Characterization of a Strain of *Cryphonectria parasitica* Doubly Infected with Hypovirulence-Associated dsRNA Viruses

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ABSTRACT

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To examine the effect of double infection by hypovirulence-associated viruses on the phenotype of *Cryphonectria parasitica,* dsRNAs from two Michigan hypovirulent isolates were cytoplasmically transferred into a common nuclear background. The coinfected strain had a dsRNA banding pattern profile that represented the sum total of the dsRNA segments of the parental strains. The douby infected strain was less virulent than either of the singly infected strains. Asexual segregation of the dsRNA viruses was followed through single-conidial isolates. The resulting asexual progeny contained one or both parental dsRNAs without any detected exchange of dsRNA segments. These findings indicate that field isolates of *C. parasitica* containing more than one dsRNA segment may be coinfected and that asexual segregation patterns relating dsRNA segments to hypovirulence phenotypes should be pursued before assuming the observed hypovirulence phenotype is due to a single virus.

The American chestnut (*Castanea dentata* (Marsh) Borkh.), a co-dominant hardwood species of the eastern forest, was reduced to a population of immature understory sprouts throughout its natural range by the accidental importation of the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr (1). The European chestnut (*C. sativa* Miller) in Italy and France, and the American chestnut in a few locations in North America, including Michigan, are surviving despite repeated infections (9,13). The isolates of *C. parasitica* recovered from surviving chestnut trees in Europe and North America harbor double-stranded RNA (dsRNA) molecules that are associated with reduced virulence, altered culture morphology, a reduced growth rate, and changes in pigmentation, when compared with isogenic strains without dsRNA. These isolates are termed hypovirulent and are capable of transferring these traits through hyphal anastomosis to virulent strains conferring hypovirulence on the new strain (15).

A dsRNA virus of French origin from *C. parasitica* strain EP713 was recently designated as the type species of a newly established family of unencapsidated dsRNA viruses termed "*Hypoviridae*" (11,12). This dsRNA virus, designated Cryphonectria hypovirus 1-EP713 (CHV1-EP713), was shown to be directly responsible for conferring a hypovirulent phenotype on its fungal host (3). EP713 harbors several dsRNAs including the largest dsRNA segment designated L-dsRNA (12.7 kb) and several smaller segments designated M-dsRNA (8–10 kb) and S-dsRNA (0.6–1.7 kb). Transformation studies with a full-length cDNA clone of L-dsRNA, which contains two open reading frames (ORF), known as ORF A and ORF B (18), proved that L-dsRNA is solely responsible for the specific hypovirulence phenotype observed in EP713 (3). The smaller dsRNA segments observed in EP713 are internally deleted forms of L-dsRNA (18). These defective dsRNAs contribute to the overall complexity of the dsRNA populations within hypovirulent strains but their role in hypovirulence, if any, is unknown.

While Michigan and European dsRNA viruses share little sequence homology, the complexity of the dsRNA population within a fungal strain, as resolved through gel electrophoresis, is similar (4,16,19). The Michigan hypovirulent strain, GH2, contains at least three dsRNA segments of different size including L-dsRNA (>9.6 kilobase pairs, kb), M-dsRNA (3.5 kb) and S-dsRNA (0.8 kb) (4,19). Whereas M-dsRNA sequence is derived from L-dsRNA, S-dsRNA has a unique sequence. It has been postulated that M- and S-dsRNAs represent defective and satellite-like dsRNAs of the L-dsRNA, respectively (19). This is further corroborated in field release studies of GH2 in which L-dsRNA can be isolated without M- and S-dsRNA, but M- and S-dsRNA have never been found without L-dsRNA (10).

It is important to understand the complexity of dsRNA populations in hypovirulent isolates, so that it may be determined whether or not a single isolate is coinfected with more than one virus. Elliston (5,6) reported evidence that two dsRNA viruses can coexist in EP-60, a bark isolate from a surviving American chestnut tree in Michigan. Single-conidial isolates were collected from this isolate and, after an exhaustive process of relating phenotypes, virulence, and dsRNA electrophoretic banding patterns, it was determined that two viruses were present in the cytoplasm of EP-60 and were responsible for its hypovirulence phenotype. Elliston (6) reported nine and 11 dsRNA segments were associated with the two viruses, respectively; however, 15 segments were observed in the coinfected strain. Our laboratory has obtained similar results with another Michigan hypovirulent isolate, GHU4, but fewer dsRNA segments were involved (8,9,16). Thus, strains with more than one dsRNA segment may be coinfected or they may be infected with one virus with associated defective or satellite dsRNA.

Obviously, such interpretations could be misleading in terms of both genetic and field studies in which the simple presence or absence of dsRNA viruses may not provide sufficient details relating to the outcome of successful biocontrol of chestnut blight. The work presented in this paper was initiated to increase our understanding of coinfections in *C. parasitica.* By purposely transferring dsRNAs from two separate and distinct Michigan *C. parasitica* strains to a virulent strain, we have been able to measure the effect of coinfection on the virulence and oxalic acid production of the strain (20). In this paper we report on the segregation patterns of dsRNA segments from douby infected strains of *C. parasitica* in single-conidial isolates.

MATERIALS AND METHODS

Cultures and growth conditions. The *C. parasitica* cultures used in this study are listed in Table 1. The virus-infected isolate GH2 has been classified as a tentative species in the genus *Hypovirus*

and has been designated CHV3-GH2 (12). This virus consists of three dsRNA molecules designated L-dsRNA (>9.6 kb), M-dsRNA (3.5 kb) and S-dsRNA (0.8 kb). We have not characterized the other dsRNA virus used in this study and, therefore, will only refer to it as RC1 dsRNA (9). The hypovirulent strain, RC1, contains only two relatively small segments of dsRNA, dsRNA-1 (2.8 kb) and dsRNA-2 (1.6 kb).

All fungal strains used in this work were vegetatively compatible and all additional strains used for this work were derived from these cultures. The fungus was grown on potato-dextrose agar (PDA; Difco, Detroit, MI) at room temperature under cool-white fluorescent lights with a 16-h photoperiod (10). A mutant of *C. parasitica* resistant to pentachloronitrobenzene (PCNB, Olin-Terracoat L205) was previously isolated (10). This strain was cultured on Endothia complete agar supplemented with 100 μg PCNB/ml. Cultures were stored on PDA slants at 4 C. Cultures used for dsRNA isolation were grown on cellophane-covered PDA plates for 7 days or in stationary culture in Endothia complete broth without glucose (17) for 14–21 days.

Strain construction. To reduce the potential for interference resulting from different nuclear backgrounds, the dsRNA viruses from the two hypovirulent fungal isolates (GH2 and RC1) were transferred to the common nuclear background of CL1-16, a virulent single-conidial isolate of the bark isolate CL1 (9,20). Transfer of dsRNA from each of the hypovirulent isolates to CL1-16 was accomplished by pairing a hypovirulent isolate with CL1-16 on PDA as previously described (2,20). The construction of a coinfected strain from two singly infected strains was accomplished by pairing the two converted CL1-16 isolates (20). Conversion of either strain was deduced by noting a significant decrease in growth of one or both isolates at the margin of the two colonies (20). Subcultures of the putative coinfected strains were made on PDA. In all cases, transfer of dsRNA was confirmed by polyacrylamide gel electrophoresis (PAGE) of the dsRNA molecules. Also, culture morphology changes were noted and virulence assays were performed. Converted strains were given designations listing the source of the dsRNA in parentheses (Table 2).

To ensure that differences in fungal morphology and virulence were due to the presence of the virus and not due to a nuclear effect, dsRNA was transferred from the coinfected strain CL1-16(GH2/RC1) to a strain that carried a nuclear marker. Transfer of dsRNA from the coinfected strain in the CL1-16 nuclear background into the nuclear background of the CL1 PCNB-resistant strain (CL1-PCNB^R) was accomplished on PDA by pairing the cultures as described above. Subsequent subcultures from the CL1-PCNB^R(GH2/RC1) strain were grown on Endothia complete agar supplemented with PCNB and were compared with growth of the PCNB-sensitive strain CL1-16(GH2/RC1).

Single-conidial isolation and virulence assays. Single pycnidiospores (conidia) were isolated from the singly infected strains CL1-16(GH2) and CL1-16(RC1) as well as from the coinfected strains CL1-16(GH2/RC1) and CL1-PCNB^R(GH2/RC1) by removing 10-mm-diameter plugs from 10-day-old cultures and placing them in sterile distilled water. Ten-fold serial dilutions of the resulting spore suspensions were used to inoculate PDA plates. After 3 days, individual germinating spores were identified, were cut from the agar surface, and were subcultured on PDA. Virulence was determined by inoculating Golden Delicious apple fruit with mycelial plugs removed from PDA plates and then measuring the resulting lesion 3 wk after inoculation (8,20).

Double-stranded RNA isolation. Double-stranded RNA was isolated as described by Morris and Dodds (14) and modified by Fulbright et al (9). The extract was loaded on 5% polyacrylamide gels, was electrophoresed, and then stained with ethidium bromide or silver stained according to manufacturer's protocol (Bio-Rad, Hercules, CA).

TABLE 1. Virulent and hypovirulent isolates and strains of *Cryphonectria parasitica* used to construct singly and doubly infected hypovirulent strains

Isolate designation	dsRNA[y]	Virulence[z]	Reference
CL1-16	−	V	16,20
GH2	+	H	8,9,10,16,19,20
RC1	+	H	8,9,16,20
CL1-PCNB^R	−	V	10

[y]+, dsRNA is present; −, dsRNA is not detectable.
[z]V, determined virulent in virulence assays; H, determined hypovirulent in virulence assays.

TABLE 2. Characteristics of virulent *Cryphonectria parasitica* strains after infection with dsRNA

Strain[y]	Virulence tests[z] (mm^2)	dsRNA molecules detected	
		CHV3-GH2	RC1
CL1-16	3,125 a	−	−
PCNB^R	2,982 a	−	−
CL1-16(GH2)	1,062 b	+	−
CL1-16(RC1)	831 b	−	+
CL1-16(GH2/RC1)	224 c	+	+
PCNB^R(GH2/RC1)	227 c	+	+
CL1-16(GH2/RC1)SCI1	1,024 b	+	−
CL1-16(GH2/RC1)SCI2	745 b	+	−
CL1-16(GH2/RC1)SCI7	4,359 a	−	−
CL1-16(GH2/RC1)SCI11	932 b	−	+
CL1-16(GH2/RC1)SCI17	165 c	+	+
CL1-16(CH2/RC1)SCI19	982 b	+	−
CL1-16(GH2/RC1)SCI23	134 c	+	+
CL1-16(GH2/RC1)SCI25	2,835 a	−	−
PCNB^R(GH2/RC1)SCI26	3,080 a	−	−
PCNB^R(GH2/RC1)SCI42	355 c	+	+
PCNB^R(GH2/RC1)SCI48	1,059 b	+	−
PCNB^R(GH2/RC1)SCI50	791 b	−	+

[y]Strains in parentheses served as the source of dsRNA, which was transferred into the virulent strain CL1-16 or CL1-PCNB^R (designated here as PCNB^R).
[z]Virulence data represent mean lesion areas of inoculated Golden Delicious apple fruit from three replicates per strain. Means followed by the same letter do not differ significantly ($P = 0.05$) according to Tukey's honestly significant difference test.

RESULTS

Co-infection studies. Transfer of dsRNA into CL1-16 from fungal isolates GH2 and RC1 was verified by dsRNA electrophoretic banding patterns (Fig. 1; Fig. 2 step 1). As previously reported (20), the transfer was accompanied by a significant reduction in virulence (Table 2). Upon dsRNA analysis, the band intensities of individual dsRNA segments after PAGE varied between different dsRNA extractions. This phenomenon was characteristic of all strains utilized in this study. We occasionally observed a fourth band in some dsRNA preparations from strains containing CHV3-GH2, as has been previously reported (20). Pairing the singly infected strains CL1-16(GH2) and CL1-16(RC1) resulted in strain CL1-16(GH2/RC1), which contained dsRNA from both sources (Fig. 1; Fig. 2 step 2). This coinfected strain was more debilitated in culture than either singly infected parent. When grown on PDA, CL1-16(GH2) and CL1-16(GH2/RC1) were a bright orange color similar to that of CL1-16, while CL1-16(RC1) was dark orange. Similarly, both CL1-16(GH2) and CL1-16(GH2/RC1) had aerial hyphae; however, CL1-16(RC1) was flat, with no aerial hyphae. All of the strains used in this study produced conidia when grown on PDA. Upon transfer of dsRNA from strain CL1-16(GH2/RC1) into strain CL1-PCNB^R, dsRNA banding patterns identical to those of CL1-16(GH2/RC1) were observed after PAGE (Fig. 3). This strain, CL1-PCNB^R(GH2/RC1), which was resistant to PCNB, demonstrated a culture morphology, virulence (Table 2), and dsRNA electrophoretic banding pattern (Fig. 3) identical to that of CL1-16(GH2/RC1).

Characterization of single-conidial isolates. To determine whether dsRNA viruses or their individual segments from two different hypovirulent strains can segregate independently from a coinfected strain, single-conidial isolates were obtained from CL1-16(GH2/RC1) (Fig. 2, step 3), as well as from strains CL1-16, CL1-16(RC1),

CL1-16(GH2), CL1-16(GH2/RC1)SCI23, and CL1-PCNB^R (GH2/RC1) (Tables 2 and 3). The isolates from each strain were classified according to phenotype and two to five representatives of each class were chosen for virulence and dsRNA assays. Strain CL1-16 produced single-conidial isolates that were all highly virulent and did not contain dsRNA, while CL1-16(RC1) and CL1-16(GH2) produced asexual progeny with either no dsRNA or with dsRNA banding patterns similar to those of RC1 dsRNA or CHV3-GH2. Segregation of the dsRNA viruses in single-conidial isolates from CL1-16(GH2/RC1) resulted in four classes of strains: those without dsRNA; those containing dsRNA with electrophoretic banding patterns similar to CHV3-GH2; RC1 dsRNA; or both CHV3-GH2 and RC1 dsRNA (Fig. 2 step 3; Fig. 4). The single-conidial isolates displayed morphological and growth characteristics consistent with the dsRNA they contained. For example, the isolates containing RC1 dsRNA showed a phenotype like that of CL1-16(RC1), even though they were progeny of CL1-16(GH2/RC1). The isolates containing CHV3-GH2, RC1 dsRNA and CHV3-GH2, or no dsRNA all showed phenotypes like the strains CL1-16(GH2), CL1-16(GH2/RC1), or CL1-16 respectively. Forty-six single-conidial isolates were also collected from CL1-16(GH2/RC1)SCI23 (Fig.4, lane 3), a coinfected single-conidial isolate of strain CL1-16(GH2/RC1). The 46 resulting cultures segregated in similar proportion as observed for single-conidial isolates of the coinfected parental strain CL1-16(GH2/RC1). Similar segregation patterns were also seen in strain CL1-PCNB^R(GH2/RC1), in which, again, four classes of single-conidial isolates were identified. Morphological and growth characteristics of these single-conidial isolates were also consistent with the dsRNA they contained. No exchange of dsRNA segments was observed to occur between CHV3-GH2 and RC1 dsRNA. Virulence of the various single-conidial isolates with the different dsRNA electrophoretic banding patterns was comparable with the respective singly infected hypovirulent strains (Table 2).

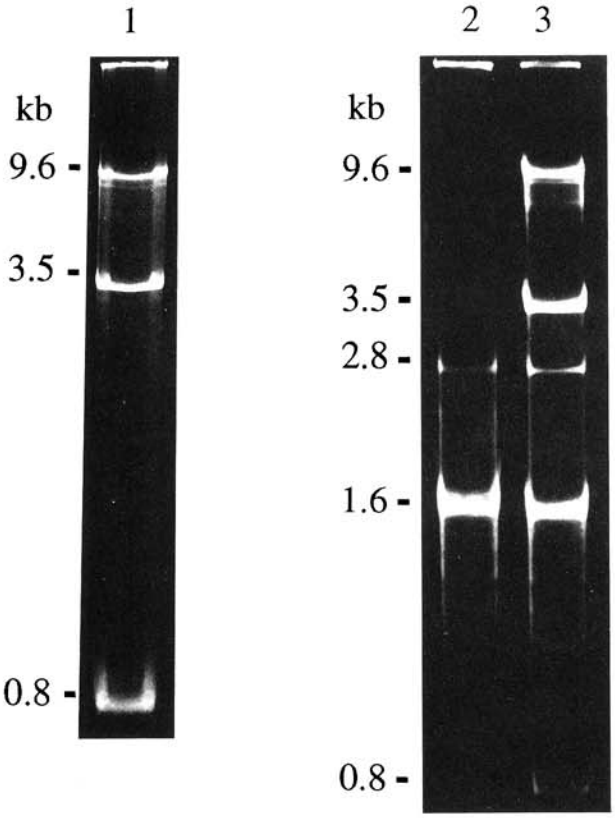

Fig. 1. Banding patterns of dsRNA isolated from converted strains of *Cryphonectria parasitica* electrophoresed in a 5% polyacrylamide gel and stained with ethidium bromide. Lanes: 1, CL1-16(GH2); 2, CL1-16(RC1); 3, CL1-16(GH2/RC1). Sizes of dsRNA molecules present in strains RC1 and GH2 are indicated in kilobase pairs (kb).

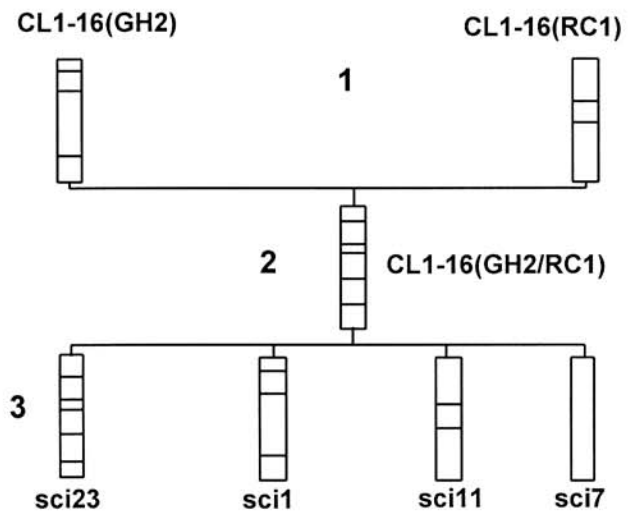

Fig. 2. Diagrammatic representation of dsRNA banding patterns in stained polyacrylamide gels. Thick horizontal lines represent dsRNA bands in a gel. Numbers designate steps referred to in text.

DISCUSSION

The primary goal of this study was to analyze the maintenance and segregation of two distinct dsRNA viruses associated with hypovirulence phenotype when they were introduced into a single nuclear background. The effects on virulence and oxalic acid production have also been studied (20). We found that the dsRNAs associated with isolates GH2 and RC1 segregated independently from coinfected strains without a detectable mixing of individual dsRNA segments during transmission to conidia. When dsRNA was present in single-conidial isolates, the dsRNA banding pattern profiles always included the known segments of the complete viral genomes.

The coinfected strain CL1-16(GH2/RC1) was less virulent than singly infected strains CL1-16(GH2) and CL1-16(RC1), suggesting

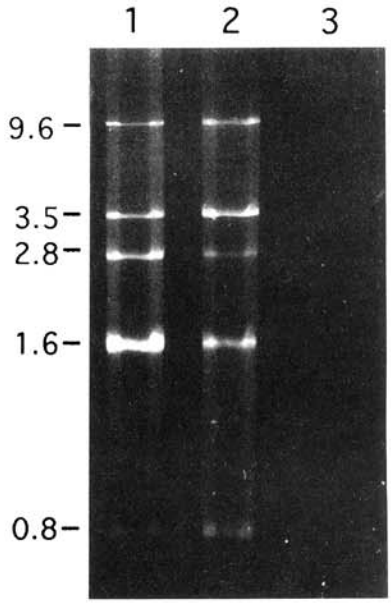

Fig. 3. Banding patterns of dsRNA isolated from the converted pentachloronitrobenzene-resistant (PCNB^R) strain of *Cryphonectria parasitica* electrophoresed in a 5% polyacrylamide gel and stained with ethidium bromide. Lanes: 1, CL1 PCNB^R(GH2/RC1); 2, CL1-16(GH2/RC1); 3, CL1-PCNB^R. Sizes of dsRNA molecules are indicated in kilobase pairs (kb).

TABLE 3. Double-stranded RNA viruses present in single-conidial isolates collected from various singly or doubly infected strains of *Cryphonectria parasitica*

Strain	Classification of single-conidial isolates[z]				Total number examined
	Without dsRNA	CHV3-GH2	RC1-dsRNA	CHV3-GH2/RC1-dsRNA	
CL1-16	47	0	0	0	47
CL1-16(GH2)	25	25	0	0	50
CL1-16(RC1)	51	0	19	0	70
CL1-16(GH2/RC1)	101	107	14	20	242
CL1 PCNB^R(GH2/RC1)	90	86	5	9	190
CL1-16(GH2/RC1)SCI23	18	21	3	4	46

[z]dsRNA types were based on dsRNA-banding patterns on polyacrylamide gel electrophoresis.

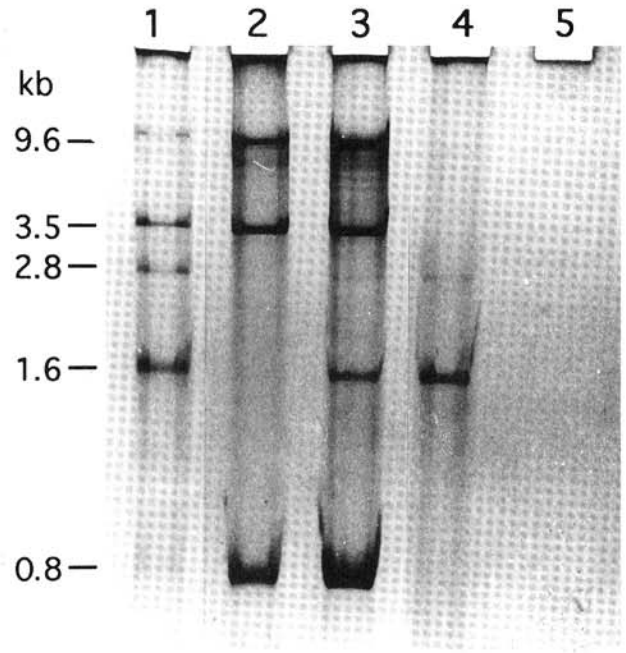

Fig. 4. Banding patterns of dsRNA isolated from single-conidial isolates (SCI) of *Cryphonectria parasitica* strain CL1-16(GH2/RC1). The dsRNA was electrophoresed in a 5% polyacrylamide gel and silver stained. Lanes: 1, CL1-16(GH2/RC1); 2, CL1-16(GH2/RC1)SCI1; 3, CL1-16(GH2/RC1)SCI23; 4, CL1-16(GH2/RC1)SCI11; 5, CL1-16(GH2/RC1)SCI7. Sizes of dsRNA molecules are indicated in kilobase pairs (kb).

that CHV3-GH2 and RC1 dsRNA act additively. This is in contrast to the studies of Elliston (5,6), who did not observe an additive effect upon coinfection. We also observed distinct culture morphologies and virulence states that could be identified with the individual dsRNA virus upon isolating single conidia from each coinfected strain.

The contributions of mixed infection may be important when hypovirulent strains are functioning as biocontrol agents in surviving chestnut stands. If two viruses were present, theoretically three hypovirulent phenotypes could be expected, resulting from infection by each virus separately and by coinfection of the two. While there is no evidence to date that three viruses can infect one strain, the presence of three viruses in the population of the fungus could potentially produce seven hypovirulent phenotypes when the various coinfection combinations are considered.

Hypovirulent strains recovered from infected but surviving chestnut trees often harbor more than one segment of dsRNA (16). That fact, when considered with the results of this study, the work of Elliston (6), and the work of Nuss and co-workers (3,18), indicates that the hypovirulence phenotype of an unknown strain harboring a population of dsRNA segments may be a result of a single dsRNA virus infection or of multiple infections. This work raises the point that a hypovirulent strain with multiple dsRNAs could be coinfected by two distinct viruses, each contributing to the hypovirulence phenotype. Therefore, to determine whether the phenotype of a hypovirulent strain is caused by one or more dsRNA viruses, it is important to study the asexual segregation of viral dsRNA segments and the phenotypic effects arising from different combinations of viral genomes.

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