Interspecific Transmission of Double-Stranded RNA and Hypovirulence from *Sclerotinia sclerotiorum* to *S. minor*

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ABSTRACT

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Interspecific transmission of a hypovirulence-associated double-stranded RNA (dsRNA) and hypovirulent phenotype was attempted from hypovirulent isolate Ss275 of *Sclerotinia sclerotiorum* to five virulent isolates of *S. minor.* dsRNA and the hypovirulent phenotype were successfully transmitted to one of the five isolates, Sm10. Three putative converted isolates of Sm10 were slow growing and developed atypical colony morphologies characteristic of the hypovirulent phenotype. These isolates were assayed for virulence and produced significantly smaller lesions than isolate Sm10 on detached leaves of Romaine lettuce. One of these putative converted isolates, designated Sm10T, was tested to confirm interspecific transmission of dsRNA. In northern hybridizations, dsRNA isolated from Sm10T hybridized with a digoxigenin-labeled cDNA probe prepared from dsRNA isolated from Ss275. Random amplified polymorphic DNA analysis confirmed that isolate Sm10T was derived from Sm10 and not from Ss275 or a hybrid of the two species. The dsRNA and hypovirulent phenotype were subsequently transmitted intraspecifically from Sm10T to Sm8. To our knowledge, this is the first report of interspecific transmission of dsRNA and an associated hypovirulent phenotype between fungal plant pathogens by hyphal anastomosis.

Additional keywords: fungal virus, horizontal transmission, intraspecific transmission.

Hypovirulence associated with double-stranded RNA (dsRNA) in plant pathogenic fungi is gaining increased attention because of the opportunity for an increased understanding of mechanisms of action by which these agents influence the physiological or regulatory pathways of their fungal hosts, and because of their possible utility in the management of plant diseases through biological control. Hypovirulence associated with dsRNA has been reported in several plant pathogenic fungi but most intensively studied in *Cryphonectria parasitica* (9,15,37,38,44,52) where two unencapsidated, hypovirulence-associated dsRNAs were classified within the virus family *Hypoviridae* (19). Full-length synthetic cDNA transcripts of one hypovirulence-associated dsRNA (CHV1-713) conferred the hypovirulence phenotype when introduced into virulent strains of *C. parasitica* by DNA-mediated transformation (12,13). In addition, a new genus of fungal viruses, *Mitovirus*, has been established for unencapsidated viral genomes associated with hypovirulence and mitochondria in fungal hosts (45).

Little information is available on the host range of hypovirulence-associated dsRNAs because these agents are not externally infectious, and protocols for initiating infections with purified dsRNAs are not available for filamentous fungi although dsRNA viruses infect *Saccharomyces cerevisiae* in vitro (16). Hypovirulence-associated dsRNAs are horizontally transmitted between compatible isolates of individual species through hyphal anastomosis, and vertically transmitted through conidia but not ascospores (1,2,11). Barriers to transmission have been associated with vegetative or mycelial compatibility groups (MCG) (17) and a negative correlation was observed between the frequency of hypovirus transmission and the number of vegetative incompatibility (*vic*) genes, such that isolates with the same *vic* loci or that differed by only one *vic* locus were able to frequently transmit hypoviruses between isolates (29). Isolates that differed by two or more *vic* loci had an increasingly restricted ability to transmit hypoviruses. In addition, individual *vic* genes were associated with specific effects such as epistatic interactions and unidirectional transfer of dsRNA among isolates of *C. parasitica* (20,29). An infectious synthetic cDNA transcript of CHV1-713 conferred the hypovirulence phenotype when introduced into virulent strains of *C. parasitica* and several related *Cryphonectria* spp., confirming that this dsRNA can incite hypovirulence symptoms in other hosts (10,12). However, symptom expression varied somewhat according to host genotype. All fungal species tested were able to intraspecifically transmit the hypovirulence-associated dsRNA by anastomosis following transfection, expanding the possible utility of selected dsRNAs for biological control of other pathogens.

Transmissible hypovirulence associated with dsRNA has been reported in *Sclerotinia sclerotiorum* (3,5) and *S. homoeocarpa* (53,54), and may also occur in *S. minor* (32). *S. sclerotiorum* has high levels of mycelial incompatibility (25,27), which would restrict the movement of hypovirulence-associated dsRNAs in populations of this pathogen, thus reducing its effectiveness as a biocontrol strategy. In comparison, *S. minor* has fewer MCGs, and therefore, transmission of dsRNA and hypovirulence would be expected to be higher (32,39). The successful transfer of a hypovirulence-associated dsRNA from *S. sclerotiorum* to *S. minor* would demonstrate a broader utility of these agents for biological control than was previously considered. Hypovirulence-associated dsRNAs from *Sclerotinia* spp. are unique, because individual dsRNAs did not hybridize with each other or with selected dsRNAs from other fungi (6). Continued evaluation of the influence of these dsRNAs on the physiological or regulatory pathways of their infected hosts could be enhanced by direct comparison within a common host genotype. To our knowledge, there are no reports of transmission of dsRNA between fungal species by hyphal anastomosis. Therefore, the objective of this study was to attempt interspecific transmission of hypovirulence-associated dsRNA by hyphal anastomosis from *S. sclerotiorum* to *S. minor*.

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MATERIALS AND METHODS

Fungal isolates. For interspecific transmission experiments, isolate Ss275 of *S. sclerotiorum*, which contains a hypovirulence-associated dsRNA (3; now referred to as SsV1/Ss275), was selected as the donor isolate. Five recipient virulent isolates (Sm5, Sm8, Sm9, Sm10, and Sm30) of *S. minor*, all isolated from lettuce, were selected as recipient isolates, representing the three MCGs described by Melzer and Boland (32). Isolates Sm8 and Sm10 were from MCG I, Sm5 from MCG II, and Sm9 and Sm30 from MCG III. All isolates were stored or grown on potato dextrose agar (PDA) at 4 or 20 to 22°C, respectively. Cultures were transferred by mass transfer.

Transmission of hypovirulent phenotype. Agar disks (6-mm-diameter) from the actively growing margins of colonies of individual isolates of *S. minor* were placed on the actively growing margin of an established colony of Ss275 to allow hyphal interaction (3). Colonies of *S. minor* with an atypical colony morphology that subsequently grew away from the established colony of Ss275 were subcultured to PDA. Subcultures that continued to grow atypically were assessed for virulence. The distinct colony morphology and smaller sclerotia of *S. minor*, and the slow growth and larger sclerotia of isolate Ss275 of *S. sclerotiorum*, permitted clear identification of the two colonies when subculturing.

Virulence assay. All subcultures exhibiting an atypical morphology were assessed for virulence using a detached lettuce leaf assay (32). Detached leaves of Romaine lettuce (*Lactuca sativa* L.) were inoculated with agar disks (6-mm-diameter) from actively growing colony margins of atypical subcultures. Each leaf was inoculated with two disks, one on each side of the midvein. Inoculated lettuce leaves were incubated at 20 to 22°C in plastic crisper boxes and sprayed daily with water to maintain high humidity. Diameter of lesions resulting from each disk was measured 48 h postinoculation. There were three replications of each treatment. These experiments were repeated with four replications for isolates that showed significant reductions in lesion diameter after conversion. Lesion diameters were compared using a Kruskal-Wallis one-way analysis of variance (Statistix6, Analytical Software, Tallahassee, FL).

Isolation of dsRNA. Selected isolates exhibiting a statistically significant reduction in lesion diameter were assessed for the presence of dsRNA according to the method described in Melzer et al. (34).

Northern hybridization analysis. Total dsRNA extracted from Ss275, the original virulent isolate, and the corresponding converted isolate of *S. minor* were electrophoresed in 0.8% agarose at 0.5 V/cm for 1.5 to 2 h. The gel was soaked in 0.25 M NaOH for 15 min and Tris-borate-EDTA (TBE) buffer (90 mM Tris base, 90 mM boric acid, and 2 mM EDTA) for 10 min and transferred to nylon membrane using electrophoretic transfer (Mini Trans-Blot, Bio-Rad, Richmond, CA) in 0.5× TBE at 80 V for 2 h. The Trans-Blot unit was surrounded by ice during transfer, and a magnetic stir bar inside the unit circulated buffer. Prehybridization was conducted in high sodium dodecyl sulfate (SDS) hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2.0% blocking reagent, 50 mM sodium phosphate [pH 7.0], 0.1% *N*-lauroylsarcosine, 7.0% SDS, and 50% formamide) for 4 to 6 h at 42°C according to the method of the manufacturer (Boehringer Mannheim, Laval, Canada). Probes were constructed from 11-dUTP digoxigenin (DIG)-labeled cDNA synthesized from total dsRNA recovered from isolate Ss275 using *Avian myeloblastosis virus* reverse transcriptase and a random prime reaction according to the method of the manufacturer (Boehringer Mannheim). Hybridization was conducted overnight at 42°C with 5 ng of probe

TABLE 1. Lesion length of hypovirulent isolate Ss275 and converted isolates of Sm10 (Sm10G, Sm10I, and Sm10T) in comparison to virulent isolate Sm10 on detached leaves of Romaine lettuce 48 h postinoculation[a]

	Lesion length (mm)							
	Sm10	Ss275	Sm10	Sm10G	Sm10	Sm10I	Sm10	Sm10T
Leaf 1	46.2	0.0	37.3	0.0	41.3	0.0	55.6	1.0
Leaf 2	37.8	0.0	25.3	0.0	42.8	2.0	44.3	0.0
Leaf 3	43.3	0.0	34.8	0.0	36.3	0.0	29.7	0.0
Mean	42.4	0.0	32.5	0.0	40.1	0.7	43.2	0.3
	$P = 0.007$		$P = 0.007$		$P = 0.017$		$P = 0.017$	

[a] Lesion length incited by Sm10 and test isolates Ss275, Sm10G, Sm10I, and Sm10T differed significantly at the *P* value listed (Kruskal-Wallis analysis of variance).

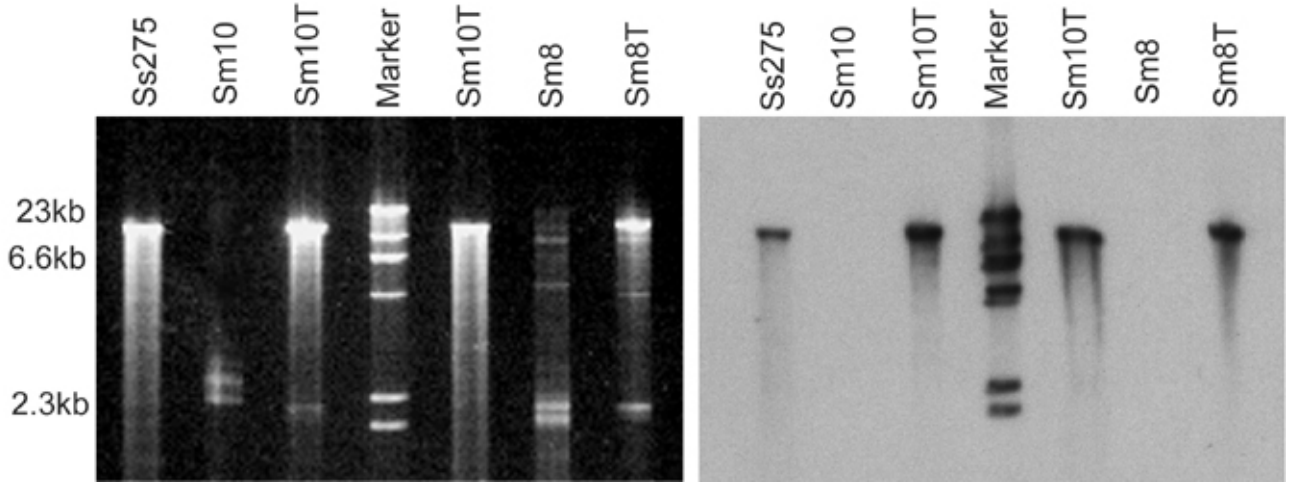

Fig. 1. Double-stranded RNA (dsRNA) banding patterns for isolates Ss275, Sm10, Sm10T, digoxigenin (DIG)-labeled λ DNA/*Hin*dIII marker, Sm10T, Sm8, and Sm8T in lanes 1 to 7, respectively (left panel). Northern hybridization of membrane with dsRNA transferred from gel shown in left panel and probed with a DIG-labeled cDNA probe prepared from dsRNA of Ss275 (right panel). Sm10T was derived from Sm10 after it was paired with Ss275 and Sm8T was derived from Sm8 after it was paired with Sm10T.

per ml of hybridization buffer. The membranes were washed twice in 2× washing solution (2× SSC, 0.1% SDS) for 15 min at 20 to 22°C and twice in 0.5× washing solution (0.5× SSC, 0.1% SDS) for 15 min at 50°C. A chemiluminescent product, Lumigen PPD, was used for detection according to the method of the manufacturer (Boehringer Mannheim). Membranes were autoradiographed with Kodak film (X-OMAT; Kodak, Rochester, NY) using an intensifying screen.

Random amplified polymorphic DNA analysis. Random amplified polymorphic DNA (RAPD) analysis was conducted on isolate Ss275, the original virulent isolate Sm10, and the corresponding converted isolate Sm10T to confirm that the converted isolate was *S. minor* and not Ss275 or a hybrid of the two species. Colonies were grown on cellophane membranes overlain on PDA, and DNA was extracted and purified according to the methods of Melzer and Boland (33).

Amplification reactions were performed according to the method of Williams et al. (48) with 25 ng of genomic DNA, 0.5 units of *Taq* DNA polymerase (Perkin-Elmer, Foster City, CA), and 0.2 μM (of one) 10-mer oligonucleotide primer (UBC RAPD Primer Synthesis Project, University of British Colombia) in a 25-μl reaction volume. Amplification was performed (Perkin-Elmer GeneAmp PCR System 2400) with initial denaturation for 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C followed by a final extension at 72°C for 7 min.

Intraspecific transmission in *S. minor*. A converted isolate of *S. minor* (Sm10T) containing dsRNA transmitted from isolate Ss275 was used to attempt conversion of isolates Sm5, Sm8, Sm9, and Sm30 to the hypovirulent phenotype. Transmission of hypovirulence and dsRNA was assessed by the lettuce leaf assay, dsRNA extraction and purification, and northern hybridization analysis as described previously.

RESULTS

Transmission of hypovirulent phenotype. Most colonies of *S. minor* developed atypical colony morphologies when paired with isolate Ss275, but the majority of these colonies regained normal morphologies after subculture to PDA. However, in 9 of 39 attempts, subcultures of isolate Sm10 retained an atypical colony morphology after subculture.

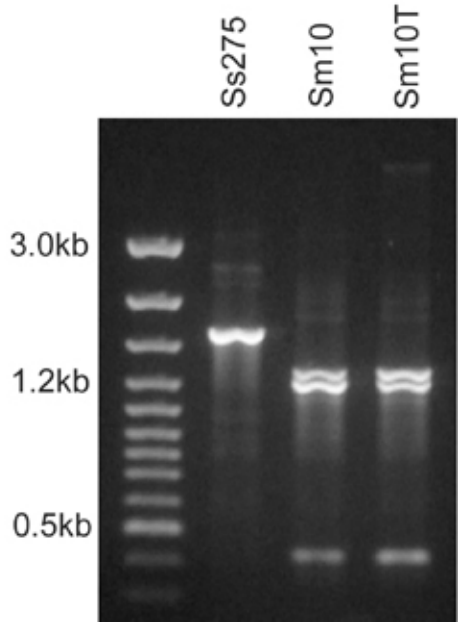

Fig. 2. Polymerase chain reaction amplification products of DNA extracted from *Sclerotinia sclerotiorum* isolate Ss275 and *S. minor* isolates Sm10 and Sm10T with a random amplified polymorphic DNA primer (ACG ACG TAG G), demonstrating that Sm10T was derived from Sm10 and not from Ss275.

Virulence assay. Three of the nine putative converted isolates of Sm10 were randomly selected for evaluation in the lettuce leaf virulence assay. Sm10G, Sm10I, Sm10T, and Ss275 produced significantly smaller lesions ($P \leq 0.05$) on detached leaves of Romaine lettuce than the original Sm10 in the first virulence assay (Table 1). In the second virulence assay, Sm10T and Ss275 produced significantly smaller lesions ($P = 0.047$ and 0.004, respectively) than Sm10, but no significant differences ($P \leq 0.05$) were detected among Sm10G, Sm10I, and Sm10 (data not shown).

Isolation of dsRNA. Sm10G, Sm10I, and Sm10T were evaluated for the presence of dsRNA and all three isolates contained a band of dsRNA consistent in size with the dsRNA in isolate Ss275. dsRNA banding patterns for Sm10T and Ss275 are shown in Figure 1. This dsRNA was not found in the original Sm10.

Northern hybridization analysis. dsRNA isolated from Sm10T hybridized with a DIG-labeled cDNA probe prepared from dsRNA isolated from Ss275 but did not hybridize with dsRNA from the original isolate Sm10 (Fig. 1).

RAPD analysis. Comparison of the amplified segments of DNA of Ss275, Sm10, and Sm10T indicated that Sm10T was derived from Sm10 and not from Ss275 or a hybrid of the two species (Fig. 2).

Intraspecific transmission. Subsequent intraspecific transmission of dsRNA from Sm10T (MCG I) to Sm5 (MCG II), Sm8 (MCG I), Sm9 (MCG III), and Sm30 (MCG III) was attempted 14, 20, 14, and 12 times, respectively. Sm5, Sm9, and Sm30 did not develop atypical morphologies following transmission attempts and subculturing, but in 17 of 20 attempts, Sm8 developed atypical colony morphologies. One of these 17 putative converted isolates, designated Sm8T, was chosen for further study and produced significantly smaller lesions than Sm8 in two virulence assays ($P = 0.047$ and 0.003) (Table 2). A dsRNA similar in size to the dsRNA in Ss275 was isolated from Sm8T (Fig. 1) and this dsRNA hybridized with a cDNA probe prepared from dsRNA isolated from Ss275 (Fig. 1).

DISCUSSION

To our knowledge, this is the first report of interspecific transmission of a hypovirulence-associated dsRNA and hypovirulence phenotype in plant pathogenic fungi by hyphal anastomosis. Transmission was confirmed through assessment of reduced virulence in recipient isolates, detection of dsRNA from Ss275 in recipient isolates of *S. minor* using gel electrophoresis and northern hybridization, and determination of the genotypic background of recipient converted isolates of *S. minor* through RAPD analysis.

Transmission of dsRNA and the hypovirulent phenotype from isolate Ss275 of *S. sclerotiorum* to Sm10T of *S. minor* is presumed to have occurred during hyphal anastomosis. Hyphal anastomosis is the only known route by which dsRNA is intraspecifically transmitted among fungal isolates (8), and the methods used in the present study have previously been used to intra-

TABLE 2. Lesion length of Sm8 and Sm8T on detached leaves of Romaine lettuce 48 h postinoculation[a]

	Lesion length (mm)	
	Sm8	Sm8T
Leaf 1	42.2	0.0
Leaf 2	43.2	0.0
Leaf 3	41.6	0.0
Leaf 4	44.1	2.0
Mean	42.8	0.5

[a] Isolate Sm8T is isolate Sm8 after being converted to the hypovirulent phenotype by Sm10T. Lesion length incited by Sm8 and Sm8T differed significantly at $P = 0.003$ according to Kruskal-Wallis analysis of variance.

specifically transmit dsRNA and hypovirulent phenotypes within *Sclerotinia* spp. through anastomosis. *S. sclerotiorum* and *S. minor* are thought to be closely related species but mycelial incompatibility responses are known to occur between these species (47,50). Although they are closely related, there are numerous reports determining *S. sclerotiorum* and *S. minor* to be distinct species (14,24,26,42,46,49,51).

Little information is available on the host range of hypovirulence-associated dsRNAs because methods for initiating infections with purified dsRNAs are not available for filamentous fungi and interspecific transmission of hypovirulence-associated dsRNAs through hyphal anastomosis has not previously been reported. However, fungi have been artificially infected by hypoviruses from other species. Hypovirus-free isolates of *C. parasitica*, *C. radicalis*, *C. havanensis*, *C. cubensis*, and *Endothia gyrosa* were infected by electroporation of sphearoplasts with synthetic transcripts of a full-length hypovirus, CHV1-713, from *C. parasitica* (10,12). Chen et al. (10) reported that the same hypovirus can have different phenotypic effects on different host species, and that some phenotypic traits associated with hypovirulence are dependent on the genetics of the hypovirus while others are influenced by the genetics of the host. Transmission of a hypovirulence-associated dsRNA from isolate Ss275 of *S. sclerotiorum* to isolates of *S. minor* was accompanied by the development of a phenotype similar to that previously reported for hypovirulent isolate Ss275. These results establish that *S. minor* is also susceptible to the hypovirulence-associated dsRNA from isolate Ss275 of *S. sclerotiorum*.

Instability was observed in some isolates where dsRNA and the hypovirulent phenotype were transferred between isolates and species. In these instances, isolates initially displayed the hypovirulent phenotype but, with subsequent subculturing, regained the typical wild-type phenotype. These instances were interpreted as either noninfected sectors of the culture outgrowing infected sectors or the possible development of latent infections. We have previously observed virulent sectors growing from hypovirulent colonies. Latent infection of hypovirulence-associated dsRNAs have been reported in *Ophiostoma ulmi* (40) and *Rhizoctonia solani* (28). Although some isolates regained the typical wild-type phenotype, isolate Sm10T retained the hypovirulent phenotype throughout all experiments and appeared to be a stable recipient isolate that was hypovirulent and contained dsRNA.

To date, the interspecific transferral of hypovirulence-associated dsRNAs or cDNA transcripts of these dsRNAs have only been reported from in vitro experiments. Transfer of dsRNA between Sm10 and Ss275 was attempted in vivo but was unsuccessful because isolate Ss275 was unable to grow on lettuce tissue. In fungi, there have been reports of transfer of genetic material between genetically isolated clones of the same species, between a mycoparasite and its host, and between two unrelated species that inhabit the same ecological niche. Masel et al. (31) reported evidence of transfer of a chromosome between two biotypes of *Colletotrichum gloeosporioides.* The biotypes were considered genetically isolated clones. Kellner et al. (22) demonstrated in vitro transfer of genetic material from the mycoparasite *Parasitella parasitica* to its host *Absidia glauca*. This was a specific case where a plasma bridge formed between hyphae of the two species. Researchers were able to transfer genetic information of *Parasitella parasitica* to the host and to transform *Parasitella parasitica* with a plasmid that they were then able to transfer to *Absidia glauca*. Kempken (23) demonstrated in vitro transfer of a mitochondrial plasmid from *Ascobolus immersus* to *Podospora anserina*. These species inhabit the same ecological niche and are unrelated. Hyphal anastomosis was not observed between *Ascobolus immersus* and *Podospora anserina* but may occur at a low frequency. Rare interspecific hybrids of *O. ulmi* and *O. novo-ulmi* have been identified in natural populations of these fungi where their distributions overlap, and may serve as genetic bridges facilitating the transfer of genetic traits between species (7). Transfer of other elements such as introns, repetitive DNA elements, and mitochondria among isolates and species of fungi have also been reported (18,30,43).

The results of these studies and the present study suggest that hypovirulence-associated dsRNAs may be transmitted between fungal species in the field. The species examined in this study, *S. sclerotiorum* and *S. minor*, share many hosts (4,36), are often present in the same agricultural fields (21,35,41), and have been observed on the same plant (41). Therefore, the potential for interaction and possible transferral of hypovirulence-associated dsRNAs in vivo also exists between these two species and possibly others. As part of a risk assessment of this strategy for biological control, evaluations of interspecific transferral of hypovirulence-associated dsRNAs or cDNA transcripts of these dsRNAs to target and nontarget microorganisms should be considered.

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