Association of Distinct Double-Stranded RNAs with Enhanced or Diminished Virulence in *Rhizoctonia solani* Infecting Potato

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A virulent field isolate of *Rhizoctonia solani* AG 3, Rhs 1AP, has given rise to three sectors in a period of several years. Subculturing of these sectors resulted in three hypovirulent isolates—Rhs 1A1, Rhs 1A2, and Rhs 1A3. We reported previously that five genetically different double-stranded (ds) RNAs occur in these four isolates, with each isolate containing a unique combination of two or more dsRNAs. We report here that all five dsRNA elements occur in the cytoplasm, and none in the nucleus. The mitochondria contains low to moderate concentrations of the four larger dsRNAs. The four isolates were paired in selected combinations in an attempt to transmit specific dsRNAs to cultures lacking these dsRNAs. This approach generated groups of near-isogenic lines possessing the same dsRNA elements. As many as six cultures possessing the same dsRNA genotype were found to have the same degree of pathogenicity. Consistently, acquisition of a 6.4-kb dsRNA brought about increased virulence, whereas the presence or acquisition of a 3.6-kb dsRNA resulted in diminished virulence in the recipient culture. All cultures had the same mitochondrial DNA haplotype as that of the original field isolate, Rhs 1AP. This, in conjunction with the fact that all cultures in this study had a single nuclear DNA origin (Rhs 1AP), indicates a strong correlation between certain phenotypic characters or changes thereof and dsRNA profiles or acquisition of particular dsRNAs, respectively.

Rhizoctonia solani (teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk) is a cosmopolitan, soilborne, plant-pathogenic basidiomycete that causes economically important diseases on several vegetable and field crops, turfgrasses, and trees (Sneh et al. 1996). It is a collective species comprising at least 13 anastomosis groups (AG) (Carling 1996). In general, AGs are genetically isolated, noninterbreeding populations with distinct host ranges (Carling 1996). A cytoplasmically controlled degenerative disease of *R. solani* was reported in 1978 (Castanho and Butler 1978; Castanho et al. 1978). The

above condition was characterized by loss of mycelial pigmentation, reduced growth rate and sclerotia production, presence of double-stranded (ds) RNA, and hypovirulence similar to that of *Cryphonectria parasitica* (Van Alfen et al. 1975). Three out of 13 strains had dsRNA and were hypovirulent, whereas the 10 virulent isolates contained no detectable dsRNA (Castanho et al. 1978). Subsequent studies, however, were not in accordance with these findings (Hyakumachi et al. 1985; Zanzinger et al. 1984; Finkler et al. 1985; Kousik et al. 1994). In the late 1980s and early 1990s, our research group generated several lines of indirect evidence suggesting that although the mere presence of dsRNA was not associated with specific phenotypes, particular dsRNAs might affect the degree of pathogenicity (virulence) in *R. solani* (Tavantzis 1988; Bharathan and Tavantzis 1990, 1991; Tavantzis and Lakshman 1995). More importantly, we showed that dsRNAs from *R. solani* isolates obtained from the same field as well as those from different continents were quite diverse genetically (Bharathan and Tavantzis 1990, 1991). This work shed some light on the controversy, as it became evident that dsRNA elements, carrying different genetic information and occurring in different fungal genotypes, might have varying effects, direct or indirect, on the virulence of the respective hosts. In the last few years, we have focused on a particular set of dsRNAs, some of which appeared to be associated with distinct phenotypes (Lakshman and Tavantzis 1994; Tavantzis and Lakshman 1995). Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3, the four “parental” AG 3 isolates used in this study, have been described previously (Lakshman and Tavantzis 1994). Rhs 1AP is a virulent field isolate. Over a period of years, we selected three morphologically distinct sectors of Rhs 1AP, which gave rise to the hypovirulent (essentially nonpathogenic) cultures Rhs 1A1, Rhs 1A2, and Rhs 1A3. Rhs 1AP possesses two dsRNAs of 23 kb (L2) and 6.4 kb (M1). Rhs 1A1 has three dsRNAs in addition to the two dsRNAs found in the “parental” Rhs 1AP. The apparent sizes of the novel dsRNAs are 25 kb (L1), 3.6 kb (M2), and 1.2 kb (S1). In contrast, Rhs 1A2 and Rhs 1A3 lack the two dsRNAs (L2 and M1) of Rhs 1AP, but have M2 and S1, and L1 and S1, respectively. The dsRNAs with corresponding sizes (L2 and M1) occurring in Rhs 1AP and Rhs 1A1 cross-hybridize with each other, and are probably identical, but the five dsRNAs are not related (e.g., they do not cross-hybridize to one another) (Lakshman and Tavantzis 1994).

It is extremely difficult to induce *R. solani* to sporulate in

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vitro (Adams 1996) in order to conduct experiments involving segregation of dsRNAs. In addition, Castanho et al. (1978) failed to transmit dsRNA to different genotypes within the same AG of *R. solani*. Our data on the high degree of genetic diversity in dsRNAs from isolates of the same AG (Bharathan and Tavantzis 1990, 1991) was in accordance with the above observations. In order to test the hypothesis that specific dsRNAs are associated with different degrees of pathogenicity, we needed to circumvent the above problems. Thus, we created different lines of *R. solani* that share the same genetic background, with each line containing specific dsRNAs. This process involved transmission or elimination of particular dsRNAs by hyphal anastomosis or hyphal tipping, respectively. We report here that lines of *R. solani* that share the same gene pool and contain the same dsRNAs have the same degree of pathogenicity. Furthermore, the data suggest that, in this genetic background, a 3.6-kb dsRNA is associated with a drastic decrease in virulence, whereas a 6.4-kb dsRNA is associated with virulence. Parts of this study have been reported previously (Jian et al. 1995, 1996).

RESULTS

Cellular distribution of dsRNAs.

Nuclear and mitochondrial fractions were treated with RNase to ensure that dsRNA bands or radioactive signals were not due to dsRNA that adhered externally to the organelles. Results of dsRNA analyses of subcellular fractions from ethidium bromide-stained gels and Northern (RNA) blot analyses showed that none of the five dsRNAs obtained from Rhs 1A1 are located in the nucleus (Table 1). Four of the five elements (L1, L2, M1, and M2) were found in the mitochondria at low (L1) to high (L2 and M1) concentrations. Segment S1 was detected only in the supernatant following high-speed centrifugation (cytosol). A high concentration of M2-specific signal was also found in the high-speed supernatant. It should be noted that the bulk of the amount of both "original" (Rhs 1AP) dsRNAs (L2 and M1) was also evenly distributed in the mitochondrial and high-speed pellet fractions.

Transmission of dsRNA by hyphal anastomosis.

A comparison of phenotypic characters, including degree of pathogenicity, and dsRNA content of the four parental isolates (Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3) led to the following two observations. First, hypovirulence coincided with the induction of one or more of the three nascent dsRNAs in Rhs 1A1. Second, loss of vigor apparently leading to dimin-

ished virulence in Rhs 1A2 and Rhs 1A3 correlated with the suppression of L2 or M1 or both. Since none of the four cultures could be induced to sporulate in vitro to bring about dsRNA segregation, we paired them in selected combinations in attempts to transmit particular dsRNAs to cultures lacking them. The goal was to generate groups of isolates having the same dsRNA content or a single-dsRNA difference, and compare their respective virulence.

For clarity of presentation, data from a single pairing (Rhs 1AP with Rhs 1A3) are shown in Figures 1, 2, and 3, followed by a compilation of all the data given in Table 2. The dsRNA transmission data are shown in Figure 4. Rhs 1AP and Rhs 1A3 were paired to transmit the M1 (6.4 kb) dsRNA from Rhs 1AP to Rhs 1A3. Mycelial explants were isolated from the Rhs 1A3 colony, away from the hyphal fusion zone (Fig. 1). Transmission of M1 to Rhs 1A3 was associated with increased growth rate and pigmentation. Subculture (of Rhs 1A3) P3-3b was typical in this respect. Figure 1 shows the position of the mycelial explant that gave rise to P3-3b, and Figure 2 shows that P3-3b has the same cultural characteristics as Rhs 1AP. The M1 dsRNA was transmitted to isolate P3-3b (Fig. 4, panel II, lane E).

All five dsRNAs were transmitted from one isolate to another (Fig. 4, Table 2). M2 was barely detectable following transmission into Rhs 1AP (cultures P1-Pa and P1-Px; Fig. 4, panel II, lanes J and K, respectively, and Table 2). In contrast, M2 was at relatively high concentrations upon transmission to Rhs 1A3 (cultures 23-3b and 13-3c; Fig. 4, panel I, lanes I and J, respectively, and Table 2).

Fig. 1. Pairing of Rhs 1AP (A) and Rhs 1A3 (B). In this case, Rhs 1A3 was used as a potential recipient of double-stranded RNA. To maximize the distance between anastomosis zone and the location of the transfer plugs, Rhs 1A3 was allowed to grow for a few days, before transferring the Rhs 1AP plug. Plug b and other plugs (not shown here) were taken 48 h after hyphal anastomosis. Control plates were used to monitor the unobstructed growth of Rhs 1AP during the above 48-h interval (see text). Plug b gave rise to culture P3-3b.

Table 1. Subcellular location of the five double-stranded (ds) RNA elements found in isolate Rhs 1A1 of *Rhizoctonia solani*

dsRNA element	Cell fraction				
	Nuclei	Mitochondria	30,000 × g pellet	120,000 × g pellet	120,000 × g supernatant
L1	0 ^a	1	1	3	0 to 1
L2	0	4	2	5	1
M1	0	4	1	4	2
M2	0	2	3	1	10
S1	0	0	0	0	1

^a Numbers indicate relative intensity of autoradiogram signal specific to the particular dsRNA element. Vertical quantitative comparisons are not valid.

Changes in virulence correlated with transmission of distinct dsRNA elements.

All subcultures that did not acquire a dsRNA they previously lacked, retained both the cultural characteristics (pigmentation, mycelial texture, radial rate of growth) and degree of pathogenicity of the culture from which the respective mycelial explant was taken after hyphal anastomosis (data not shown).

Isolate P3-3b was a typical case in which increased virulence was associated with the acquisition of the M1 dsRNA (Fig. 3). This conclusion stems from the fact that when L2 (22 kb) was the only dsRNA transmitted, no changes in virulence or morphological characteristics were observed (Fig. 4, panel I, lane E; J. Jian and S. M. Tavantzis, *unpublished data*). Similar observations indicated that acquisition of L1 (25 kb) or S1 (1.2 kb) was not associated with phenotypic changes.

All cultures lacking M1 (6.4 kb) are hypovirulent (Table 2),

whereas all isolates with M1 but not M2 (or the M2-related, 2.4-kb dsRNA) are virulent (Table 2). In cultures in which both M1 and M2 were present, the degree of pathogenicity of the respective culture is determined by the relative concentrations of the two dsRNA species (Table 2). It appears that M2 counteracts the effect of M1 even when it is present at a low concentration (Table 2).

Of six different cultures that lack M1 and are hypovirulent, four were obtained through hyphal anastomosis (13-3c, 21-2b2, 23-2a, and 23-2b), and two by hyphal tipping (1A1-HT1 and 1A1-HT2) (Table 2). Thus, independent of the method by which these cultures were generated, elimination or absence of M1 results in drastically diminished virulence.

mtDNA restriction analysis.

Mitochondrial (mt) DNA from isolates Rhs 1AP, Rhs 1A1,

Fig. 2. Four-day-old mycelial cultures of Rhs 1AP (A), Rhs 1A3 (B), and P3-3b (C). Growth rate and appearance of P3-3b are similar to those of Rhs 1AP.

Fig. 3. Sprouts of potato cultivar Katahdin infected with Rhs 1AP (top panel), Rhs 1A3 (middle panel), and P3-3b (bottom panel). Virulence of P3-3b is comparable to that of Rhs 1AP.

Rhs 1A2, Rhs 1A3, and P3-3b showed no evidence of polymorphism upon digestion with four different restriction endonucleases, *Bst*EII, *Cla*I, *Eco*RI (data not shown), and *Pst*I (Fig. 5). These results suggest that the changes in virulence following hyphal anastomosis could not be attributed to migration of mitochondria from donor to recipient cultures. To our knowledge, this is the first report on mtDNA analysis in *R. solani*. It appears that the size of the mitochondrial genome in this basidiomycete is approximately 200 kb.

DISCUSSION

This study has provided evidence suggesting that, in the genetic background of the field isolate of *R. solani*, Rhs 1AP, discrete dsRNAs affected the phenotype of the host in different ways. In two cases, as many as six independently produced cultures with the same dsRNA profile had the same degree of pathogenicity and cultural characteristics (Table 2).

Table 2. Correlation between specific double-stranded (ds) RNA elements and up- or down-regulation of virulence in *Rhizoctonia solani*

Isolate	dsRNA ^a			Virulence ^b
	M1	M2	2.4 kb	
Rhs 1AP ^c	++++			8 to 10 (V)
P1-Pb2				
P3-3a				
P3-3b				
P3-Pe1				
P3-Pa				
P3-Pc2	++			7 to 9 (V)
P1-Pa	++++	+	+	7 to 9 (V)
P1-Px	+++	+	++	2 to 3 (WV)
Rhs 1A1	++++	+++	++	2 to 3 (WV)
Rhs 1A2		++++	++++	1 to 2 (HV)
1A1 HT1	-	++++	+++	1 to 2 (HV)
1A1 HT2				
13-3c				
21-2b2				
23-2a				
23-2b				
21-2a		+++		1 to 2 (HV)
Rhs 1A3				1 to 2 (HV)

^a dsRNAs that did not exhibit an apparent correlation with degree of pathogenicity were not included. For complete dsRNA patterns of most isolates included here, see Figure 4. Concentration of a dsRNA species in a particular culture or group of cultures ranged from undetectable (no mark) or very low (+, barely visible dsRNA band) to high (++++, thickness of dsRNA band was 2 mm or greater).

^b Based on percentage of infected area in a 8-cm-long segment of the sprout surrounding the point of inoculation (see text). A rate of 7 to 10 is designated as virulent (V), 5 to 6 moderately virulent (MV), 3 to 4 weakly virulent (WV), 1 to 2 hypovirulent (HV), and zero, non-pathogenic (NP).

^c The four parental cultures, Rhs 1AP, Rhs 1A1, Rhs 1A2, and Rhs 1A3, were paired in selected combinations (see text) in a manner similar to that of Castanho and Butler (1978). About 48 h after hyphal anastomosis, mycelial explants were taken away from the anastomosis zone and subcultured. These subcultures were assigned designations, the first portion of which indicated the identity of the paired cultures, and the second the parent from which the explant was obtained. For example, the designation P3-3b indicates that the respective culture was generated by pairing Rhs 1AP and Rhs 1A3, and then taking a mycelial explant from Rhs 1A3 at position b.

Removal of M1 by hyphal tipping resulted in loss of virulence (1A1 HT1 and 1A1 HT2), whereas its acquisition by hyphal anastomosis coincided with increased virulence (P3-3b and others) (Table 2). Moreover, transmission of low amounts of M2 resulted in a dramatic attenuation of the virulence associated with M1. This work has shown that a single culture (genotype) of *R. solani* can harbor two dsRNAs that have diametrically opposed effects on the same characteristic (degree of pathogenicity). This is in accordance with earlier data on the genetic diversity of dsRNA in this important plant pathogen (Bharathan and Tavantzis 1990, 1991).

M1 cannot be transmitted without the concomitant presence of L2 (22 kb), but L2 can be transmitted (and replicate) alone without affecting the virulence or morphological characters of the cultures (e.g., 21-2b2; Fig. 4, panel I, lane E, and others not shown) acquiring it. It should be noted that L2 possesses an open reading frame encoding a putative RNA-dependent RNA polymerase (RDRP) (Tavantzis and Lakshman, 1995; A. J. Lambert, D. K. Lakshman, and S. M. Tavantzis, *unpublished data*), whereas M1 lacks an RDRP gene of its own (Jian 1997). The small-sized dsRNAs migrating just below the S1 (1.2 kb) dsRNA (Fig 4, panel I) cross-hybridize with M2-specific probes, and they are presumed to be replication intermediates or deletion mutants of M2. Their size and presence are variable, and have no apparent relationship to virulence or phenotype.

In the ascomycete *C. parasitica*, cytoplasmically transmitted hypovirulence and dsRNA were transmitted simultaneously from a hypovirulent dsRNA-containing strain to a virulent dsRNA-free isolate (Day et al. 1977). Recently, Choi and Nuss (1992) provided evidence demonstrating a cause-and-effect relationship between dsRNA and hypovirulence in *C. parasitica*. A virulent strain was converted to hypovirulent by transfection with a complementary DNA or single-stranded RNA copy of a hypovirus dsRNA. It has been shown that dsRNA-induced hypovirulence in *C. parasitica* involves down-regulation of specific fungal mRNAs and their respective polypeptides, some of which are involved in a signal transduction pathway (Choi et al. 1995; Kazmierczak et al. 1996). In other host-pathogen systems, a relationship between dsRNA and degree of pathogenicity has been deduced through indirect experimental evidence (for review, see Nuss and Koltin 1990).

The potential effects of M1 and M2 were studied without interference of a diverse nuclear gene pool. Interactions between nuclear genes and cytoplasmic dsRNA have been shown to be quite complex in yeast (for recent review, see Wickner 1996). The fact that two groups of six isolates, generated by different methods, with the same dsRNA (M1 or M2) had the same virulence (Table 2) suggests that differences in nuclear genes among these isolates either did not exist or had no observable effect on virulence. Preliminary data on electrophoretic karyotyping revealed that the four parental cultures did not exhibit differences at the chromosomal level (A. J. Lambert and S. M. Tavantzis, *unpublished data*). It has been reported that in *R. solani* the diameter of septal pores is 0.1 to 0.2 μ m whereas that of nuclei appears to be 2 to 3 μ m (Bracker and Butler 1964). It might be possible for nuclei to enter septal pores in a constricted state, but their migration from cell to cell and against the apparently unidirectional flow of protoplasmic streaming (Bracker and Butler 1964), if at all feasible, must be a very slow process. In this re-

gard, Snider (1962) reported that it took at least 96 h for nuclei to migrate 2 to 4 cm in the direction of protoplasmic streaming, to only 1% of the tested points in mycelia of the basidiomycete *Schizophyllum commune*. Cubeta et al. (1993) reported that out of 30 randomly selected pairings of AG 4 *R. solani* isolates, 12 pairings exhibited bidirectional and three unilateral nuclear migration. Mycelial explants were excised 4 cm from either side of the hyphal interaction zone after 15 days of incubation in the dark. In the present study, mycelial explants were taken 2 cm from the hyphal interaction zone 48 h after hyphal fusion.

Restriction analysis of mtDNA from Rhs 1AP, Rhs 1A1, Rhs 1A2, Rhs 1A3, and P3-3b revealed no differences (Fig. 5), thus suggesting that the isolates used in this study shared the same mitochondrial genome. This finding confirms the claim that only one mitochondrial haplotype exists in individual field heterokaryons (such as Rhs 1AP) of different basidiomycetous fungi (for review, see Adams 1996).

The subcellular fractionation experiments provided information that helped interpret the dsRNA transmission data. The fact that all five dsRNAs occur in the cytoplasm is in agreement with the finding that they were easily transmitted from one culture to another. The only exception to this phenomenon was that, following its transmission to Rhs 1AP, the concentration of M2 did not reach levels comparable to those of the other three cultures (Fig. 4, panel II, lanes J and K, and Table 2). In contrast, L1, upon transmission into Rhs 1AP, attained high titers not indicative of suppression (Fig. 4, panel II, lanes

J and K). Both L1 and M2 are suppressed (not detectable by ethidium bromide staining) in the original Rhs 1AP culture (Fig. 4, panel II, lane I, and Lakshman and Tavantzis 1994). The genetic event that brought about derepression of these two dsRNAs in the sector (Rhs 1A1) of the original culture (Rhs 1AP) is of unknown nature, since it occurred spontaneously on typical culture media (potato dextrose yeast extract agar [PDYA]) and under routine incubation conditions (22°C, in the dark).

The presence of the M1 and M2 dsRNAs in the mitochondria of *R. solani*, and their effect on virulence, is a phenomenon that has been observed previously in other plant-pathogenic fungi. The ascomycetes *C. parasitica* and *Ophiostoma ulmi*, incitant of Dutch elm disease, possess mitochondrial dsRNAs associated with reduced virulence (Polashock and Hillman 1994; Rogers et al. 1987). M1 is the first mitochondrial dsRNA found to be associated with enhancement of virulence.

Direct evidence concerning the involvement of M1 and M2 in virulence regulation can only be obtained through transfection of *R. solani* cells with DNA or RNA copies of the respective dsRNA. A transformation system for this basidiomycete is currently under development through the collaborative efforts of our group and that of Victor Rubio (Madrid, Spain).

It is important to note here that we are not advocating that M1 or M2 have a direct effect on virulence, rate of growth, pigmentation, or other features. It is possible that they inter-

Fig. 4. Ethidium bromide-stained, agarose gel (1%) electrophoretogram of double-stranded (ds) RNAs extracted from the four “parental” cultures Rhs 1AP (panel II, lane I), Rhs 1A1 (panel I, lane B), Rhs 1A2 (panel I, lane F), and Rhs 1A3 (panel I, lane K, and panel II, lane C), and selected plug-derived cultures (obtained as described in the text) as follows. Panel I: 21-2a (lane D), 21-2b2 (lane E), 23-2a (lane G), 23-3b (lane I), 13-3c (lane J); panel II: P3-3a (lane D), P3-3b (lane E), P3-Pe1 (lane G), P3-Pc2 (lane H), P1-Pa (lane J), and P1-Px (lane K). Lanes C and H of panel I, and B and F of panel II are blank. Lane A of both panels contains the DNA standards of the 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, MD). Numbers on the right indicate approximate sizes of the five dsRNA elements. Sequencing data (J. Jian, D. K. Lakshman, and S. M. Tavantzis, *unpublished*) has shown that the sizes of M1 and M2 are 6.4 and 3.6 kb, respectively, compared with the 6.5- and 3.7-kb estimates reported previously (Lakshman and Tavantzis 1994).

fere with routine metabolic processes that affect expression of the above characteristics directly or indirectly, as is the case in *C. parasitica* (Nuss 1996).

MATERIALS AND METHODS

Fungal isolates and culture conditions.

All of the fungal cultures were maintained on PDYA at room temperature. Mycelial mats were grown in 1-liter flasks containing 150 ml of malt extract broth (30 mg/liter), for 3 weeks at room temperature unless otherwise indicated. Mycelium was harvested by draining the liquid medium and rinsing the mat with sterile, deionized water. It was used fresh for isolation of mitochondria, or frozen in liquid N₂ for dsRNA extraction and nuclei isolation. If necessary, mycelial mats were stored at -80°C until used.

Subcellular fractionation.

Nuclei were purified according to Gealt et al. (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 methods of Specht et al. (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 × g, 20 min) for further purification, the resulting supernatant was centrifuged at 30,000 × g for 30 min to obtain the membrane fraction. The supernatant was then centrifuged at 120,000 × g for 3 h to obtain the high-speed pellet, and the cytosolic fraction from the supernatant.

dsRNA extraction from different fractions and Northern blot hybridization analysis.

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 onto the outer membrane, organelle preparations were treated with pancreatic RNase A (25 µg/ml) before lysing, as described previously (Specht et al. 1983; Syminis 1989). After adjusting to 0.3 M NaCl, all five fractions were treated with proteinase K (10 µg/ml) and Sarcosyl (0.1%) at 37°C for 2 h with gentle shaking. Samples were then phenol extracted and ethanol precipitated. Subsequent dsRNA purification, agarose gel fractionation, and Northern blot hybridization analyses were as described previously (Lakshman and Tavantzis 1994).

dsRNA transmission experiments.

The four cultures were paired in a manner similar to that of Castanho and Butler (1978). Approximately 48 h after hyphal anastomosis, mycelial explants were taken 2 or more centimeters away from the anastomosis zone (Fig. 1) and subcultured. These subcultures were assigned designations, the first portion of which indicated the identity of the paired cultures, and the second the "parent" from which the mycelial explant was obtained. For example, the designation P3-3b (Fig. 1) indicates that the respective culture was generated by pairing Rhs 1AP with Rhs 1A3, and then taking an explant from Rhs 1A3 at position b (Fig. 1). To monitor the extent of the 48-h post-anastomosis hyphal growth, mycelial explants of the re-

spective paired isolates had been transferred individually on control plates at the same distance as their respective paired isolates. Within this 48-h period, hyphal growth on control plates covered only a small fraction of the respective distance between the anastomosis zone and the position of the mycelial explants taken for subculturing. dsRNA extraction from all the isolates was carried out as described by Morris and Dodds (1979) with minor modifications.

The two cultures Rhs 1A1-HT1 and Rhs 1A1-HT2 (Table 2) were obtained through several cycles of hyphal-tip isolation from Rhs 1A1 mycelium.

Virulence assessment.

Degree of pathogenicity tests were conducted as described previously (Bandy et al. 1988). Briefly, 8- to 10-cm-long attached potato sprouts, placed on sterile soil in sterile plastic flats, were inoculated with a single 5-mm mycelial plug obtained from the respective PDYA culture. Inoculum was kept in place with a small piece of cellophane, and sprouts were covered with moist sterile soil and aluminum foil. Flats were incubated at 22°C. Infection progress was monitored daily by direct observation of inoculated sprouts in control flats. Virulence groupings were assigned as follows. Raw data were ex-

Fig. 5. *Pst*I restriction profiles of mitochondrial DNA from the *Rhizoctonia solani* isolates Rhs 1AP (lane C), Rhs 1A1 (lane D), Rhs 1A2 (lane E), Rhs 1A3 (lane F), and P3-3b (lane G). Lanes A and B contain DNA standards, 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, MD) and *Hind*III-restricted λ DNA, respectively.

pressed as percentage of sprout stem area covered by lesions over the entire area of an 8-cm-long segment extending 4 cm on either side of the point of inoculation. The infected-area percentages were then transformed to numbers on a scale of 0 to 10, where 0 was no visible lesion, 5 was 50% lesioned area, and 10 was 100% lesioned area.

Restriction fragment length polymorphism of mitochondrial DNA.

Purification and lysis of mitochondria were as described above. mtDNA was treated with DNase-free RNase A (20 µg/ml), fractionated on agarose gels to remove plasmid DNA and other impurities, gel eluted, phenol extracted, and ethanol precipitated as described by Sambrook et al. (1989). Purified mtDNA from Rhs 1AP, Rhs 1A1, Rhs 1A2, Rhs 1A3, and P3-3b was digested with restriction endonucleases *Cla*I, *Pst*II, *Bst*EII, and *Eco*RI (New England Biolabs, Beverly, MA). Restriction mtDNAs were fractionated on 1% agarose gels and stained with ethidium bromide.

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