in 1 × Tris-acetate/EDTA buffer. As positive controls, isolated dsRNA and *in vitro*-produced positive- and negative-stranded RNA from pDV3 were included. The latter were produced by the T7 or the SP6 RNA polymerase from pDV3, which was linearized with *SpeI* or *NcoI* (Fig. 1A), respectively. The agarose gel was denatured in 50 mM NaOH/150 mM NaCl for 30 min and subsequently neutralized in 1 M Tris-HCl (pH 7.5)/1.5 M NaCl twice for 15 min. The nucleic acids were then transferred by a capillary blot onto positively charged nylon membrane (Roche Molecular Biochemicals). Hybridization with DIG-labelled, strand-specific RNA probes was carried out according to the manufacturer's protocols in DIG Easy Hyb buffer at 68 °C (Roche

Molecular Biochemicals). Colorimetric detection was done with NBT/BCIP.

Results

Synthesis and sequencing of cDNA from dsRNA of *D. ambigua*

Single-sized dsRNA elements of about 4 kb were isolated from the hypovirulent *D. ambigua* isolate CMW3407 using the dsRNA isolation protocol described in Methods. DsRNA elements of the same size from other isolates of this fungus had been isolated and studied previously (Smit *et al.*, 1996b). In an initial attempt to obtain some sequence information for this dsRNA element, mixed hexanucleotides were used to randomly prime cDNA synthesis from heat-denatured, gel-purified dsRNA based on the method of Gubler & Hoffmann (1983). To avoid the low efficiency of blunt-end clonings, the cDNA products were restricted by the frequently cutting restriction enzyme *Sau3AI*. The fragments were then cloned into the compatible *BamHI* site of the vector pGEM-3Zf(+). The transformation yielded nine clones with inserts of 100 bp to 500 bp. A preliminary sequence analysis suggested that at least four of the inserts might have been derived from a viral genome.

Primers were designed from the short cDNA sequences. Different primer combinations were then randomly applied in RT-PCR to amplify overlapping products to determine gaps between the sequenced parts of the putative genome. This worked in a few cases. In other cases RT-PCR products were obtained from reactions in which only one specific primer amplified a product from its specific binding site. These products, which were very valuable for establishment of the sequence, were apparently due to nonspecific binding of the primer to a homologous region on the template close to its specific site. The second primers in such reactions were found to have been derived from a cDNA clone that was not of viral origin. Using the sequences of the RT-PCR products, the cDNA sequence of the whole dsRNA element could be completed, with the exception of the terminal sequences. The distal 5' and 3' termini of the dsRNA were then determined by a 5' RACE approach (Frohman, 1994). For both ends, single PCR products were amplified with an oligo(dT) primer and a terminus-specific primer. The products were directly sequenced with nested primers. The termini did not show any variation in sequence or length.

The described sequencing strategy established the complete cDNA sequence of the dsRNA genetic element of *D. ambigua*. The ability to determine distinct ends of the dsRNA genetic element shows that the dsRNA is a linear molecule. The sequence consists of 4113 bp with a GC content of 53%. The accuracy of the sequence was confirmed through sequencing both strands of the full-length cDNA clone pDV3 (construction described in Methods; Fig. 1A). Only two sequence variations were observed, namely at positions 389 (G → A) and 1743 (G → A).

Sequence analysis of the complete cDNA of the dsRNA genetic element

Analysis of the complete cDNA sequence revealed the presence of two large open reading frames (ORFs). Translation of the first ORF might be initiated at the AUG start codon at position 576 and be terminated at the UAG stop codon at position 2085. Translation of the second ORF might be internally initiated at the AUG start codon at position 2202 to the stop codon UAG at position 3678. However, it is common in the translation of RNA viruses for ribosomes to readthrough UAG (amber) stop codons and to produce a fusion protein (Skuzeski *et al.*, 1991). A readthrough of UAG at position 2085–2087 is supported by the fact that both ORFs are in the same frame, which is essential for a readthrough translation. Furthermore, the flanking region of this amber codon (²⁰⁸²UUU-UAG-GGA²⁰⁹⁰) is similar to the consensus sequence AAA-UAG-G (^{G/U})(^{G/A}) around readthrough codons found in carmoviruses (Skuzeski *et al.*, 1991). Therefore, the two ORFs are most probably translated as a fusion protein from the AUG at position 576. Translation of this fusion protein might be extended over two more UAG codons at positions 3678 (the end of the second ORF) and 3846 and might stop at the UGA stop codon at position 3954. No other ORFs of significant length can be detected either upstream of position 576 or downstream of position 3954.

Considering the different possibilities for readthrough translations, proteins of 503, 1034, 1090 and 1126 amino acids might be translated from the genetic element of *D. ambigua*. Their predicted molecular masses would be 56.0, 114.9, 120.7 or 124.3 kDa, respectively. These predictions do not take the unknown substitutes for the amber codons into account. In the following characterization, the longest translation product, p125, which is 1126 amino acids long, will be considered. However, the shorter translation product p56 (N-terminal part of p125) might be the major translation product because readthrough events occur at a lower rate than translation termination (Skuzeski *et al.*, 1991).

The PSORT program, employing the method of Klein *et al.* (1985), predicts eight possible transmembrane helices for p125 (Fig. 2). Six of these are predicted to occur at the N terminus, which is also part of p56, and two at the C terminus. Therefore, p56 and p125 could be associated with membranes of the host cell.

In BLAST searches against the SWISS-PROT and TrEMBL protein databases, protein p125 shows the highest homology to non-structural proteins encoded by positive- single-stranded (ss)RNA viruses in the plant virus family *Tombusviridae*. These proteins contain the RNA-dependent RNA polymerase (RDRP) domain. Using the SIM program for binary protein alignments, the nonstructural proteins of the carmoviruses *Turnip crinkle virus* (TCV; Carrington *et al.*, 1989) and *Carnation mottle virus* (CarMV; Guilley *et al.*, 1985), both members of *Tombusviridae*, were separately aligned to p125. The PRSS

program was used to evaluate the statistical significance of the alignments, which all were confirmed as significant. The RDRPs of TCV and CarMV show an identity of 28.0% and 24.4%, respectively, to p125. The two carmoviral RDRPs share an identity of 43.6% between each other. The RDRPs of TCV and CarMV are also readthrough translation products with sizes of 775 and 869 amino acids. In Fig. 2, p125 is aligned with the nonstructural proteins of TCV and CarMV. The conserved motifs of RDRP domains (O'Reilly & Kao, 1998) are present in the C-terminal half of all three proteins (Fig. 2, Motifs A–D). However, the p125 sequence shows some important differences from the normal consensus: (1) a glutamate (E⁷⁴²) is present at the place of a second aspartate in motif A (...-D-X-X-X-X-D-...); (2) in the highly conserved motif C (...-G-D-D-...), the second aspartate is replaced by an asparagine (N⁸³²); (3) the lysine in motif D is not present but there would be an alternative lysine (K⁸⁷⁹) seven amino acids closer to the C terminus. To confirm these deviations from the consensus sequences, the region from nucleotides 2648–3247, containing motifs A–D, was independently amplified in 12 separate experiments from *D. ambigua* dsRNA by RT-PCR using the primer pair Oli64/Oli80 (Fig. 1A). The sequences of the RT-PCR products are identical to the initial cDNA sequence (data made available to reviewers) and, therefore, confirm that the sequence of the RDRP of DaRV deviates from the common consensus sequences.

A reduced but still significant homology can be found outside the RDRP domain in the alignment of the three gene products (Fig. 2). However, the N-terminal part of p125 down to the potential readthrough codon, which might also be translated as p56, is N-terminally extended by 220 amino acids compared to the TCV and CarMV proteins (Fig. 2). On its own, the protein p56 does not produce any significant homology to proteins in the SWISS-PROT and TrEMBL protein databases.

The potential translation product p125 shows clear homology to viral RDRPs. It can, therefore, be assumed that the dsRNA in *D. ambigua* is of viral origin. We have named the virus *Diaporthe ambigua RNA virus* (DaRV). Interestingly, the sequence of the DaRV genome does not include an ORF encoding a coat protein. In the cases of TCV and CarMV (Guilley *et al.*, 1985; Carrington *et al.*, 1989), an ORF for a coat protein is located at the 3' end of their genomes, downstream of the coding region for the RDRP (Fig. 1B).

Relative abundance of viral ssRNA versus dsRNA in *D. ambigua*

The dsRNA isolated from the infected *D. ambigua* isolate may represent the genomic or the replicative form of DaRV. In order to test if the DaRV genome is maintained in an ssRNA or in a dsRNA form, a Northern blot hybridization study was done. This also enabled determination of the relative abundance of viral ssRNA versus dsRNA.

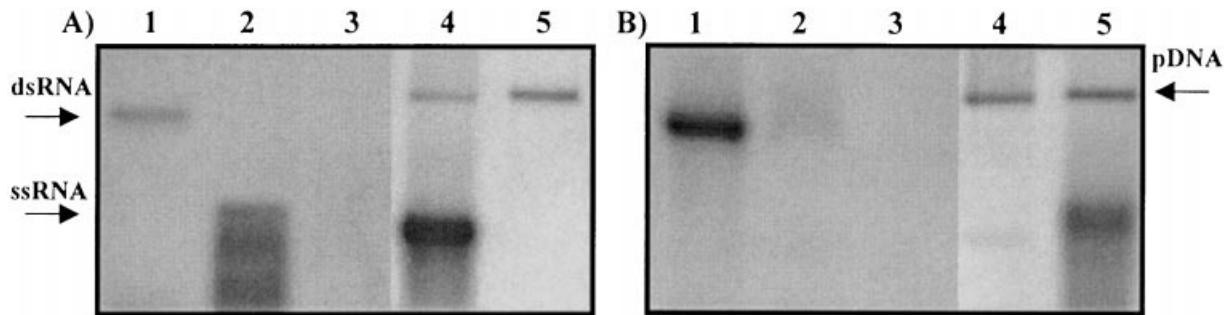


Fig. 3. Detection of viral ssRNA and dsRNA of DaRV by Northern blot hybridization. Detection of positive-stranded (A) and negative-stranded (B) RNA from DaRV with digoxigenin-labelled, strand-specific RNA probes. The following samples were separated by nondenaturing, 1% agarose gel electrophoresis and blotted onto nylon membranes: isolated DaRV dsRNA (lane 1); total nucleic acid from virus-infected (lane 2) and from virus-free (lane 3) *D. ambigua* isolate; *in vitro*-produced positive-stranded (400 bp shorter than full-length, lane 4) and negative-stranded (full-length, lane 5) RNA transcribed from pDV3. Parts of the blots were incubated for different times for colour development: lanes 4 and 5 for 2 h, lanes 1–3 of blot A for 4 h and of blot B overnight. The positions of the dsRNA and the ssRNA forms of DaRV as well as the linearized pDV3 (used in the *in vitro* transcription reaction) are indicated on the left and right sides, respectively.

the position where the positive-strand RNA control migrated was very strong, while the slower migrating dsRNA could hardly be detected (Fig. 3 A). A smear of RNA migrating faster than the full-length DaRV RNA was also observed. This band most probably represents shorter transcripts or different conformations of the positive-strand RNA of DaRV. With the negative-strand RNA probe, only a faint signal of dsRNA was observed in the total nucleic acid preparation from the virus-infected isolate (Fig. 3 B). No signal was observed with either probe in the lanes with nucleic acid from the virus-free isolate.

This result shows that positive-strand RNA of DaRV is present in much larger amounts than dsRNA in the virus-infected fungal isolate. Therefore, it is possible that the positive-strand RNA is in fact the genome of DaRV. The dsRNA might in this case represent only a replication form of the RNA virus genome.

Discussion

The linear dsRNA genetic element isolated from hypovirulent isolates of *D. ambigua* most probably represents a replicative form of a positive-strand RNA virus, which we have named DaRV. The putative protein p125 is possibly translated from the 4.1 kb DaRV genome by reading through up to three amber stop codons (Fig. 2). Its C-terminal half shows the highest sequence similarities to the nonstructural proteins of the plant viruses TCV and CarMV, which contain the RDRP domain (Guilley *et al.*, 1985; Carrington *et al.*, 1989). Both are classified in the genus *Carmovirus* of the family *Tombusviridae*. They contain positive-strand RNA genomes of a size similar to that of the genome of DaRV and translate the nonstructural protein containing the RDRP domain by reading through amber stop codons.

Mycoviruses other than DaRV have been reported to show distant sequence relationship to *Tombusviridae*. Koonin & Dolja (1993) studied the tentative phylogeny of positive-strand

RNA viruses based on multiple alignments of the conserved RDRP domains. In their study, the *Saccharomyces cerevisiae* 20S and 23S narnaviruses grouped with tombusviruses among others in supergroup II. Recently, the fungal mitochondrial RNA replicons, the mitoviruses, were also reported to be part of this lineage (Hong *et al.*, 1998, 1999). Because DaRV shows the highest sequence similarities to the *Tombusviridae*, we propose that DaRV is a member of supergroup II of positive-strand RNA viruses.

DaRV is among a number of mycoviruses that show distinct relatedness to plant viruses (Ghabrial, 1998). In addition to the narnaviruses and mitoviruses, it was previously shown that the hypoviruses of *C. parasitica* have common ancestry with plant potyviruses (Koonin *et al.*, 1991). *Mushroom bacilliform virus*, a barnavirus with an ssRNA genome, was also reported to share sequence similarity with plant viruses of the genus *Polerovirus* and to sobemoviruses (Revell *et al.*, 1999). However, there is no obvious relationship based on sequence similarity and genome organization between these viruses and DaRV, except around the consensus sequences in the RDRP domain.

The deviations from the conserved motifs observed in the DaRV RDRP are of particular interest with respect to the activity of the polymerase. These motifs are present in all classes of polymerases and certain polymerase functions are attributed to them (O'Reilly & Kao, 1998). Motif A is possibly involved in the coordination of divalent metal cofactors and the selection for NTP or dNTP. It is therefore interesting that the modified Motif A of DaRV (-D⁷³⁷-X-X-X-E⁷⁴²-; Fig. 2), with a glutamate instead of an aspartate at position 742, resembles more the active site of DNA polymerases than that of an RDRP (O'Reilly & Kao, 1998; Patel & Loeb, 2000). The -G-D-D- consensus sequence of Motif C, which is also involved in the coordination of divalent metal ions, is highly conserved in RDRPs, especially the two aspartates (O'Reilly & Kao, 1998). Therefore, it might be important for the function of the

DaRV RDRP that the second aspartate in DaRV Motif C is replaced by an asparagine (Fig. 2). The same, modified -G-D-N- sequence can only be found in the functional RDRP domain of the L proteins of nonsegmented negative-strand RNA viruses, like *Rabies virus* or *Measles virus* (Schnell & Conzelmann, 1995).

These few but significant differences in the conserved motifs of the DaRV RDRP might cause an altered polymerase activity. However, the possibility that the viral sequence represents a defective RNA cannot be ruled out. This RNA could be in such a high abundance that the active viral genome could not be detected in the several, independent RT-PCR amplifications that we did. Only a successful transfection or transformation of *D. ambigua* with *in vitro*-produced viral RNA or with a cDNA construct from DaRV, respectively, can definitely indicate that the described viral genome is functional.

The most significant difference in the genome organization of DaRV and the carmoviruses is that the DaRV genome encodes neither a coat protein (p38) nor small movement proteins (p7, p8 or p9) such as are found for TCV or CarMV (Fig. 1B). Movement proteins are essential for the intercellular spread in plants (Hacker *et al.*, 1992) and, therefore, might be dispensable in a mycovirus. In carmoviruses, coat proteins make up a large number of isometric virus particles of about 25 nm in diameter (Russo & Martelli, 1982). A possibility remains that p56 or a cleaved part of it, for example the N-terminal extension, might function as a coat protein. However, no significant sequence similarity can be found between p56 and coat proteins of carmoviruses or other viruses. Without coat proteins, DaRV probably has no means to form virus particles. Experiments with a TCV mutant lacking the coding region for a coat protein showed that the mutant viral RNA was still able to replicate in the cells of inoculated plant leaves (Hacker *et al.*, 1992).

It might be assumed that the DaRV genome is either present as RNA-p56/p125 nucleoprotein complexes in the cytoplasm or that the virus might be associated with membrane vesicles. While ribonucleoprotein complexes were reported for yeast narnaviruses (García-Cuellar *et al.*, 1997), the unencapsidated hypovirus of *C. parasitica* was found to be associated with fungal vesicles (Hansen *et al.*, 1985; Fahima *et al.*, 1993). The N-terminal, hydrophobic part of p56/p125 could favour a membrane association of DaRV. Furthermore, it has been shown that the replication of positive-stranded RNA virus genomes commonly takes place in close association with membranes (Schaad *et al.*, 1997). The N terminus of p56/p125 could ideally anchor the viral protein in a membrane through the six predicted transmembrane helices. Interestingly, the non-structural proteins of the tombusviruses *Cymbidium ringspot virus* and *Carnation Italian ringspot virus* target the peroxisomal or mitochondrial membranes based on their N-terminal sequences (Rubino & Russo, 1998; Rubino *et al.*, 2000). The targeting is determined by the leader sequence as well as the transmembrane segments. Further studies will be needed to clarify the localization of DaRV in the cell.

The hypovirulence-associated mycovirus DaRV, with its relatively small genome size, is of great interest for the development of a biological control system for *D. ambigua* and other related plant pathogens. This study has characterized the virus at sequence level, which is an important step towards any advanced use of this virus in biological control. The development of protocols for transfection and transformation of the fungus with DaRV is under way. Such studies are essential to understand the mechanism involved in hypovirulence, as well as for a practical application of this virus in biological control.

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References

- Anagnostakis, S. L. & Waggoner, P. E. (1981).** Hypovirulence, vegetative incompatibility, and the growth of cankers of chestnut blight. *Phytopathology* **71**, 1198–1202.
- Carrington, J. C., Heaton, L. A., Zuidema, D., Hillman, B. I. & Morris, T. J. (1989).** The genome structure of turnip crinkle virus. *Virology* **170**, 219–226.
- Fahima, T., Kazmierczak, P., Hansen, D. R., Pfeiffer, P. & van Alfen, N. K. (1993).** Membrane-associated replication of an unencapsidated double-strand RNA of the fungus, *Cryphonectria parasitica*. *Virology* **195**, 81–89.
- Frohman, M. A. (1994).** On beyond classic RACE (rapid amplification of cDNA ends). *PCR Methods and Applications* **4**, S40–S58.
- García-Cuellar, M. P., Esteban, R. & Fujimura, T. (1997).** RNA-dependent RNA polymerase activity associated with the yeast viral p91/20S RNA ribonucleoprotein complex. *RNA* **3**, 27–36.
- Ghabrial, S. A. (1998).** Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* **16**, 119–131.
- Gubler, U. & Hoffmann, B. J. (1983).** A simple and very efficient method for generating cDNA libraries. A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263–269.
- Guilley, H., Carrington, J. C., Balazs, E., Jonard, G., Richards, K. & Morris, T. J. (1985).** Nucleotide sequence and genome organization of carnation mottle virus. *Nucleic Acids Research* **13**, 6663–6677.
- Hacker, D. L., Petty, I. T. D., Wei, N. & Morris, T. J. (1992).** Turnip crinkle virus genes required for RNA replication and virus movement. *Virology* **186**, 1–8.
- Hammar, S., Fulbright, D. W. & Adams, G. C. (1989).** Association of double-stranded RNA with low virulence in an isolate of *Leucostoma peroonii*. *Phytopathology* **79**, 568–572.
- Hansen, D. R., van Alfen, N. K., Gillies, K. & Powell, W. A. (1985).** Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. *Journal of General Virology* **66**, 2605–2614.
- Heiniger, U. & Rigling, D. (1994).** Biological control of chestnut blight in Europe. *Annual Review of Phytopathology* **32**, 581–599.
- Hong, Y., Cole, T. E., Brasier, C. M. & Buck, K. W. (1998).** Evolutionary relationships among putative RNA-dependent RNA polymerases encoded by a mitochondrial virus-like RNA in the Dutch elm disease fungus, *Ophiostoma novo-ulmi*, by other viruses and virus-like RNAs and by the *Arabidopsis* mitochondrial genome. *Virology* **246**, 158–169.

- Hong, Y., Dover, S. L., Cole, T. E., Brasier, C. M. & Buck, K. W. (1999).** Multiple mitochondrial viruses in an isolate of the Dutch elm disease fungus *Ophiostoma novo-ulmi*. *Virology* **258**, 118–127.
- Huang, S. & Ghabrial, S. A. (1996).** Organization and expression of the double-stranded RNA genome of *Helminthosporium victoriae* 190S virus, a totivirus infecting a plant pathogenic filamentous fungus. *Proceedings of the National Academy of Sciences, USA* **93**, 12541–12546.
- Klein, P., Kanehisa, M. & DeLisi, C. (1985).** The detection and classification of membrane-spanning proteins. *Biochimica et Biophysica Acta* **815**, 468–476.
- Koonin, E. V. & Dolja, V. V. (1993).** Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Critical Reviews in Biochemistry and Molecular Biology* **28**, 375–430.
- Koonin, E. V., Choi, G. H., Nuss, D. L., Shapira, R. & Carrington, J. C. (1991).** Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. *Proceedings of the National Academy of Sciences, USA* **88**, 10647–10651.
- Liu, Y.-C. & Milgroom, M. G. (1996).** Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* **86**, 79–86.
- Nuss, D. L. (1992).** Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. *Microbiological Reviews* **56**, 561–576.
- O'Reilly, E. R. & Kao, C. C. (1998).** Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. *Virology* **252**, 287–303.
- Patel, P. H. & Loeb, L. A. (2000).** DNA polymerase active site is highly mutable: evolutionary consequences. *Proceedings of the National Academy of Sciences, USA* **97**, 5095–5100.
- Preisig, O., Wingfield, B. D. & Wingfield, M. J. (1998).** Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. *Virology* **252**, 399–406.
- Revell, P. A., Davidson, A. D. & Wright, P. J. (1999).** Identification of a subgenomic mRNA encoding the capsid protein of mushroom bacilliform virus, a single-stranded RNA mycovirus. *Virology* **260**, 273–276.
- Rubino, L. & Russo, M. (1998).** Membrane targeting sequences in tombusvirus infections. *Virology* **252**, 431–437.
- Rubino, L., Di Franco, A. & Russo, M. (2000).** Expression of a plant virus non-structural protein in *Saccharomyces cerevisiae* causes membrane proliferation and altered mitochondrial morphology. *Journal of General Virology* **81**, 279–286.
- Russo, M. & Martelli, G. P. (1982).** Ultrastructure of turnip crinkle- and saguaro cactus virus-infected tissues. *Virology* **118**, 109–116.
- Schaad, M. C., Jensen, P. E. & Carrington, J. C. (1997).** Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *EMBO Journal* **16**, 4049–4059.
- Schnell, M. J. & Conzelmann, K.-K. (1995).** Polymerase activity of *in vitro* mutated rabies virus L protein. *Virology* **214**, 522–530.
- Shapira, R., Choi, G. H. & Nuss, D. L. (1991).** Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. *EMBO Journal* **10**, 731–739.
- Skuzeski, J. M., Nichols, L. M., Gesteland, R. F. & Atkins, J. F. (1991).** The signal for a leaky UAG stop codon in several plant viruses includes the two downstream codons. *Journal of Molecular Biology* **218**, 365–373.
- Smit, W. A., Viljoen, C. D., Wingfield, B. D., Wingfield, M. J. & Calitz, F. J. (1996 a).** A new canker disease of apple, pear and plum rootstocks caused by *Diaporthe ambigua* in South Africa. *Plant Disease* **80**, 1331–1335.
- Smit, W. A., Wingfield, B. D. & Wingfield, M. J. (1996 b).** Reduction of laccase activity and other hypovirulence-associated traits in dsRNA-containing strains of *Diaporthe ambigua*. *Phytopathology* **86**, 1311–1316.
- Smit, W. A., Wingfield, B. D. & Wingfield, M. J. (1998).** Integrated approach to controlling *Diaporthe* canker of deciduous fruit in South Africa. *Recent Research Developments in Plant Pathology* **2**, 43–62.
- Steenkamp, E. T., Wingfield, B. D., Swart, W. J. & Wingfield, M. J. (1998).** Double-stranded RNA and associated virulence in South African isolates of *Sphaeropsis sapinea*. *Canadian Journal of Botany* **76**, 1412–1417.
- Valverde, R. A., Nameth, S. T. & Jordan, R. L. (1990).** Analysis of double-stranded RNA for plant virus diagnostics. *Plant Disease* **74**, 255–258.

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