A Hypovirulent Isolate of *Cryphonectria parasitica* with Multiple, Genetically Unique dsRNA Segments

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Many different dsRNA-containing isolates of *Cryphonectria (Endothia) parasitica*, the chestnut blight fungus, have been identified in surveys conducted in the central Appalachians. Isolate C-18, collected in 1989 from a virulent canker in southern West Virginia, was found to contain 11 segments of dsRNA ranging from 1 to 5 kb in size. Virulence experiments, using Golden Delicious apples, excised dormant American chestnut stems, and American chestnut sprouts, determined that the dsRNA associated with isolate C-18 significantly reduced virulence and altered cultural morphology when isogenic dsRNA-containing and dsRNA-free asexual progeny were compared. The 11 dsRNA segments were transmitted into other isolates of *C. parasitica* via hyphal anastomosis and into conidia in an all-or-none fashion. Complementary DNA clones from a library representing C-18 dsRNA did not hybridize to other dsRNAs associated with hypovirulent isolates D2 (Pennsylvania), Ep-713 (Europe), NB58-88 (New Jersey), to another multisegmented dsRNA from West Virginia (9B-2-1), or to wound tumor virus. None of the cDNA clones tested hybridized to more than one dsRNA segment by Northern blot analysis. Eight of the 11 dsRNA segments were demonstrated to be genetically unique. Using a phosphate buffer extraction protocol, icosahedral particles approximately 60 nm in diameter were purified from the mycelial tissue of isolate C-18. The dsRNA virus associated with hypovirulence of isolate C-18 is therefore fundamentally different from other hypovirulence-associated viruses characterized to date.

Additional keywords: Endothia, chestnut blight, reovirus.

Since the discovery of swollen, superficial cankers and chestnut trees recovering from chestnut blight in Europe (Biraghi 1953) and Michigan (Elliston *et al.* 1977; Day *et al.* 1977; Day 1978), many different types of dsRNA have been found associated with *Cryphonectria (Endothia) parasitica*

(Murrill) Barr, the chestnut blight fungus. The number of dsRNA segments, their relative sizes, titers, and sequence relationships, as well as the effects these dsRNAs have on the fungus with respect to virulence and morphology, vary significantly among the dsRNAs examined (Fulbright *et al.* 1983; Paul and Fulbright 1988; Hillman *et al.* 1992; Hiremath *et al.* 1986).

Hypovirulence-associated dsRNAs affiliated with *C. parasitica* are often multisegmented. Typically these multisegmented dsRNAs consist of a larger 12–13-kb segment along with smaller segments that range in size from 1 to 10 kb (L'Hostis *et al.* 1985; Paul and Fulbright 1988; Shapira *et al.* 1991a). These smaller segments are derived by internal deletions from the largest segment so that dsRNA segments within isolates examined have conserved regions and share terminal sequences (Hiremath *et al.* 1986; Tartaglia *et al.* 1986; Shapira *et al.* 1991b). The largest segment contains all genetic information required for virus replication and maintenance (Choi and Nuss 1992).

The only members characterized to date belong to the recently described Hypoviridae family (Hillman *et al.* 1994a), which are ancestrally related to the Potyviridae family of plant viruses. Transmission electron microscopy examination of freeze-substituted hyphae of virulent and hypovirulent isolates reveals dsRNA associated with hypovirulent isolates in viruslike particles (Newhouse *et al.* 1983, 1990). Members of the Hypoviridae are not encapsidated in a protein coat but are encapsulated in lipid vesicles derived from host membranes (Hansen *et al.* 1985).
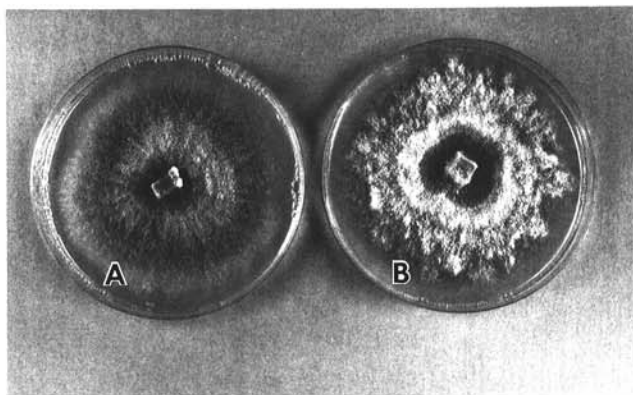

Fig 1. Cultural morphology of A, dsRNA-containing isolate C-18 and B, dsRNA-free single conidial isolate C18-23. Isolate C-18 produces a brownish-orange pigment on PDAmb, spreads more slowly across the plate, and produces fewer aerial hyphae. Isolate C18-23 produces an orange pigment on PDAmb, spreads more rapidly across the plate, and produces both diurnal zonations and aerial hyphae.

Recently, isolate C-18 was found to contain multiple segments of dsRNA ranging in size from 1 to 5 kb. The relative size and the number of segments of this dsRNA were unusual with respect to dsRNA from other North American or European isolates (Dodds 1978; Paul and Fulbright 1988; Shapira et al. 1991b). Since this dsRNA was atypical of many dsRNAs associated with *C. parasitica*, a series of experiments was conducted to examine isolate C-18 more closely. Specifically, the goals of this experimentation were to examine the effects of this dsRNA on morphology, virulence, and transmissibility and to compare the dsRNA associated with isolate C-18 to other known dsRNAs associated with hypovirulence. Preliminary reports of this work have been published (Enebak and MacDonald 1990; Enebak et al. 1991).

RESULTS

Influence of dsRNA on cultural morphology and sporulation.

Two distinct cultural morphologies resulted from the 60 single conidial isolates of C-18. One set, which looked like the original isolate C-18 with brownish orange pigment, grew more slowly and produced fewer aerial hyphae. The second set was orange pigmented and grew across the plate with diurnal zonations and aerial hyphae (Fig. 1). The presence of dsRNA also significantly reduced the number of conidia produced by isolate C-18. A 10-fold increase in the number of spores produced by dsRNA-free isolate C18-23 over isolate C-18 was observed (Fig. 2). The number of conidia produce by isolate C18-23 was similar to those produced by the dsRNA-free virulent control isolate Ep-155. In addition to reducing sporulation, the presence of dsRNA significantly reduced the production of laccase (data not shown).

Sixty single conidial isolates derived from C-18 were analyzed for the presence of dsRNA. Isolates that were similar in morphology to isolate C-18 (14 of 60) contained dsRNA, whereas those isolates that did not resemble C-18 were dsRNA-free. All 11 dsRNA segments found in isolate C-18 were present in each of the 14 dsRNA-containing single conidial isolates.
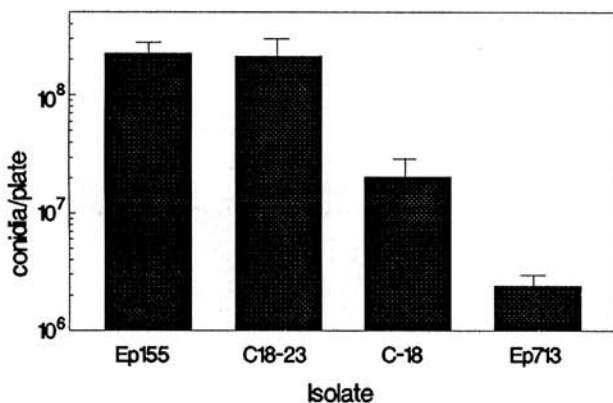
Fig. 2. Sporulation of *C. parasitica* isolates. Conidia from two virulent, dsRNA-free isolates (Ep155 and C18-23) and two hypovirulent, dsRNA-containing isolates (C-18 and Ep713) were counted as described in the text. Five replicates of each isolate were counted.

Transmission of dsRNA into vegetatively compatible and incompatible isolates.

Pairings of isolate C-18 with the dsRNA-free progeny C18-23 and C18-37 resulted in morphological alteration of colony growth to the C-18 phenotype. Double-stranded RNA analysis of subcultures from the leading edge of the pairings yielded 11 segments of dsRNA. When isolate C-18 was paired with the 37 vegetative compatibility testers, it converted three different vegetative compatibility groups to C-18
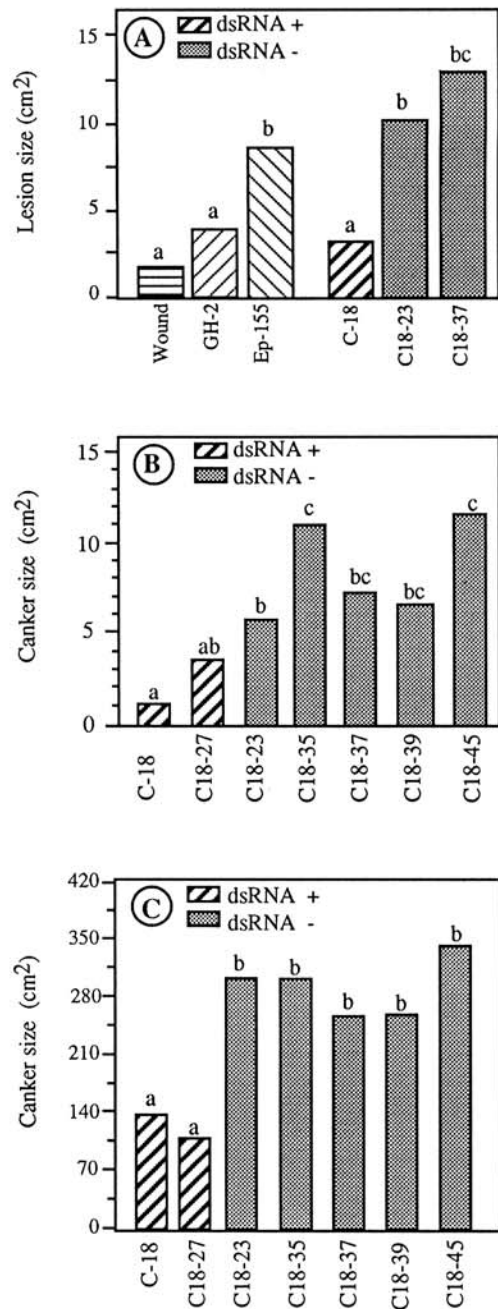
Fig. 3. Effect of cytoplasmic dsRNA on the size of lesions produced by single conidial isolates from strain C-18. **A,** Fourteen days after inoculation on Golden Delicious apples. **B,** Four weeks after inoculation on excised, dormant American chestnut stems. **C,** One year after inoculation on American chestnut sprouts in the field. Isolates GH-2 and Ep-155 illustrated in A are dsRNA positive and negative control isolates, respectively. Bars with the same letter are not significantly different according to Fisher's LSD ($P = 0.05$).

morphology. Transmission of dsRNA from isolate C-18 to the different isolates was confirmed by dsRNA analysis. The remaining 34 pairings also were analyzed for dsRNA, and none contained dsRNA.

Influence of dsRNA on virulence.

In the Golden Delicious apple inoculations, dsRNA-free progeny C18-23 and C18-37 were significantly more virulent than isolate C-18 (Fig. 3A). The size of lesions produced by these two dsRNA-free isolates was comparable to the virulent control Ep-155. In contrast, isolate C-18 produced lesions as small as the hypovirulent control isolate GH-2. Similar results were obtained when dsRNA-containing isolates C-18 and C18-27 were inoculated into excised dormant chestnut stems. Relative sizes of cankers are shown in Figure 3B, which represents data collected 4 wk after inoculation. Canker size ranged from 1.2 cm² for C-18 to 11.7 cm² for C18-45. Although C18-27 was not significantly smaller than three of the dsRNA-free isolates tested, it and C-18 did produce the smallest cankers. Canker size on American chestnut sprouts 12-mo after inoculation resulted in the dsRNA-free progeny producing lesions that were significantly larger than their dsRNA-containing parent (C-18) and dsRNA-containing progeny C18-27 (Fig. 3C).

Cloning and analysis of dsRNA.

Random priming and cloning of C-18 dsRNA yielded a cDNA library totaling 360 white colonies. Digestion of randomly selected plasmids with the restriction enzyme *Pst*I liberated cDNA inserts ranging in size from less than 0.5 to more than 3 kb. Dilutions of denatured dsRNA or plasmid preparations were spotted to nylon membranes and probed with a ^{32}P-labeled cDNA clone of C-18 dsRNA. The recombinant plasmid hybridized only to the C-18 template dsRNA and to the control pUC9, but not to the other North American (D2, NB58-88) or European (Ep-713, Ep-747) dsRNAs tested (Fig. 4). Sixteen cDNA clones were further characterized by Northern blot analysis to determine their segment specificities. C-18 dsRNA separated through denaturing agarose gels was blotted to nitrocellulose membranes and probed with a number of ^{32}P-labeled cDNA clones. In each experiment, a
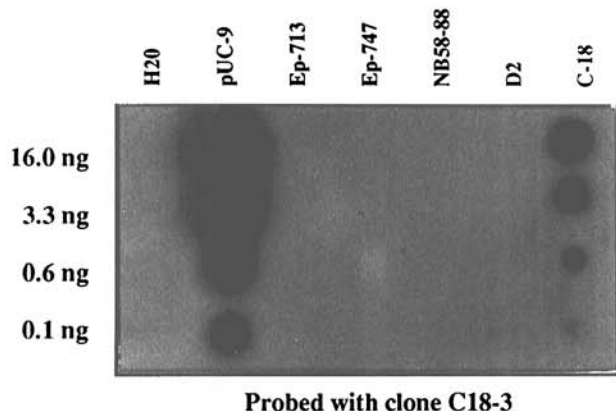

Probed with clone C18-3

Fig. 4. Dot blot hybridization of dsRNA preparations from North American and European isolates probed with a cDNA clones of isolate C-18. Approximate amounts of dsRNA spotted are listed on the left; dsRNA preparations are listed at the top of the membrane. Lanes 1 and 2 are water and pUC9, negative and positive controls; lanes 3 through 7 are dsRNA preparations from isolates Ep-713, Ep-747 (Italy), NB58-88 (New Jersey), D2 (Pennsylvania), and C-18 (West Virginia).
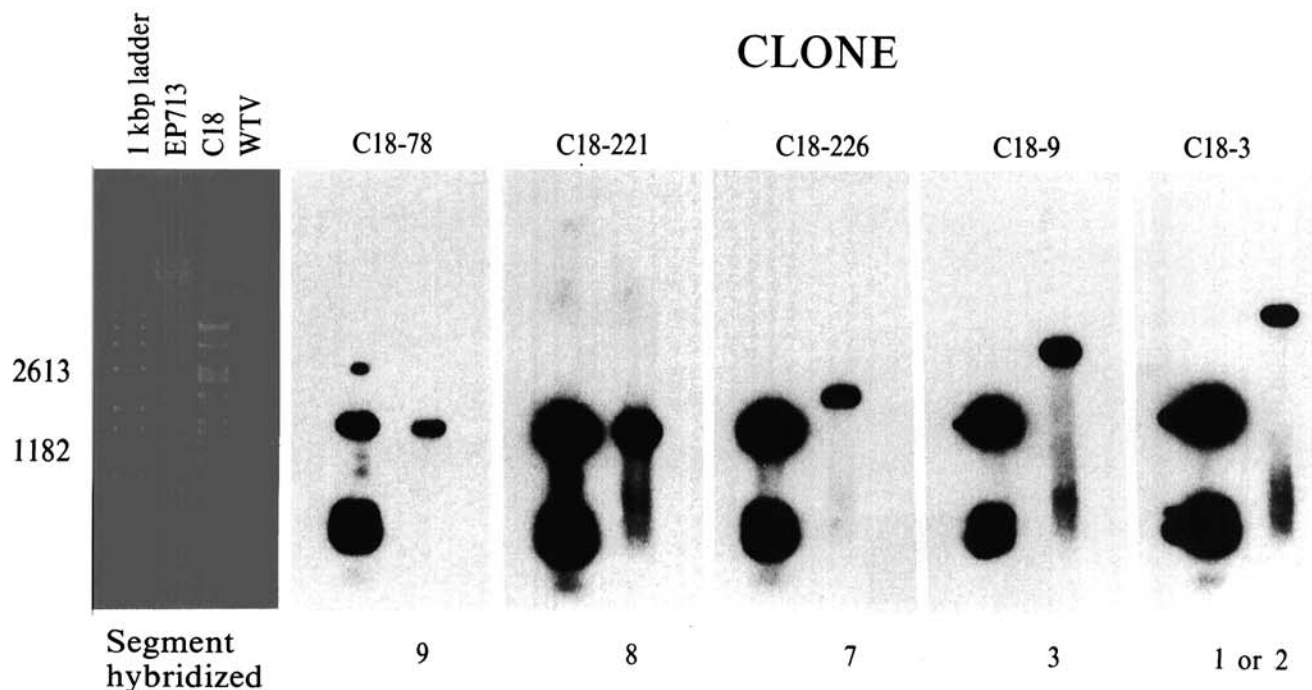

Fig. 5. Northern blot hybridization of dsRNA preparations from isolates C-18, wound tumor virus (WTV), and Ep-713 probed with five cDNA clones from isolate C-18. The clone used as a probe and the corresponding segment to which it hybridized are listed above and below each membrane, respectively. For each membrane blotted, lanes 1 and 2 are 1-kb ladder and dsRNA from Ep-713, positive and negative controls, respectively. Lanes 3 and 4 are C-18 and WTV. Hybridization to the 1-kb ladder lane represents the pBR322-derived fragments of the ladder hybridizing with the pUC9-derived portion of cDNA clones.

single cDNA clone hybridized to only one dsRNA segment within the blot (Fig. 5). Clones representing eight of the 11 dsRNA segments have been identified to date.

Virus particle extraction.

Double-stranded RNA analysis of the phosphate and Mg-histidine buffer extractions of C-18 mycelia yielded 11 segments of dsRNA in approximately equal molar amounts (Fig. 6). Approximately 1 μg of dsRNA per gram of mycelium was recovered from the phosphate buffer extract; considerably less was recovered from the histidine extract. When samples of the particle extractions using the phosphate buffer were stained with 2% phosphotungstic acid and examined by transmission electron microscopy, a small population of icosahedral particles, approximately 60 nm in diameter, was revealed (Fig. 7). We have so far been unable to identify specific proteins associated with this particle fraction.

DISCUSSION

We report here the partial characterization of a hypovirulent isolate of the chestnut blight fungus with multiple genetically unique segments of dsRNA. The dsRNAs and hypovirulence-associated properties of isolate C-18 can be compared to dsRNAs and properties of other, more thoroughly investigated, hypovirulent *C. parasitica* isolates.

Like many dsRNA-containing *C. parasitica* isolates from Europe and North America, C-18 is greatly reduced in virulence. Also like many previously characterized hypovirulent *C. parasitica* isolates, presence of dsRNA in isolate C-18 is associated with a distinctive morphology and downregulation of fungal attributes such as sporulation and laccase production. The dsRNA and its associated properties are acquired by uninfected strains following successful hyphal anastomosis with C-18. Therefore, based on its morphology

and many of its fungal features, C-18 is quite similar to other hypovirulent *C. parasitica* isolates.

From a virological perspective, C-18 dsRNAs are dissimilar from other hypovirulence-associated dsRNAs characterized to date. Two other types of hypovirulence-associated dsRNAs have been examined at the level of primary nucleotide sequence. The most thoroughly characterized belong to the newly described virus family Hypoviridae (Shapira *et al.* 1991b; Hillman *et al.* 1994a,b). Members of the Hypoviridae contain a single genomic dsRNA segment of 10–3 kb, which behaves like the genome of a positive-sense RNA virus (Fahima *et al.* 1993) and is ancestrally related to the Potyviridae family of RNA plant viruses (Koonin *et al.* 1991; Hillman *et al.* 1994a). Deletions of the genomic segment (Shapira *et al.* 1991a) or satellite RNAs (Tartaglia *et al.* 1986) may be associated with the large genomic segment but are not required for viral replication (Choi and Nuss 1992). Another recently characterized hypovirulence-associated dsRNA element is 2.7 kb in size and phylogenetically related to similar sized dsRNAs of *Saccharomyces cerevisiae* (J. J. Polashock and B. I. Hillman, unpublished). In contrast, C-18 dsRNAs are genetically unique, as determined by Northern blot hybridizations. Furthermore, there is no dsRNA segment larger than 5 kb in isolate C-18. Thus, it is highly unlikely that C-18 dsRNA is closely related ancestrally to members of the Hypoviridae.

Properties of C-18 dsRNAs are reminiscent of the Reoviridae family of dsRNA viruses (for review, see Joklik 1983). Whereas reoviruses have been characterized from invertebrate, vertebrate, and plant hosts, none has been characterized from a fungal host. The number, relative sizes, and approximately equimolar concentrations of C-18 dsRNA segments are all consistent with the Reoviridae family. Individual dsRNA segments of reoviruses are genetically unique, each encoding a single gene product or, in some cases, two gene products (Nuss and Dall 1990). Such a genome organization
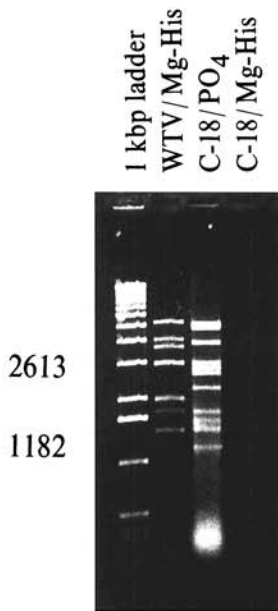

Fig. 6. Double-stranded RNA analysis of the phosphate and Mg-histidine buffer extractions of C-18 mycelium. Phosphate buffer yielded approximately 1 μg of dsRNA per gram of mycelium extracted.
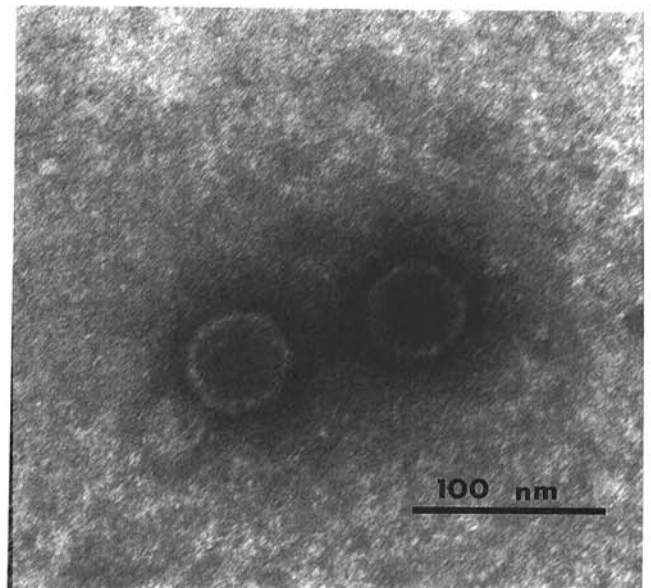

Fig. 7. Transmission electron micrograph of phosphate buffer–extracted mycelium of isolate C-18 stained with 2% phosphotungstic acid showing two icosahedral viral particles approximately 60 nm in diameter.

would be predicted for C-18 based on its dsRNA properties. Preliminary sequence analysis of C-18 segments 3 and 9 has revealed no clear similarity to other viruses (B. I. Hillman, unpublished). Properties of particles associated with isolate C-18 remain to be determined, so it is not known whether they are similar to members of the Reoviridae (Joklik 1983).

Isolate C-18 was recovered from a canker in West Virginia in 1989. Although the particular dsRNA constitution of C-18 has not been found in other hypovirulent *C. parasitica* isolates, other isolates with similar dsRNA compositions have been recovered from the central Appalachians (Paul and Fulbright 1988; W. L. MacDonald, unpublished). Although dsRNAs associated with some of these isolates have proven to be unstable in long-term culture, the dsRNA of one particular isolate, 9B-2-1, has been stably maintained since its isolation in 1982. Interestingly, the morphology of isolate 9B-2-1 is quite distinct from that of C-18. Furthermore, electrophoretic mobilities of dsRNAs of the two isolates differ noticeably.

Preliminary characterization of hypovirulent isolate C-18 has led to interesting questions, among them: 1) What is the relationship between dsRNAs of isolate C-18 and other hypovirulent *C. parasitica* dsRNAs? 2) What is the particle structure of the C-18 virus? 3) Is the C-18 virus ancestrally related to members of the Reoviridae family of viruses? 4) What is the ecological and epidemiological importance of viruses such as the C-18 agent? We are seeking answers to these and other questions through further molecular and biological characterization of isolate C-18 and its virus.

MATERIALS AND METHODS

Influence of dsRNA on cultural morphology and sporulation.

To examine the effect of the C-18 virus on *C. parasitica*, cultures derived from dsRNA-free and dsRNA-containing single conidial progeny were derived as described by Dodds and Elliston (1978). Dilute conidial suspensions were allowed to germinate on Difco potato-dextrose agar amended with methionine (100 mg/L) and biotin (5 mg/L) (PDAmb), and individual germlings were transferred to fresh plates as single conidial isolates. After incubation, 60 such isolates were examined for cultural morphology and dsRNA content. Double-stranded RNA was isolated from fungal tissue grown on cellophane-covered PDAmb plates essentially as described by Morris and Dodds (1979). Sporulation was measured as described previously (Hillman *et al.* 1990). Colonies were grown under cool white fluorescent lights (220 μmol · m^{-2} · s^{-1}), for 7 days. Conidia were washed from the plates with 0.015% Tween 80 and counted with a hemacytometer.

Transmission of dsRNA into vegetatively compatible and incompatible isolates.

Double-stranded, RNA-free single conidial isolates were paired with isolate C-18 to determine transmission properties of the dsRNA segments via hyphal anastomosis (Anagnostakis and Day 1979). In addition to that homologous pairing, isolate C-18 was paired with 37 vegetative compatibility tester isolates to determine the ability of isolate C-18 to transfer dsRNA to different isolates. Mycelium from the leading edge of the 37 pairings was subcultured to fresh

plates and examined for colony morphology and dsRNA content.

Influence of C-18 dsRNA on virulence.

To determine the effect of C-18 on the virulence of *C. parasitica*, fungal inoculations to Golden Delicious apples, excised dormant stems, and American chestnut sprouts were performed. The apples were inoculated with isolate C-18 and two dsRNA-free progeny (C18-23, C18-37) along with a hypovirulent and virulent isolate (GH-2 and Ep-155, respectively) as controls (Fulbright 1984). Isolates were replicated eight times (three per apple) in a completely random design. For inoculations, wounds were made on apples with a no. 11 leather punch (8 mm) and inoculated with agar plugs (8 mm) cut from the margins of actively growing cultures. Lesion size was measured 3 wk after inoculation.

Dormant stem tests of virulence were performed following the procedure of Elliston (1978). American chestnut stems, 5–10 cm in diameter, were inoculated with isolate C-18, five dsRNA-free single conidial isolates (C18-23, C18-35, C18-37, C18-39, C18-45), and one dsRNA-containing single conidial isolate (C18-27). Isolates were replicated eight times in a completely random design, with each stem receiving four wounds and inoculated as described above for the apple tests. Subsequent cankers were measured 4 wk after inoculation, and canker size was determined using the formula for the area of an ellipse (A = $\prod$ [½ × ^{w}/2]).

Field experiments to determine the effect of C-18 dsRNA on fungal virulence were performed with American chestnut near Parsons, WV, in a 6-yr-old clear-cut that contained numerous chestnut sprouts ranging from 1 to 20 cm in diameter. The isolates chosen for the study were those used in the excised dormant chestnut stem experiment above, inoculated with 10 replications per isolate, with the subsequent cankers measured 12-mo after inoculation.

The effect of dsRNA on the virulence of *C. parasitica* was determined by comparing the mean lesion size for each dsRNA-free progeny to the dsRNA-containing progeny and to isolate C-18. Significant differences among isolates in all virulence studies were determined using ANOVA (STATISTIX, NH Analytical Software, St. Paul, MN) and Fisher's LSD (Dowdy and Wearden 1983).

Cloning and analysis of dsRNA.

To analyze the relationship of C-18 dsRNA to other dsRNAs associated with *C. parasitica*, a cDNA library was constructed using dsRNA from C-18 as a template. The synthesis and analysis of the cDNA library were conducted as described by Hillman *et al.* (1992). Spot and Northern blot hybridization analysis of isolated dsRNA was performed as previously described (Rae *et al.* 1989; Hillman *et al.* 1992).

Viral particle extraction.

Extraction of a particle-containing fraction from the mycelium of C-18 was performed with both phosphate and histidine buffer solutions (Kimura and Black 1971). Two 10-g aliquots of C-18 mycelium were frozen in liquid nitrogen and ground to a fine powder. Forty milliliters of either 0.1 M K-NaPO$_4$, pH 7.2, or 0.1 M histidine, 0.01 M MgCl$_2$, pH 7.0, was added to the ground mycelium of each. Each sample was mixed for 10 min and centrifuged at 8,000 × *g* for 20 min,

and the supernatant was decanted through a cheesecloth filter. Supernatants were adjusted to 6% polyethylene glycol, placed on ice for 30 min, and then centrifuged at 8,000 × g for 20 min. The supernatants were decanted, and each pellet was resuspended with 20 ml of the appropriate buffer solution. Samples were centrifuged at 150,000 × g for 1.5 hr, after which pellets were resuspended in 500 µl of their respective buffers.

After particle extraction, subsamples were examined for the presence of dsRNA using a sodium dodecyl sulfate/phenol extraction protocol as described by Morris and Dodds (1979). Nucleic acids collected from this procedure were resuspended in 50 µl of water, and 15 µl of each sample was run on a 1% agarose gel. The remaining virus extract samples were prepared for transmission electron microscopy by placing a drop of each buffer sample onto copper grids and stained with 2% phosphotungstic acid, pH 7.0. Grids were allowed to dry and then examined in the electron microscope for viral particles.

ACKNOWLEDGMENTS

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