A Virulence-Associated, 6.4-kb, Double-Stranded RNA from *Rhizoctonia solani* Is Phylogenetically Related to Plant Bromoviruses and Electron Transport Enzymes

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We have recently shown that acquisition of a 6.4-kb, double-stranded (ds) RNA (M1) by hyphal anastomosis is associated with enhanced vigor and virulence, whereas its removal by hyphal tipping correlates with diminished virulence in the plant-pathogenic basidiomycete *Rhizoctonia solani*. The M1 dsRNA is not encapsidated by a typical nucleocapsid, has a circular and/or concatemeric form, and is associated with the mitochondrial and cytosolic fractions. M1 possesses six open reading frames (ORFs) the longest of which (ORF 2) is located on the (+) strand, and encodes a putative polypeptide consisting of 1,747 amino acids or 199.4 kDa. This polypeptide has a significant amino acid sequence similarity, including six conserved helicase domains and an ATP/GTP binding motif, with the 1A protein of broad bean mottle virus (BBMV) and other bromoviruses. ORF 5, which is located on the (–) strand of M1 and is complementary to a region of ORF 2, codes for a putative polypeptide that has a significant amino acid sequence similarity with the cytochrome c oxidase assembly factor. This complementarity provides direct evidence suggesting that the long-standing hypothesis of viruses evolving from cellular genes may be valid.

Rhizoctonia solani is a ubiquitous, soilborne, plant-pathogenic basidiomycete that causes economically important diseases on numerous crop, turfgrass, and tree species worldwide (Sneh et al. 1996). It is thought to be a collective species comprising several anastomosis groups (AG), which are, in general, genetically isolated, noninterbreeding populations with distinct host ranges (Anderson 1982). Thirteen AGs have been described to date (Caroling 1996). The question as to whether double-stranded (ds) RNA moieties affect the degree of pathogenicity (virulence) in *R. solani* has been debated among a number of research groups globally in the last 20 years. An initial study linking dsRNA to hypovirulence (Castanho et al. 1978) in this plant pathogen was followed by

studies that did not corroborate this observation. One research group reported that dsRNA conferred virulence (Finkler et al. 1985), whereas other groups, working independently, found no apparent relationship between the mere presence of dsRNA and degree of pathogenicity in *R. solani* (Zanzinger et al. 1984; Hyakumachi et al. 1985; Washington and Martin 1991; Kousik et al. 1994). In the early 1990s, we reported that dsRNA elements from the same isolate, cultures obtained from the same field, or isolates from different AGs were genetically different (Bharathan and Tavantzis 1990, 1991). This work suggested the existence of a high degree of genetic variation in dsRNA populations, which, combined with the genotypic variability of the host fungus, could explain, at least in part, the conflicting data arising from the above studies.

In recent years, we have focused on a particular set of dsRNAs, some of which appeared to be associated with a number of phenotypic characters including degree of pathogenicity (Lakshman and Tavantzis 1994; Tavantzis and Lakshman 1995). The four AG 3 isolates, Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3, have been described previously (Lakshman and Tavantzis 1994). Rhs 1AP is a virulent field isolate. Three morphologically distinct sectors of Rhs 1AP gave rise to the hypovirulent (essentially nonpathogenic) cultures Rhs 1A1, Rhs 1A2, and Rhs 1A3. We have reported that five genetically different dsRNAs (25, 22, 6.4, 3.6, and 1.2 kb) are found in these four isolates, with each culture containing a unique combination of two or more of these dsRNAs (Lakshman and Tavantzis 1994).

In this paper, we present data on the characterization of one of these five dsRNAs, a virulence-enhancing, 6.4-kb molecule (M1) that is found in the mitochondria and cytoplasm, has a circular or concatemeric form, and has no typical protein capsid. Sequence analysis showed that M1 possesses a helicase gene that has a significant sequence similarity with the helicase of bromoviruses. A portion of the helicase gene is complementary to an open reading frame (ORF) that has a strong similarity in amino acid sequence with the cytochrome c oxidase assembly factor (CcOAF). A portion of this work has been presented previously (Jian et al. 1996).

RESULTS

Subcellular distribution of the M1 dsRNA.

dsRNA analysis of subcellular fractions by Northern (RNA) blot hybridization showed that the nuclease-treated mitochon-

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drial fraction (Fig. 1, lane 1) and the high-speed pellet fraction (Fig. 1, lane 4) contained relatively high concentrations of M1. In contrast, nuclei (Fig. 1, lane 2) contained no detectable amount of M1. The membrane and cytosolic fractions (Fig. 1, lanes 3 and 5, respectively) had small amounts of this dsRNA.

Partial characterization of M1-containing structures.

Initial work showed that, following ultracentrifugation ($105,000 \times g$, 4 h), M1 was associated with a soft, membrane-like layer separated easily from the lower and more compact portion of the high-speed pellet. This M1-containing material was very difficult to resuspend in relatively small volumes (up to 5 ml) of various aqueous buffer solutions in spite of the use of sucrose cushions (20%, wt/vol) containing dithiothreitol (1 to 2 mM) in high-speed centrifugation, overnight stirring of the high-speed pellets, and other methods that often facilitate resuspension of viruslike particles (VLP) from high-speed pellets (Tavantzis and Bandy 1988). Spectrophotometric scanning (320 to 220 nm) of resuspended preparations (clarified or not) failed to generate absorbance profiles indicative of a typical nucleoprotein. Examination of samples resulting from resuspension of high-speed pellets by electron microscopy showed amorphous entities of various sizes but not VLPs of any shape. Electrophoretic fractionation of the same samples on 1% agarose gels and staining with ethidium bromide revealed slow-migrating entities (Fig. 2, lane 1) that, upon disruption with protein denaturing agents (Tavantzis and Bandy 1988), released the M1 and L2 dsRNAs (Fig. 2, lane 2). The top band in lane 1 of Figure 2 represents RNA complexes that did not migrate into the gel. The protein content of M1-carrying moieties was considerably lower than that of typical VLPs (data not shown). The M1 dsRNA was protected from pancreatic RNase (20 $\mu\text{g}/\text{ml}$ in 0.3 M NaCl) in samples not subjected to denaturation treatment.

Fig. 1. Autoradiograph of a double-stranded (ds) RNA Northern blot of subcellular fractions from the *Rhizoctonia solani* isolate Rh5 1A1. Blot includes mitochondrial (1), nuclear (2), membrane (3), high-speed pellet (4), and high-speed supernatant (5) fractions (see Materials and Methods), and was hybridized with a ^{32}P -labeled, 3.9-kb cDNA clone of the M1 dsRNA. Migrating position of M1 is indicated by arrow on right.

Sequence analysis of M1.

The full sequence of M1 (cDNA form) was determined by sequencing the cDNA clones as shown in Figure 3. The linear dsRNA form contains 6,390 bp of nucleotides with the (–) strand ending in a G (Fig. 4). A circular or concatemeric form of M1 has eight G-C pairs between the two ends of the linear form (see below) but, due to the GC tailing reaction of the 5'-RACE (rapid amplification of cDNA ends) it was not possible to determine their exact distribution in the 3' or 5' end of the (+) or the (–) strand of M1. All of the eight Gs are located on the (–) strand. Base composition analysis indicated that M1 is AT-rich (AT = 59.3%). This and the fact that a large portion of the 6.4-kb dsRNA was associated with the mitochondrial fraction were the reasons for using the yeast mitochondrial genetic code for sequence analysis of M1. It should be noted, however, that the universal genetic code gave information very similar to that presented here. M1 has six ORFs (Fig. 5). The strand carrying the longest ORF (ORF 2) was designated as the positive (+) strand. ORF 1 is on frame +3 and putatively codes for a 25-kDa polypeptide. ORF 2, which is on frame +1, has 1,747 amino acids (aa) and encodes a putative 199.4-kDa polypeptide. ORF 3 to ORF 6 are on frame –1 and code for polypeptides of 29.8, 14.5, 16.2, and 14.7 kDa, respectively.

Sequence similarities.

The putative ORF 2 polypeptide has a significant sequence similarity with the 1A protein of broad bean mottle virus

Fig. 2. Fractionation of M1- and L2-associated moieties following high-speed centrifugation either untreated (1) or denatured (2) (see Materials and Methods) on 1% agarose. Numbers on right indicate size (kb) of L2 (22 kb) and M1 (6.4 kb) double-stranded RNAs. Top band of lane 1 represents RNA complexes that have not entered the gel. Arrows on left show untreated, slow-migrating structures containing above two dsRNAs.

(BBMV) and other plant bromoviruses (Dzianott and Bujarski 1991) (Fig. 6). In a region of 966 aa (positions 27 to 993 of ORF 2), the two proteins have 164 identities (15.5%), and 431 conservative substitutions (40.7%). Moreover, the degree of conservation between the ORF 2 polypeptide and the 1A protein of bromoviruses is much greater (than the above percentages) in the stretches corresponding to the six helicase domains (Fig. 6). Most interestingly, ORF 2 contains the conserved motif GAPGTGKT (amino acid positions 742 to 749) that is characteristic of purine nucleotide triphosphate-binding proteins (Walker et al. 1982).

Substantial homology exists between ORF 2 and polyprotein 1b of the lactate dehydrogenase-elevating virus (LDV) found in chronically infected mice exhibiting high levels of lactate dehydrogenase and other serum enzyme activities (Godeny et al. 1993). In a region of 910 aa (positions 472 to 1382 of ORF 2), which includes conserved regions for a zinc finger and RNA helicase from polyprotein 1b of LDV, the two proteins have 150 identities (16.5%), and 392 (43.1%) conservative substitutions (data not shown). The respective percentages of amino acid identities and similarities are much greater than the above in the regions that include the helicase domains and the zinc finger.

ORF 5, located on the (–) strand, encodes a putative polypeptide of 141 aa (16.2 kDa), which has sequence similarity with the CcOAF (Saraste et al. 1991). The two polypeptides have a significant homology in stretches ranging from 10 to 50 aa and totaling 110 aa (of the 141 aa total), with 31% identities and 19% conservative substitutions (data not shown). No extensive sequence similarities were found for ORF 1, ORF 3, ORF 4, or ORF 6.

M1-related RNAs.

Inverse reverse transcription–polymerase chain reaction (RT-PCR) with total dsRNA from Rhs 1A1 as a template gave rise to a 420-bp product (Fig. 7A, lane 1) as determined by sequencing, thus suggesting the presence of circular and/or concatemeric forms of M1. The sequence of the 420 bp con-

sisted of the expected 156 nucleotides from the 5′ end and 264 nucleotides from the 3′ end of (+) strand plus eight G-C pairs located between base positions 1 and 6390, i.e., the first and last identifiable nucleotides of the (+) strand. The 5′ RACE protocol, used to determine the sequence of the two ends of M1, prevented the distinction between the above eight G-C pairs and those added through the G and C tailing reactions included in this protocol.

Northern blot hybridization of dsRNA or total RNA from Rhs 1AP revealed M1-related RNA molecules migrating slower than M1 (Fig. 7C). An RNA species of low concentration was associated with the dsRNA-enriched fraction. This RNA migrated to a position that corresponded to that of a 18- to 20-kb linear DNA molecule (Fig. 7C, lane 1). In comparison, the total RNA sample contained M1-related complexes the sizes of which ranged from 6.4 to 20 kb (Fig. 7C, lane 2). These results suggest the presence of a dsRNA circular or concatemeric replicative form (Fig. 7C, lane 1) and replicative intermediates (Fig. 7C, lane 2) of M1. There were no M1-related subgenomic RNA forms (Fig. 7B and C).

DISCUSSION

The association of the M1 dsRNA with virulence in Rhs 1AP and derivative cultures (Jian et al. 1997) triggered questions related primarily to the genetic information carried by this element, and secondarily to its physical existence within the cell. It appears that M1 is associated with membranelike vesicles but more data is needed to demonstrate this unequivocally. The dsRNA genome of the hypovirus causing hypovirulence in the ascomycete *Cryphonectria parasitica* has been found to be packaged in membranous vesicles of host origin (Dodds 1980; Hansen et al. 1985). Approximately one-half of the M1 molecules are associated with the mitochondrial fraction (Fig. 1). Similarly, dsRNAs linked to reduced aggressiveness in the Dutch elm disease incitant *Ceratocystis ulmi* (Rogers et al. 1987) or hypovirulence in *C. parasitica* (Polashock and Hillman 1994) are located in the mitochondria.

Fig. 3. Schematic showing an overview of the strategy used in sequencing the M1 double-stranded RNA.

The M1 moiety appears to possess a circular or concatemeric replicative form as indicated by the inverse RT-PCR (Fig. 7A, lane 2) and Northern blot hybridization data (Fig. 7C, lane 1). The size range of the hybridizing probe streak on lane 2 (Fig. 7B and C) matches the expected size range for the replicative intermediates of the above putative replicative form. The function (if any) of the nine terminal Gs of the (–)

strand is unknown. Wu and Kaper (1994) showed that the 3'-terminal G of the satellite (–) RNA strand was an absolute requirement for satellite (+) strand synthesis by the cucumber mosaic virus (CMV) RNA-dependent RNA polymerase (RDRP).

The putative polypeptide encoded by ORF 1 showed no significant homology with polypeptides listed in various data

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1      GACTAAAAAT GCAATTTAGT CGCAATATCC ACACAAACAC AACCATACAA CCCAAGCATA 60
61     TATTTTCGACT AGATTGCCAT CAGGGGGAAA AGATTGATTT TCTACTCCCC CCCAGAAGAC 120
121    CCCCAAATTT GATTTAAACCA GAGGATGCTA GAGCAGCTAT TTATAGCTCT TGGTTGAAAG 180
181    GTGTAGCCTG GGGTTCCGAA GTTTTAGTCA GCCAAGAAGA AGTGATGACG CATGATAGCT 240
241    CTGTGACAAT CACAAAGAGT TTATTGGGGA CTTGCATAGG CCCGTCAGGA GTAATGAGAC 300
301    TGGGAGTAGA ATATGTGTAT ATCTGTGATC CGCAATTTGA AATTAATTTG AAGGGCCATA 360
361    ACCCAGGGAT GTACCCGATA GCACATTTTA AAGATGCACT GAAGAATCTA CACAGTATCA 420
421    AGGACGCATT CAAAGTAAAG ATCACCACAA AATCGTGCGA AGGTGAAATC CTCCCTGACA 480
481    TTACATACCT TCCAGAACCA ACAGAGATCG TGACAGTGAA ACAAACTGTG CGTTATCCAG 540
541    TCGTTAAAGA CTTGGATCGG GCCAGCTTAT TAAAGTGGAT ACTAGATAGA AGCGATGTTA 600
601    ATGCACCTAT AGTACAGCCA GCACCACAAC TAGAGGATAG ATCTTGAGCC AATAGCAATT 660
661    GTGACGACAG GTAAATTAGG GCATATCAAG AAAGGGTCCA GAATAATATC GGCCGAAGAG 720
721    TTCGACAGGG ATAAATGGAT CAAGGCCGAA AACGTGCCGT TACCAGATGA AGATGATGAA 780
781    GATTCTGGGA AAGTAATCAG TTTCAGAATG ACCAATACTT TTATACAAAA CACAGTACTG 840
841    CTAGAACTTA ACGATTGTGC TAGTTACGAG AACGACGCAC TGTTGCTTGA ACATTATTTCA 900
901    ACCTTCAAAA ATTTACATAA AGTACGACAC ATAGTGGATG AAGACAAAGT GGGCGTCAAC 960
961    AATTTTTCAA AATTTTGGTA CTGTGAAAAG AAAAAATATGA GACAAATGAT GGCCGAAGAG 1020
1021   AGACTGGTCT CTATAGTCGA AGACGATGAA GATTTTCGATA ATGAAATGAC ACTTGACCGT 1080
1081   GTGGCCAGAC TATACAACAC AAGAAAATTG GGAATTGTAA GTAACGGAGA ATGTTTAGAG 1140
1141   ACATGCTGTT TGGAATATGA CTGTGAGTGT TACCCAACCA AATATTGCTC AACTGGATGT 1200
1201   CACAACGGAG TTCGGACCTC CAATGGAGAA AAATTTAGAA GGTTTGTGTT GACACAAGAC 1260
1261   ATGGCCACTC ACATATTAAA GCAAGCAGAA GTGCGGTGCT TATCCACTAA TTTAACAAAT 1320
1321   GACCAACTGT CTAGTTTGTG TAATAAAGCA AATTCAATGT CACAAGGGGA AATAATTTTG 1380
1381   AACCAAGCTG GAAGGACCGC CATTCCATAC ATGTATAAAA TTCCTGAAAG GACGAACGAT 1440
1441   GTAGAGGTGA GGTACAAACC AGTTACAAATC AGTTTAGACG GCAGATCTTT ACTTGACAAC 1500
1501   TGGCATATAC TCGTGTATGTC CGACTCAAAAT GTTGACGCTT TATTCTTTAC AGGCAGACGA 1560
1561   CACATCAACA GAGGTTTAAA CTTTGTACAC TATAACATTG ACGGCACCTG GTTCACACAT 1620
1621   CAAATAGGAA CTAACCAAAA CACCACCTCC GGTGGCAGGA TACAGGT'TGG GAATGGTATG 1680
1681   GTTGGGTCAT CCATAACCCG ACGATTACTT AACATTAGGA CGGCAGACGT CAAAGATCTA 1740
1741   ACAGACGAAG AATATAAAGA TCTGTTCGAC CTGGACTTAG TCATAGAGCC AACCACCAAC 1800
1801   GTCCATTATT TAAAATTCTC ACTGTCTGAT ACGGCCAATA GGTATAAAGA TGGCTTGTAT 1860
1861   GACTTTTTAG TTAGATCTTT ACCATAGAG AACAGTCTCA ATAAATTTGA AGCTGAGATG 1920
1921   GAGAAATATTT CGGTCGAGAT TAATACAAAT TTAATTGAAC TGTATCCGAA AGCCACAGCT 1980
1981   GACGCTTTGA TAGCAGCGGT TGAGACGACA AGGCAGGATT TATCGTTCTG TGACCCTTAC 2040
2041   GGAAAGATAA TTAGAAGAGA ACCAATTGAG GAGTTGGTGG AACCAAGTCA CCTCGAAAAC 2100
2101   TTGGAACGTG AGATTGAGCC AATCATGATC GGCGACACAA TAGACGTCAG AGGAACATTT 2160
2161   ATGAACTACA TGGGTTTAGT CACCTCTGCG CTGAGTACTA AAGCAAAGT TCAATTTGTAT 2220
2221   ATTGAGCAAG AGCTGAATAT GCCTTTCTAT GTCTGTCATC ATAAGTCAGA GTCTACCTGT 2280
2281   TGCCTCATGT GTGCGAAATC ATTCATGAAT GCAGACAGTT TAAAATGTTT TACCAGCGTG 2340
2341   AGACTTCAAT GGTTCGCTAT CACGAAAGTT TTAGAAAGCT TAAACATGAA ACTATTATAT 2400
2401   AAGGAATGGC CAGAATTTGT AGTTCATAT GTTCCACAAA CCAGTTTCAA AACTATATTA 2460
2461   TGGGACGAGT TAATTACTAT AATATTGGCG AGTGATAAAT ACGATATGGT TGGGGGAGAG 2520
2451   AATGTTCTCT TTGATGAACA ATCTGTTTCG AATAATCCAG ACAGAATCCA CGATCCTAGG 2580
2581   AAAAACTGTG TTAATTACAA CTCAAAGTAT TTCAATATTC CAGACATCAC ACGACTCAAT 2640
2641   GAAAGAATTC ATGTGTATGG AACTTACAAC GTGGGCGAAT GCGACGATAC TTCAACTAAT 2700
2701   TTCGCATACA ACACGTGGGT TATGCGGCAA TCTAGATGTT TGATGGGTAA TGAATTCACC 2760
2761   ACGCTAGTCA AACCTGCGTA CAATGGGCTG ATTAACGTTA CAAACATGAG TAAACTAAAA 2820
2821   ACTGGCGACG TGGTGTATC GTGTTGCATT CACAGGCGAA TTTTAAACAA AATCCTACGG 2880
2881   TACGAGAATG TAATAAAACT TGCTGAAGTC CAACCGTGCT GTGGAGTAGT TTGGGTGTTT 2940
2941   CAAACTCAGC ACTCCATAGT ATCGCGCTTC CTCGACTTAA TCAATCGATT TAGGAAAAAG 3000
3001   CAAACTCACA AAACAGAGTT GAAGGCTGTA GTTGGGGCGC CAGGCACAGG GAAAACAACG 3060
3061   GCCACAAAGA CCAATATGAA AACAAACATG AAAACGTTGG CTATTGGGAA AACAAGGAAT 3120
3121   ATTACCAACA AACTCAAAAC AGAAATGCCG CAAATTGAAG TAAGCACACT TGAAAGTGCA 3180
3181   TTAATAATCTT ACAAGGGAGC AACTTACCAA CAACTGATAA TTGAAGAAGC TACTTTGCTG 3240

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(continued on next page)

Fig. 4. cDNA-derived nucleotide sequence of the (+) strand of the M1 (6.4 kb) double-stranded (ds) RNA. Nucleotides 1 to 6390 were determined through cDNA cloning of linear form of M1 (Fig. 4) whereas additional Cs (6391 to 6398) were identified by sequencing the inverse reverse transcription–polymerase chain reaction (RT-PCR) product (Fig. 7). Primers T3R5 (5'-CACTCTGGTCTGATTGAATG-3', bases 6135 to 6155), and T7R1B (5'-CTGCTCTAGCATCTCTGGT-3', bases 156 to 136), used in the inverse RT-PCR are underlined. Numbering begins with the 5'-terminal nucleotide.

bases. It should be noted, however, that the initiation codon of ORF 1 is located on a putative stem-loop structure consisting of a 7-bp stem and a 7-nucleotide loop (not shown). Stem-loop structures located on 5' end untranslated regions are thought to be involved in translational control of mitochondrial genes in yeast, *Chlamydomonas reinhardtii*, and plants (Gillham et al. 1994).

The largest ORF of M1 is ORF 2, which encodes a protein of 1,747 aa. As mentioned above, a significant sequence similarity exists between ORF 2 (positions 27 to 993) and the 1A protein of bromoviruses (Fig. 6), a group of (+) RNA plant

viruses. ORF 2 contains a motif, GAPGTGKT (positions 742 to 749), that matches the conserved NTP-binding site motif G/AXXXXGKS/T (Walker et al. 1982). The biochemical function for the ATP/GTP-binding activity would be to supply energy or substrate for RNA polymerization (Dzianott and Bujarski 1991). Another function for NTP-binding might be related to the NTP-dependent dsRNA unwinding (helicase) activity that operates during dsRNA replication (Hodgman 1988). The presence of the helicase domains and the NTP-binding motif on ORF 2 suggest that the respective putative polypeptide is involved in the replication of M1. All six con-

Fig. 4. (continued from previous page)

3241	AGCATTATG	AGAGAGACAC	TTTGATTGAT	TTGTTCAAGC	CAGAAATTTGT	TTTGCTGATA	3300
3301	GGCGACCCAA	CACAAGGATC	ATGGAAACCT	GACATTGGTG	CTAAAGTGGT	GGGTAAATTC	3360
3361	AAAAATGCAT	TGGACAACGT	TAACGCAGAG	TACATGACAA	TGAGTTACAG	ATTTCGGCAA	3420
3421	CAAGTATGTG	ACGCGCTCAA	ACACTTCGGA	GTGGATGTTA	GACCATCCGA	CGATAAAGAC	3480
3481	ACCATTGTTA	GATTTGTCAA	TGATGTTGAT	AGATTCCTCGG	AACTAGCAGA	CTCAATAGTC	3540
3541	CTAACTTGGA	CTGTTAATGA	TTACAATGAA	ATGACCGATA	AAGGATTCGA	TTGCATGATG	3600
3601	CCCGAGGCTA	CCCAAGGGTA	TGAATGGGAT	CATGTTACCA	TTTATCTCAA	TAGCGCACAC	3660
3661	AGCGTTGATT	CAGTGTATTTC	AAACCAAAAT	TGGATCGTAG	CATTGAGCAG	AGCAAAAATC	3720
3721	TCATTAACAA	TAGCCAGCCA	GGGAGAACCC	ATCACAAGCG	GGCCACCACA	TGTGGTTTTTA	3780
3781	GCACTGATCG	CAAAAATGTG	TGATATCGTG	GCTCAACCAC	CAATGTATGT	GCAAAGAGAT	3840
3841	CATCTTGATT	TATTACCTGT	GCAATGTCAA	ACAGTAATC	AGTCATACAC	AAACTTGCTT	3900
3901	GCGAGAGTTG	CAAGAACGAC	GCAAAGAATA	GCCACTCTAT	CAATGAAATA	TGGAAAAACA	3960
3961	ATAGCCTTAT	TGGCAACTAA	TCCAGCTACT	GCAGCACTAT	CTTTAGCGCT	GGCAGGATGG	4020
4021	CAATTATATT	TTGGTTTCTT	CATGAGTCTG	GGCTCAGCGT	TCAATGTTAT	CAAACCGCAG	4080
4081	ATCGAAGTTG	TGCAGTCCCT	AAGTGCAGAC	AGACCGCCCG	TTACACTAGG	GGAAAGACTAC	4140
4141	TCAACTAGTC	CACCTTTGTC	ATGGGTCCCT	GACACTGAAA	CGAAAATCGC	AGAAAAATTA	4200
4201	AGACAATACC	TTGGAGACAG	AATGGTCACG	TTAGACTCAG	CAATGAGCGT	GTTAGAAGCA	4260
4261	TCTCAGGAAG	TGGGATTGCA	AGCCACGACC	TACAATAGTT	GGGAAGAATA	CATGAAAGAC	4320
4321	AAAACATAATG	AAATGCCACC	ACTAGAAATT	ATGGATCAAT	TAACGATGAA	ATTTAACGTT	4380
4381	AAATTCAAAG	AGCCAGTTCA	ATTTGTAAAG	CCGATACTT	ACAAGGAAGA	TGAAAAAGAG	4440
4441	TATTATACAC	AATCCGCCAT	CACAGCAGCA	AGTATAGCAG	GTTACATCGT	CGGCACCAGA	4500
4501	TATGACCATT	CAATTCAAGC	TGTGGTAGAC	AAAGCCTCGG	AGCGAAAAAT	CGAGATTGGT	4560
4561	GGTTGTAAAG	CTGCCGGACT	ACTCACCCCT	AGCACCCAAA	CCAATTATGG	AGGATTCGAA	4620
4621	ACACAGAAAG	GTTGGGTAAA	ACGCAACTTG	ATGATATATT	ATGCAATCGA	TCAAATAGCA	4680
4681	GACTTGAGCA	GCTTAAACAT	AAAAAATTTG	TACAATACAA	TTTTAGGGGT	GTATCAAACCT	4740
4741	CGAGCCGTTG	AGAAATTTCAG	ACGGCAAGAA	TGGCCAGGTG	GCATCGCTAG	AGCACACACA	4800
4801	GTTAGACCGA	TCGGAGCAAT	GTTGGCTACT	AGTAGCAACG	GGAGATTGAG	ATTGACCTTC	4860
4861	AATGAACATG	ATTCCGCGCA	GTTAACCACG	CTGTCTAGAA	CACCACTTAA	CACGACACTC	4920
4921	ACAAGACAAC	AGGTTACTTA	CACCGACAAT	AAGCAGTCCG	TACAATTACA	ACCAGCCATT	4980
4981	TTCAGAACTA	AAGAAATATT	GAGAGCGCCA	GCAGATTCAA	TTTACAGGAT	AATCGAAATA	5040
5041	GACATTGGTAA	CAAGTAAATA	CATCGGAGAA	AAATACAACG	ATAAATATTG	GGCTGGCGTA	5100
5101	CTTTGGGATT	TTGAAGGTC	GGATGCCAGA	TGGGTGGATT	CGAAGGCTGG	ACAATTAGTT	5160
5161	GGCTTAGACG	CTTTCGCAAT	GCAAACCATG	CATATCGAGT	TACTAACACT	TACTACTACA	5220
5221	GTTCAAGAAG	TGCGTGAATG	GATAAGTATT	AAATCCAGAC	CCACTGAATT	TAAACATGCC	5280
5281	AGAACACACT	TAATTAAAGG	CAAAGAAGAC	TTTGACGCAA	GAAAAACCCC	GATCATCATT	5340
5341	AAATCTATAA	GTTCCAGGAA	AGTTGCAAGT	CATCCACATG	TGCCTGAAGT	TGAAGCATCA	5400
5401	ATGACGGAAA	AAATAGATTT	TGTCACACAG	TTCATTTGAA	GAGAAGAGAC	ATACAGGCAG	5460
5461	TCTATAATAC	GTCAACTTTC	AGATAAAGAA	TACGAGGTCA	ATGCAGAACA	ATACCGAAAC	5520
5521	ATCGGCGTAC	CTAAGAGCGA	CATACGAATG	ATCAATACCA	AAGAGCATGA	AGCACTACCA	5580
5581	AAACCAAGTG	CACCAATGGC	TTCAACAAGT	TGTGGGTTTC	TGGGCATGTG	GGAGCCCCCG	5640
5641	TTCCGAAGTG	AAGTAAACCT	TCCACCAGGA	TGGGAAATAG	CGGAGTCAAA	ACCAACCCCA	5700
5701	GTGAGGAAAG	TATGGAAAAT	GGGAGGTGCA	CAACTATCTG	GAATGCGGTG	GTTGTTTATG	5760
5761	AAAACGAGAG	TCACAACGAA	TCCAAATTTG	ACAAGAGTCA	AGTTAGAGGC	AGGAGCTCAG	5820
5821	CAATTTATTG	CATCACAACA	ACTGATCGTA	ATGAACAGCA	TGAATGCGAA	AGAACCATAA	5880
5881	GAATTATCAA	ATGGGAGATT	AGTGCACAAA	ACGAAAATAG	ACAAAGAAGC	TATGGCAGAA	5940
5941	TATGAAAAGA	AAATGGCAAT	CTGGAGATTG	CTAAACTGAT	GAAAGAAAAC	ACAGTATAAC	6000
6001	AAAATTGAAA	AGAAGAGGAG	ATTTCGATAT	TTACAATCCC	TCCGCGTTTA	ACAACGCCCT	6060
6061	ATAATTAGAAC	ATGTAATGTG	TTCGGCCAGA	AATAAGACAT	CAAGGTTGGG	GAACTTTGAG	6120
6121	CAACGTAAGA	GAAACACTCT	GGTCGTATTG	AATGAGTTAG	TAATAACTCA	AGCTAAATAC	6180
6181	TAAATACGGG	AACAGAAGTA	AATGCCCTAT	AAAGAGTTGA	AAAACAAGTC	AGTCAGACAA	6240
6241	TAATTGACAC	TCGTCAAGAG	TATAAACATG	GCATTAAAGG	AGACATCCTT	TAGTTTGGGA	6300
6301	GATTTAACTA	GAAAAACACT	CAAGTTTATC	ACAAGGAAAT	TAAGGGGAAC	CTTCCAATGT	6360
6361	TGAACATAT	CAACATTTAG	GGAAAAAGAC	CCCCCCCC			

served, helicase-related, amino acid domains of bromoviruses are present in ORF 2 (Fig. 6). The amino acid sequence similarity between M1 and bromoviruses is limited to one gene as opposed to the relationship between the *C. parasitica* hypovirus and the (+) RNA potyviruses, which appears to include

genomic organization (Koonin et al. 1991). Haseloff and co-workers (1984), however, reported striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that differ in genomic organization and viral gene expression. Their study included the plant virus groups

Fig. 5. Diagram showing relative positions of all the open reading frames (ORFs) of the M1 double-stranded RNA from the Rhs 1AP isolate of *Rhizoctonia solani*.

BBMV	TCSCANAKAIADACKKVSAPTCSVMVDGVAGCGKTTAIKETFRFEKDII	711
64ORF2A	-----RKKQTHKTELKAVVGAPGTGKTTATKTNMKTNMKTL	739
	. * . . . . * * * * * * * * * *	
	<u>I</u>	
BBMV	VTANRKSIEDVRKAIIFG--DASDSEVALKVVRTADSAIMHGLPECHRLIV	759
64ORF2A	AIGKTRNITNKTKEPQIEVSTTESALKFYKGAT-----YQQTMI	780
	. . . . . * . . . . . * * * . . *	
BBMV	DEAGLLHYGQLLAVADLCKCSEVLAFGDTEQISFKSR-DATFRMKYCN-I	807
64ORF2A	EEATLTISIYERDTLIDLFKPEFVLTMGDPTQGSWKPDIGAKVVGKFKNAL	830
	. * * * . . . . * * * . * * * * * * * * . * . * .	
	<u>II</u> <u>III</u>	
BBMV	EYDKRDIVSKTFRCPQDVVSAVKILKRKCANRSSKYNGWVSSSKVEKSLS	857
64ORF2A	DNVNAEYMTMSYRFGKQVCDATKHFGVDVRPSDDK----DTIVRFVNDVD	876
	. . . . . * . * . * . . . . . *	
	<u>IV</u>	
BBMV	KSRIVSINQVSMKHKFYLTMTADKAALCSRAKDVGLDKTWVESNMETV	907
64ORF2A	RFPETADSMVTTWTVDYNEMT--DKGFDCMMP-EATQGYEWDHV---TI	920
	. . . . . * . . . * * * * . * . . . . . *	
BBMV	HEAQGKAVDHVVLVRLKSTKCDLFKSEYCLVALTRHKRTFEYLYNGDLG	957
64ORF2A	YTNSAHSVDSV-----YSNQNW-IVALSRAKISLTMASQGEPI	957
	. . . . * * * . . . . . * * * * * *	
	<u>V</u> <u>VI</u>	
BBMV	GDLISFYVT	966
64ORF2A	TSGPPHVVL	966
	. . . *	

Fig. 6. Portion of a computer-deduced alignment of amino acid sequences of the 1A protein of broad bean mottle virus (BBMV) and a region (amino acids 726 to 966) of ORF 2 of the M1 double-stranded RNA (64ORF2A). The helicase-related, conserved amino acid domains, numbered according to Hodgman (1988), are single-underlined, and an ATP/GTP-binding motif is double-underlined.

cucumoviruses, bromoviruses, and tobamoviruses, and the animal alphaviruses. They argued, in a convincing manner, that "similar genes may be incorporated independently into different viral genomes from a separate common source, presumably cellular genes." The following two observations derived from analyzing the sequence of the M1 dsRNA appear to confirm their hypothesis. (i) The putative polypeptide encoded by ORF 5 of M1 and the CcOAF share 35% amino acid identities and 27% conservative substitutions over a region that covers 80% of the CcOAF amino acid chain. (ii) A significant sequence similarity was found between NADH dehydrogenase subunit 5 (Mathews and Holde 1990) and a region of M1 (amino acid positions 63 to 1746, on frame -3) interspersed with stop codons. In stretches ranging from 9 to 47 aa and totaling 436 aa, there exist 129 amino acid identities (30%), and 101 (23%) conservative substitutions.

These two observations lead to predictions that go a step beyond that made by Haseloff et al. (1984). The sequence similarity between frame -3 of M1 and NADH dehydrogenase subunit 5 and that between ORF 5 (frame -1) and the CcOAF suggest that the (-) strand of the M1 dsRNA might have originated from a segment of the mitochondrial genome carrying electron transport genes. It is fascinating, from the evolutionary viewpoint, that a putative viral helicase (ORF 2) and amino acid regions (such as ORF 5) closely related to electron transport enzymes are complementary sequences located on the same stretch of the M1 dsRNA (Fig. 5).

Fig. 7. Circular or concatemeric forms of M1. **A**, Inverse reverse transcription-polymerase chain reaction (RT-PCR) product (lane 2) with total double-stranded (ds) RNA from Rhs 1AP as a template and primers of opposite orientation as shown in Figure 4. Numbers on left indicate size (bp) of DNA standards (1 kb plus ladder, BRL). Fractionation was in 1.5% agarose/Tris-borate-EDTA buffer. **B and C**, Northern (RNA) blot hybridization analysis of dsRNA (lane 1) and total RNA (lane 2) from Rhs 1A1. Samples were fractionated under nondenaturing conditions on 1% agarose, blotted, and hybridized with a radiolabeled, 3.9-kb cDNA clone of M1. Number on left shows size (kb) and migration position of M1; asterisk indicates position of a larger-than-M1 molecule, presumed to be a replicative form of M1. Autoradiograms represent exposure times of (B) 20 h and (C) 17 days.

The hypothesis put forward by Haseloff et al (1984) is further supported by the following observations. (i) dsRNAs from two different classes of fungi (hypovirus in *C. parasitica* [Ascomycetes], M1 in *R. solani* [Basidiomycetes]) are genetically related to two groups of plant viruses (potyviruses and bromoviruses, respectively). (ii) The L2 (22 kb) dsRNA from Rhs 1AP has a high degree of sequence similarity with a 14-kb dsRNA from rice (Moriyama et al. 1995). In a region of 474 aa, L2 and the rice dsRNA have 182 identities (37.4%) and 178 conservative substitutions (37.6%) (Tavantzis and Lakshman 1995; S. M. Tavantzis and D. K. Lakshman, *unpublished data*). It is certain that the above region will be extended when more sequencing data on L2 becomes available. (iii) The L2 mitochondrial dsRNA from *R. solani* is genetically related to a mitochondrial dsRNA from strain NB631 from *C. parasitica* (Polashock and Hillman 1994) and two enzymatic domains of the pentafunctional AROM gene from yeast and filamentous fungi (Lakshman et al., *in press*).

Marienfeld et al. (1997) have recently pointed out an apparent amino acid sequence similarity between plant mitochondrial DNA, the *R. solani* M2 dsRNA, and the dsRNA from *C. parasitica* strain NB631, and suggested that (i) plant mitochondrial sequences from *Arabidopsis thaliana* and bean originated from a fungal virus and (ii) nucleic acid sequence transfers take place between fungal viruses and plants. However, as Haseloff et al. (1984) observed, direct phylogenetic relationships, as opposed to protein sequence conservation, do not readily account for the major differences in genomic organization among viral entities from phylogenetically distant organisms.

Sequence analysis showed that M1 possesses no RDRP consensus motif (GDD) (Poch et al. 1989) or other conserved regions characterizing RDRP genes of positive-stranded or double-stranded RNA genomes. It is intriguing that M1 is consistently associated with the L2 dsRNA (22 kb) (cultures Rhs 1AP, Rhs 1A1, P3-3a, P3-3b, P3-Pe1, P3-Pc2, P1-Pa, and P1-Px), whereas L2 can exist in the absence of M1 (cultures 21-2b2 and 21-2a) (Jian et al. 1997). In addition, M1 and L2 cofractionated in the mitochondria and high-speed pellet in equal proportions (Jian et al. 1997), and Rhs 1A1 was cured from both M1 and L2 simultaneously by hyphal tipping (cultures Rhs 1A1 HT1 and HT2) (Jian et al. 1997). Sequence analysis has shown the existence of a putative RDRP gene sequence on L2 (Tavantzis and Lakshman 1995; S. M. Tavantzis and D. K. Lakshman, *unpublished data*). These facts suggest that replication of M1 might depend on the L2 RDRP gene. Such a relationship would be in accordance with the fact that most mycoviruses are multipartite (Buck 1986).

Regarding the association of M1 with increased vigor and virulence in *R. solani*, the following two hypotheses emerged from the sequence analysis of M1. (i) As described in Results, substantial homology was found between ORF 2 and the conserved regions for a zinc finger and RNA helicase of polyprotein 1b of the LDV from mice. It has been shown that LDV elevates the activity of at least seven serum enzymes in mice by slowing down the turnover rate of the respective proteins (Godeny et al. 1993). Could the increased vigor and virulence of M1-containing isolates be attributed to a similar activity exhibited by M1 in the mitochondria of *R. solani*? This is a

question that can be addressed in a straightforward manner. (ii) It was mentioned above that the putative polypeptide encoded by ORF 5 of M1 and the CcOAF have 30% amino acid identities. The cytochrome c oxidase complex in fungal mitochondria is one of three sites where movement of protons from the mitochondrial matrix to the intermembrane space takes place, thus generating the proton gradient used by the ATPase for ATP synthesis in fungi (Griffin 1994). The CcOAF is thought to be a chaperonin that helps the subunits of the complex to fold correctly and assist in the assembly of the metal centers of the cytochrome c oxidase complex (Saraste et al. 1991; Quirk et al. 1993). So, the product of the ORF 5 might be involved in the upregulation of the complex, streamlining the electron transport chain, which speeds up ATP synthesis in the cells. This mechanism is also congruent with the fact that M1-containing isogenic lines are more vigorous than those lacking it (Jian et al. 1997). The above two phenomena may complement each other.

In conclusion, the data presented in this paper support an earlier hypothesis as to a potential process of virus evolution from cellular genes. Furthermore, sequencing data may also lead to testable hypotheses regarding potential mechanism(s) by which a particular dsRNA element may affect the biology of its fungal host.

MATERIALS AND METHODS

Subcellular fractionation.

Mycelium of Rhs 1A1 was used in the subcellular fractionation experiments because this culture contains all five dsRNAs. This approach allowed us to make observations regarding cofractionation of two or more dsRNAs (Jian et al. 1997). Nuclei were purified according to Gealt and co-workers (1976) with minor modifications. The purity of nuclei was assessed by fluorescence microscopy after staining with acridine orange (Bhargava and Halvorson 1971; Gealt et al. 1976). Mitochondria were purified by the method of Specht and co-workers (1983) as modified by Syminis (1989). The purity of mitochondria was evaluated by fluorescence microscopy after staining with Rhodamine-123 (Johnson et al. 1980). The other fractions were prepared as follows. After mitochondria were sedimented ($20,000 \times g$, 20 min) for further purification, the resulting supernatant was centrifuged at $30,000 \times g$ for 30 min to obtain the membrane fraction. The supernatant of the latter centrifugation was centrifuged at $120,000 \times g$ for 3 h to obtain the high-speed pellet, whereas the supernatant of the high-speed centrifugation was the cytosolic fraction.

Nucleic acids present in the cytosolic fraction were ethanol precipitated prior to further analysis. To ensure that purified nuclei and mitochondria were free from extraneous RNA adhering to the outer membrane, organelle preparations were treated with pancreatic RNase A (25 $\mu\text{g/ml}$). Organelle lysis was as described previously (Specht et al. 1983; Syminis 1989). After all five fractions were adjusted to 0.3 M NaCl, they were treated with proteinase K (10 $\mu\text{g/ml}$) and Sarkosyl (0.1%, wt/vol) at 37°C for 2 h with gentle shaking, phenol extracted, and ethanol precipitated. Subsequent dsRNA purification, agarose gel fractionation, and Northern blot hybridization to a ^{32}P -labeled, 3.9-kb cDNA (clone no. 4) of the 6.4-kb dsRNA were as described previously (Lakshman and Tavantzis 1994).

Analysis of M1-containing structures.

The subcellular fractionation experiments suggested that approximately 80% of the cytosolic portion of the M1 dsRNA was sedimented, and 20% remained in the supernatant following high-speed centrifugation. To determine whether the high-speed pelleted M1 was associated with viruslike particles, we employed virus extraction schemes that had been suitable for purification and characterization of mycoviruses with a typical nucleoprotein structure (Tavantzis et al. 1980; Tavantzis and Bandy 1988).

Sequencing and sequence analysis.

Sequencing of the 6.4-kb, dsRNA-specific cDNA clones was initiated by primer walking from both ends of clone no. 4 (3.9 kb) (Fig. 4). This clone was obtained from a cDNA library (Lakshman and Tavantzis 1994) with M1 purified from Rhs 1AP as a template to minimize potential cross contamination by other dsRNAs occurring in Rhs 1A1. The rest of the M1 sequence was determined with the clones shown in Figure 4. Plasmid DNA was prepared for cycle sequencing with a Wizard Minipreps DNA Purification System (Promega, Madison, WI) according to manufacturer's directions. Both strands were sequenced with a 373A Automated DNA sequencer (Applied Biosystems, Foster City, CA). The sequence of the two termini was determined by sequencing PCR products obtained by amplification of cDNA copies of the 6.4-kb dsRNA ends with a 5' RACE kit (Life Technologies, Gaithersburg, MD) following the directions of the manufacturer. Sequence analysis was carried out with the PCGENE program, version 6.5 (IntelliGenetics, Mountain View, CA), and the BLAST and BEAUTY programs (NCBI, Bethesda, MD).

The presence of a circular and/or concatemeric form of the M1 dsRNA was established by carrying out inverse RT-PCR of an M1-enriched sample from Rhs 1AP, with primers T3R5 (5'-CACTCTGGTCGTATTGAATG-3', bases 6135 to 6155), and T7R1B (5'-CTGCTCTAGCATCCTCTGGT-3', bases 156 to 136) (Fig. 5). The identity of the resulting PCR product was confirmed by Northern blot hybridization and sequencing analysis.

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