Phenotypic Changes Associated with Wild-Type and Mutant Hypovirus RNA Transfection of Plant Pathogenic Fungi Phylogenetically Related to *Cryphonectria parasitica*

Baoshan Chen, Chein-Hwa Chen, Barbara H. Bowman, and Donald L. Nuss

First, second, and fourth authors: Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110; and third author: Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501.
Current address of B. Chen and D. L. Nuss: Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, 2115 Agricultural/Life Sciences Surge Building, College Park 20742-3351.
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ABSTRACT

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Double-stranded RNA viruses within the genus *Hypovirus* attenuate virulence of the chestnut blight fungus *Cryphonectria parasitica*. The recent development of an infectious synthetic hypovirus transcript has allowed the expansion of hypovirus infection and virus-mediated virulence attenuation to several fungal species not previously shown to harbor hypoviruses. This report compared the phenotypic changes resulting from transfection-mediated hypovirus infection of *Cryphonectria parasitica*, *C. radicalis*, *C. havanensis*, *C. cubensis*, and *Endothia gyrosa*. By comparing virus-mediated phenotypic changes in different fungal hosts transfected with wild-type and mutated viral transcripts, it was possible to examine the relative contribution of viral and host genetic backgrounds for some traits. For example, hypovirus infection reduced sporulation in all fungal hosts, while it suppressed pigmentation in some species and induced pigment production in others. The hypovirus-encoded protein p29 was found to have a significant impact on both sporulation and pigmentation in different fungal hosts. All fungal species tested were able to transmit virus by anastomosis within the same species. However, considerable differences were observed in the efficiency with which different fungal species transmitted hypoviruses through conidia. A molecular phylogenetic analysis based on nuclear ribosomal DNA nucleotide sequences revealed the five fungal species to be closely related. Possible relationships between taxonomic position and hypovirus host range were discussed. The ability to introduce hypoviruses into different fungal species holds promise for the expanded utility of virus-mediated hypovirulence for understanding and controlling fungal pathogenicity.

Increased emphasis on integrated pest management of crop and forest diseases has stimulated efforts to develop more effective biological control strategies. The use of mycoviruses to attenuate fungal virulence, while of considerable theoretical interest, has seen only minimal application (2,3,6,34,36). The slow pace of development of mycovirus-based fungal control strategies can be attributed to a combination of factors including the limited number of natural examples of consistent virus-mediated virulence attenuation (37), the paucity of physical and genetic data for viruses of pathogenic fungi, general restrictions on mycovirus transmission because of the absence of an extracellular route of infection (11,47), and specific barriers to cytoplasmic transmission imposed by fungal vegetative compatibility systems (1,2). However, recent progress in the molecular analysis and manipulation of viruses of the genus *Hypovirus*, responsible for transmissible hypovirulence of the chestnut blight fungus, *Cryphonectria parasitica*, has stimulated a reevaluation of the potential of mycoviruses as biological control agents (13,14,15,17,18,19,22, 26,36). Of particular significance has been the development of an infectious hypovirus cDNA clone (17) and the more recent development of infectious hypovirus synthetic transcripts (14).

Hypovirus double-stranded RNA (dsRNA) can be transmitted from infected strains of *C. parasitica* to vegetatively compatible strains during anastomosis. The transmission results in the conversion of the recipient strain to hypovirulence, thus, providing the basis for biological control by natural hypovirus-containing *C. parasitica* strains (2). *C. parasitica* strains transformed with a recently developed infectious hypovirus cDNA contain both a chromosomally integrated copy of the viral cDNA and a cDNA-derived cytoplasmically replicating RNA form (17). As a result, these engineered hypovirulent strains have the capacity to transmit hypoviruses via nuclear inheritance to ascospore progeny, a novel mode of transmission not observed for hypovirus RNA in natural hypovirulent *C. parasitica* strains (13). This property should result in enhanced virus dissemination and persistence in the field. By modifying the hypovirus genome in the context of the infectious viral cDNA clone, it has also been possible to construct hypovirulent *C. parasitica* strains that exhibit specific phenotypic traits. For example, deletion of the coding region for the hypovirus p29 protease resulted in increased levels of asexual sporulation, while retaining the ability to confer hypovirulence (19). The more recent development of an infectious hypovirus synthetic transcript, the first for any fungal virus, has facilitated the expansion of hypovirus host range and virus-mediated virulence attenuation to fungal species other than the natural host, *C. parasitica* (14). This development provides new opportunities for the broader application of hypovirus-mediated hypovirulence for purposes of understanding and controlling fungal pathogenesis. It also provides a firm foundation for continuing efforts to develop synthetic infectious transcripts for other interesting and potentially efficacious mycoviruses. This report presents a detailed description of the hypovirus transfection system and surveys the phenotypic changes resulting from the hy-

Corresponding author: D. L. Nuss; E-mail address: nuss@mbimail.umd.edu

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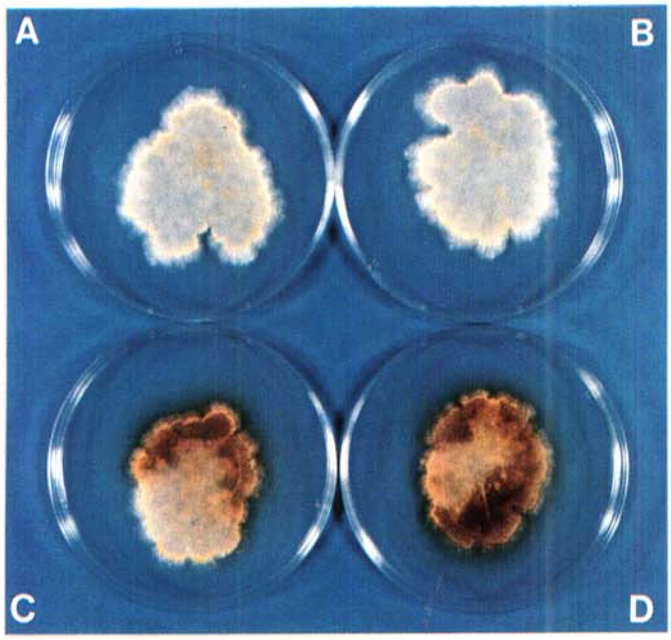

povirus infection of five different fungal species. A molecular phylogenetic analysis based on nuclear ribosomal DNA gene nucleotide sequences was also initiated to provide a more precise basis for judging the evolutionary relatedness of these five fungal species as it may relate to hypovirus host range.

MATERIALS AND METHODS

Fungal strains and growth conditions. *C. parasitica* (Murrill) Barr, strain EP155, a virulent, hypovirus-free strain (27), was provided by S. Anagnostakis (Connecticut Agricultural Experi-

Fig. 1. Hypovirus movement through regenerating transfected mycelia illustrated with the aid of regenerating transfected *Endothia gyrosa*. Regenerating colonies marked A and B were transfected with water, while colonies marked C and D were transfected with the CHV1-713 synthetic coding strand transcripts. Hypovirus infection of *E. gyrosa* resulted in the production of a diffusible dark brown pigment that provides a convenient marker for visualization of virus spread. The photograph records virus-mediated pigment production, indicative of virus spread through the regenerating colony, 5 days (colonies A and C) and 8 days (colonies B and D) posttransfection.

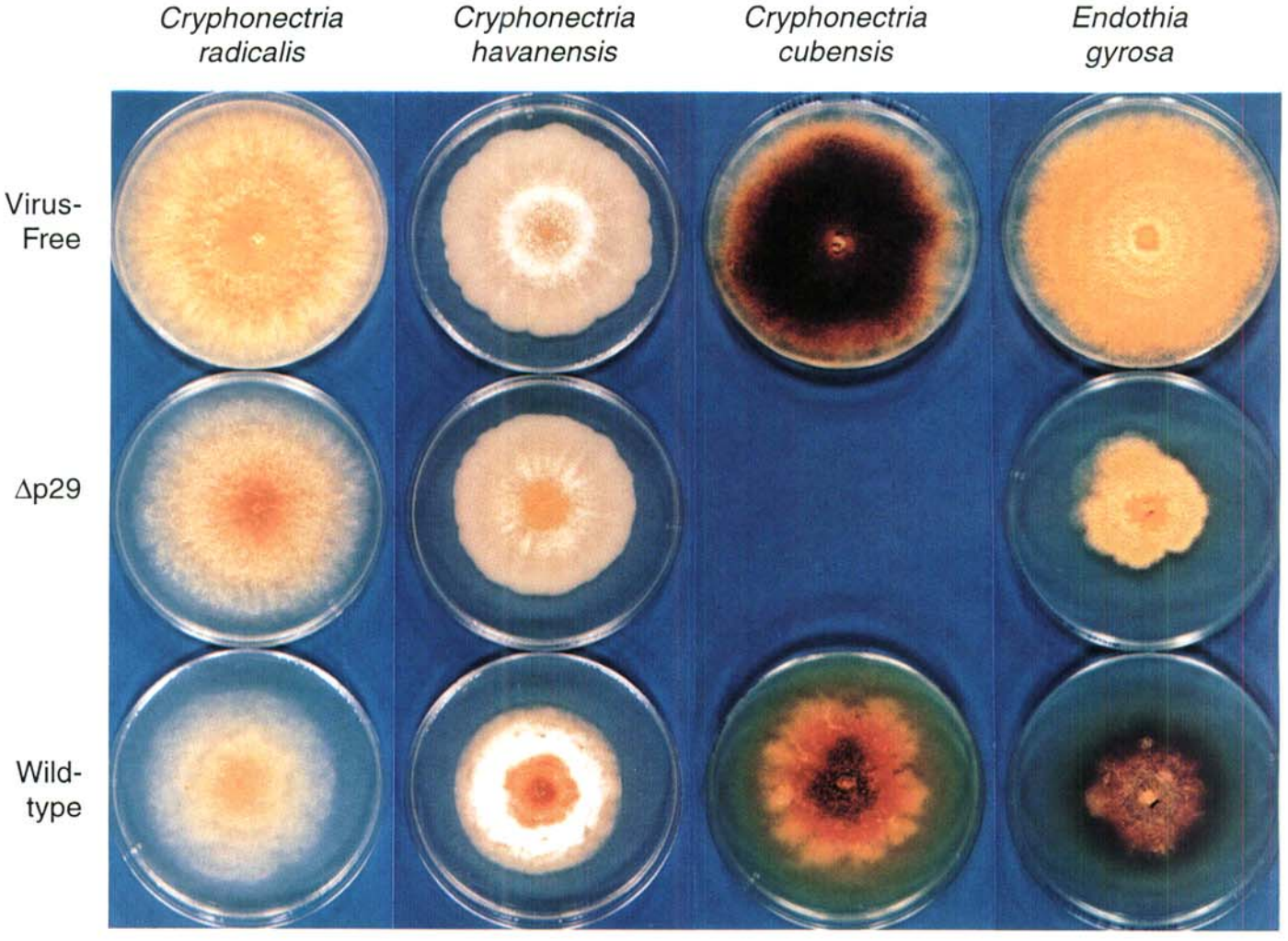

Fig. 2. Phenotypic changes associated with hypovirus infection of different fungal species mediated by transfection with synthetic CHV1-713 infectious transcripts. The fungal species designations are indicated at the top of the figure. As indicated at the left, virus-free colonies are displayed in the top row, while colonies transfected with the Δp29 mutant CHV1-713 transcript or with the wild-type hypovirus transcript are displayed in the middle and bottom rows, respectively. We were unable to successfully transfect *Cryphonectria cubensis* with the Δp29 mutant transcript for unknown reasons. Detailed descriptions of the species-specific virus-mediated phenotypic changes are presented in the text.

ment Station) who obtained the original isolate in 1977 from a canker on *Castanea dentata* (Marshall) Borkh. in a field plot in Connecticut. *C. radicalis* (Schwein.:Fr.) Barr, strain 3K/87, was also provided by S. Anagnostakis. This strain was originally isolated by S. Xenopoulos from *Castanea sativa* Miller in Keravaso, Greece, in 1987, and the species designation was independently confirmed by S. Anagnostakis. As predicted for the species *C. radicalis*, this isolate failed to form cankers of any significant size on chestnut stems (data not shown). *C. cubensis* (Bruner) C. S. Hodges was obtained from the American Type Culture Collection (ATCC) as accession 64159 deposited by E. L. Barnard and isolated from a basal canker on *Eucalyptus grandis* A. W. Hill ex Maiden in south Florida (7). *C. havanensis* (Bruner) Barr (formally *Endothia havanensis* Bruner) was obtained from ATCC as accession 56124, deposited by E. M. Davison and was isolated from a canker on *Eucalyptus marginata* Sm. in western Australia (20). *Endothia gyrosa* (Schwein.:Fr.) Fr. was also obtained from ATCC as accession 48192. It was deposited by R. J. Stipes and was isolated from pin oak (*Quercus palustris* Muenchh.) in Virginia (45). Stock cultures were maintained on potato-dextrose agar (PDA; Difco Laboratories, Detroit) on the laboratory bench: light = 7.6 to 13 μmol m^{-2} s^{-1}, temperature = 22 to 24°C. Experimental cultures were initiated by placing 3- × 3- × 3-mm agar cubes, excised from the margins of 7- to 10-day-old stock cultures, at the center of 85-mm-diameter petri dishes containing 28 ml of PDA. All fungal strains were inspected for the presence of hypovirus dsRNA by standard means (27) and found to be negative.

Phenotypic measurements. Radial growth and sporulation levels were measured as described by Hillman et al. (27). Efficiency of virus transmission through asexual spores was determined by scoring individual conidial isolates for distinctive colony morphology associated with virus infection. The physical presence of hypovirus dsRNA was confirmed for a portion of each set of conidial isolates as described (27). Relative virulence was assessed with the Granny Smith apple assay as previously described by Fulbright (24) with the modifications of Chung et al. (18).

Transfection. Full-length coding strand transcripts of wild-type hypovirus CHV1-713 L-dsRNA (42) were produced from plasmid pLDST using T7 RNA polymerase-dependent cell-free synthesis reagents from Promega Corporation (Madison, WI) as described by Chen et al. (14). Mutant CHV1-713 transcripts that

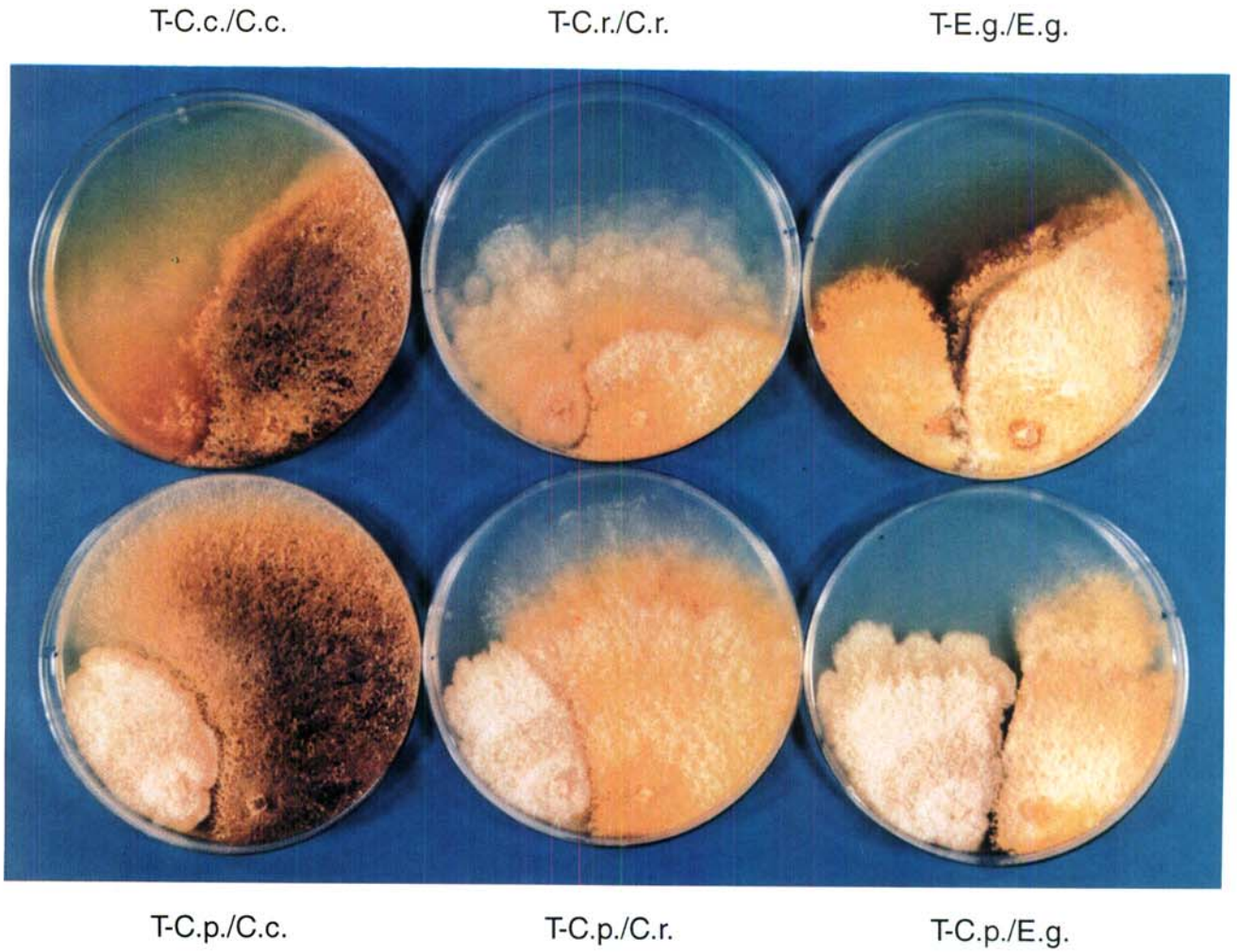

Fig. 3. Cytoplasmic transmission of hypovirus RNA via anastomosis by different transfected fungal species. Anastomosis-mediated conversion of a hypovirus-free fungal strain by a compatible virus-containing strain can be monitored visually in vitro after paired inoculation on agar plates. For each plate in this figure, the virus-containing donor strain is indicated to the left of the slash line with a T prefix indicating that hypovirus infection was initiated by transfection and the two letter abbreviation of the species designation (*Cryphonectria cubensis*, C.c.; *C. radicalis*, C.r.; *C. parasitica*, C.p.; and *Endothia gyrosa*, E.g.). The hypovirus-free recipient strains are indicated at the right of the slash lines. Conversion as a result of anastomosis appears as a wedge of mycelia with characteristic virus-associated phenotypic alterations that initiates at the interface between the two colonies and extends along the periphery of the recipient colony. Note that each of the transfected species was readily able to convert isolates of the same species, but that virus was not observed to be transmitted from infected *C. parasitica* to any of the other species even after prolonged incubation in multiple trials. Plates were photographed 12 days after paired inoculation. Anastomosis-mediated virus transmission by transfected *C. havanensis* to a hypovirus-free strain was also observed (data not shown).

contained a 657-nucleotide (nt) deletion of the p29 coding region (Δp29) were produced similarly from plasmid pXHΔ1, previously described by Craven et al. (19). Following agarose gel analysis of transcript integrity, 10 to 15 μg of transcript RNA was amended with 200 U of RNasin (Promega Corp.), gently mixed with 100 μl of sorbitol-washed fungal spheroplasts (approximately 3×10^6) and exposed to electroporation at settings of 1.5 kV, 200 ohm, and 25 μF with constant time in a Gene Pulser (Bio-Rad Laboratories, Richmond, CA). Spheroplasts surviving the electroporation procedure were regenerated as described by Chen et al. (14) and characterized for virus-mediated phenotypic changes and/or presence of viral dsRNA (27).

Ribosomal DNA sequenee determination and phylogenetic analysis. DNA was isolated from fungal mycelia as described by Lee and Taylor (32). Polymerase chain reaction (PCR) amplification of the nuclear 18S ribosomal RNA gene was performed using primers and conditions described by White et al. (46). The first internal transcribed spacer region (ITS-1) was amplified independently using primers ITS1 and ITS4 (46). In most cases, PCR products were subjected to preparative agarose gel electrophoresis and the recovered purified double-stranded fragments were sequenced directly using the Applied Biosystems, Inc. (Foster City, CA), *Taq* DyeDeoxy terminator cycle sequencing kit and an Applied Biosystems, Inc., Model 373A automated sequencer. Sequences reported were based on both strands from multiple independent amplifications.

The 18S ribosomal DNA sequences were aligned by hand using the Eyeball Sequence Editor (ESE version 2.00) (12). The rDNA alignment included GenBank database sequences from *Leucostoma persoonii* (LEARR18S), *Ophiostoma ulmi* (OPHRR18S), *Pseudallescheria boydii* (PUCRRNA), and *Neurospora crassa* (NEURRNAS), plus the plectomycetes *Histoplasma capsulatum* (=*Ajellomyces capsulatus*; HC18SR) and *Coccidioides immitis* (CI18SR), that served as outgroups. In all species, 1,679 positions were unambiguously aligned and used in analysis. Bootstrapped genetic distance trees were constructed using programs from the PHYLIP suite version 3.41 (23). SEQBOOT was used to create 500 replicate data sets; DNADIST created a pairwise distance matrix for each data set with application of a Kimura multiple-hit correction; NEIGHBOR was used to draw a phylogenetic tree from each distance matrix; and CONSENSE created the bootstrap consensus tree.

RESULTS

Transfection system. The conditions selected for *C. parasitica* transfection (1.5 kV, 200 ohm, and 25 μF) resulted in approximately 2% survivability with a fairly high transfection rate on the order of 30%. The same conditions were satisfactory for hypovirus transfection of four other fungal species as initially reported by Chen et al. (14) and expanded upon in this report. These species included two pathogens of *Eucalyptus* spp., *C. cubensis* and *C. havanensis*; a nonpathogenic species of the same taxonomic family, *C. radicalis*; and a pathogen of *Quercus* spp. from a separate taxonomic genus, *Endothia gyrosa*.

A key element in the successful development of a convenient hypovirus transfection system is the propensity of filamentous fungi to undergo hyphal anastomosis. Following electroporation, a portion of the transfected spheroplasts were placed in the middle of a petri dish and gently surrounded by molten regeneration medium. Since the hyphal structures that were regenerated from the transfected spheroplasts fuse at a very high frequency, the resulting dense colony was, in essence, a large cytoplasmic network. Consequently, the replicating hypovirus RNA that were introduced into an individual spheroplast by electroporation was able to effectively migrate unobstructed throughout the colony. This process could be conveniently followed in transfected *E. gyrosa*, in which virus infection resulted in the pronounced induction of brown pigment production (Fig. 1). Even when virus-mediated phenotypic changes were minor, as occurred for hypovirus-infected *C. parasitica* on regeneration medium, virus-containing hyphae were readily recoverable from the margin of the regenerated colony without reliance on a selectable marker and at low transfection efficiencies.

Species-specific phenotypic changes resulting from hypovirus infection. The phenotypic changes resulting from hypovirus infection of the natural host, *C. parasitica*, are well documented (2,27). As indicated in Figure 2 and Tables 1 and 2, transfection-initiated infection of fungal species other than *C. parasitica* with wild-type and mutant CHV1-713 transcripts also resulted in a variety of pronounced phenotypic changes. When grown on PDA, wild-type hypovirus-infected *C. radicalis* exhibited many of the same morphological changes that were observed for hypovirus-infected *C. parasitica* (17,27). These included a slight reduction in mycelial growth (Table 1; Fig. 2), a significant reduction in the

TABLE 1. Effect of hypovirus transfection on radial growth of five fungal species

	Colony area (cm^2)[a]			
	Bench-top		76.2 μmol m^{-2} s^{-1}	
Isolates	8 days	11 days	8 days	11 days
Cryphonectria parasitica	51.5 ± 2.2	56.7 ± 0.0*[b]	37.1 ± 5.2	56.7 ± 0.0*
C. parasitica /Δp29[c]	31.2 ± 1.0	45.6 ± 2.4	22.1 ± 2.2	37.5 ± 4.6
C. parasitica /wt.v[d]	30.2 ± 1.0	49.9 ± 3.1	23.2 ± 1.3	40.8 ± 4.9
C. radicalis	55.0 ± 0.8	56.7 ± 0.0*	43.1 ± 4.2	53.3 ± 5.9
C. radicalis /Δp29	41.8 ± 1.2	52.4 ± 0.7	25.9 ± 2.7	35.3 ± 1.8
C. radicalis /wt.v	42.2 ± 2.8	56.7 ± 0.0*	29.6 ± 2.3	34.4 ± 5.7
C. cubensis	38.5 ± 7.8	56.7 ± 0.0*	31.5 ± 2.8	46.2 ± 2.8
C. cubensis[e]/wt.v	24.1 ± 1.3	42.2 ± 1.3	15.8 ± 3.3	31.9 ± 8.9
C. havanensis	29.9 ± 0.6	51.9 ± 1.5	22.3 ± 0.5	46.2 ± 1.8
C. havanensis /Δp29	22.3 ± 0.5	42.2 ± 0.7	16.1 ± 1.1	30.2 ± 2.6
C. havanensis /wt.v	19.4 ± 0.4	36.7 ± 0.6	14.8 ± 1.0	29.4 ± 6.0
Endothia gyrosa	33.2 ± 1.8	51.1 ± 3.9	14.8 ± 2.1	31.6 ± 4.5
E. gyrosa /Δp29	18.4 ± 1.2	30.2 ± 2.0	10.6 ± 1.7	20.7 ± 1.9
E. gyrosa /wt.v	8.2 ± 0.6	14.5 ± 1.4	3.8 ± 0.6	14.0 ± 3.0

[a] Colony area was calculated according to the formula $A = \pi(d/2)^2$. Values are reported as mean ± SE from three independent experiments.
[b] * = colonies grew to edge of plate (85 mm).
[c] Δp29 designates transfection with transcripts of the Δp29 mutant of hypovirus CHV1-713.
[d] wt.v designates transfection with transcripts of wild-type hypovirus CHV1-713.
[e] Transfection of *C. cubensis* with the Δp29 transcript failed to yield any productive infection.

production of aerial mycelia (Fig. 2), suppressed orange pigmentation (Fig. 2), and reduced sporulation (Table 2). CHV1-713-encoded protein p29 has been shown to be responsible for hypovirus-induced suppression of pigment production and to contribute to the suppression of conidiation in *C. parasitica* (19). As was also observed for *C. parasitica* (19), infection of *C. radicalis* with a CHV1-713 mutant lacking Δp29 resulted in partial amelioration of these phenotypic traits (Fig. 2; Table 2).

Wild-type hypovirus infection of *C. havanensis* also resulted in a moderate reduction in growth rate on PDA (Fig. 2; Table 1). Surprisingly, hypovirus infection was accompanied by notable increases, rather than decreases, in the production of aerial mycelia and in the production of an orange-red pigment, particularly evident in older mycelia (Fig. 2). Moreover, exposure to high light intensity significantly enhanced the virus-mediated stimulation of pigment production (data not shown), in direct contrast to the ability of high light to partially relieve virus-mediated phenotypic effects in hypovirus-infected *C. parasitica* (27). Additionally, Δp29-infected *C. havanensis* consistently exhibited less aerial mycelia and reduced orange-red pigmentation compared with wild-type-infected transfectants. Thus, p29 appeared to have the opposite effects of down-regulating or up-regulating pigment production in different fungal hosts. Sporulation was also severely suppressed in hypovirus-infected *C. havanensis* (Table 2). However, unlike the results for *C. parasitica* and *C. radicalis*, Δp29-infected *C. havanensis* did not exhibit an increased level of sporulation relative to the wild-type virus-infected transfectants (Table 2).

The effect of hypovirus infection on fungal growth and pigment production was found to be most pronounced for *C. cubensis* and *E. gyrosa*. Wild-type hypovirus-infected *C. cubensis* was distinguished by the production of a bright yellow-orange pigment not observed for the uninfected strain when grown on PDA. Additionally, the infected hyphae were observed to penetrate deep into the agar medium (Fig. 2). Wild-type virus infection of *E. gyrosa* resulted in the production of a diffusible dark brown pigment, severe reduction in growth rate on PDA, and a pronounced change in colony morphology (Fig. 2). *E. gyrosa* infected with Δp29 exhibited increased spore production compared with wild-type-infected isolates and normal pigmentation, but was still considerably reduced in growth rate on PDA (Fig. 2). Unexpectedly, transfection of *C. cubensis* with the Δp29 transcript failed upon repeated (four) attempts to result in a productive infection.

Down-regulation of laccase production is a common phenotypic change associated with hypovirus infection of the natural host, *C. parasitica* (39). To determine whether hypovirus infection also affects laccase production in the new fungal hosts, the relative ability of infected and uninfected strains to produce the characteristic color reaction on tannic acid-containing plates was examined. A reduction in apparent phenyl oxidase production, equivalent to that previously described for *C. parasitica* (19), was observed for each of the infected fungal species except *E. gyrosa*, for which even uninfected isolates failed to produce a color reaction on Rigling's medium (39) (data not shown).

Cytoplasmic and conidial hypovirus transmission by different fungal species. Given the importance of hypovirus cytoplasmic transmission for effective biological control of chestnut blight, it was of considerable interest to determine whether hypovirus RNA was efficiently transmitted via anastomosis when placed in a new host genetic background. For *C. parasitica*, anastomosis-mediated conversion of a virus-free virulent strain could be monitored visually after paired inoculation on agar plates (17). As indicated in Figure 3, each of the hypovirus-transfected fungal species was able to convert isolates of the same species after paired inoculation. In contrast, no cytoplasmic transmission of virus was observed when hypovirus-infected *C. parasitica* was paired with any virus-free isolates of the other fungal species. Results obtained from visual inspection were confirmed by extraction of viral dsRNA from the converted mycelium (data not shown).

Hypoviruses are also maintained within a strain after asexual reproduction by transmission through conidia (13,41). The introduction of the same hypovirus RNA into different fungal species provided the opportunity to examine the contribution of viral and host genetic background to conidial transmission of viral RNA. Sporulation levels and transmission efficiencies were measured for colonies grown in parallel, either on the bench-top (7.6 to 13 μmol m^{-2} s^{-1}) or under high light (76.2 μmol m^{-2} s^{-1}), a condition that partially overcomes hypovirus-mediated suppression of conidiation in *C. parasitica* (19,27). Although some variations were obtained between trials, as indicated by the representative data values presented in Table 2, several clear differences in conidial transmission of viral RNA emerged among the different transfected fungal species. As observed routinely in this laboratory, conidial transmission of CHV1-713 viral RNA by infected *C. parasitica* strain EP155 ranged between 50 and 100% when grown either on bench-top or under high light intensity. Surprisingly, conidial transmission of viral RNA was low (less that 10%) for transfected *C. radicalis* when grown on bench-top, but was found to be generally high (greater than 90%) when cultures were grown under conditions of high light. Conidial transmission of viral RNA by transfected *C. havanensis* ranged between 40 and 80% in most trials, but occasionally levels of 1 to 2% were observed. The reason for this degree of variation is presently unclear. Conidial transmission by transfected *E. gyrosa* was consistently found to be below the 10% level, while conidia recovered from transfected *C. cubensis* consistently failed to give rise to infected hyphae. As noted previously in the case of infected *C. parasitica* (19), and observed for the other fungal species except *C. havanensis*, colonies infected with the Δp29 mutant viral RNA produced a higher level of conidia. However, the frequency of virus transmission through conidia for the wild-type virus and the Δp29 mutant were not appreciably different.

Effect of hypovirus transfection on fungal virulence. We previously reported that transfection of *E. gyrosa* with the CHV1-713 synthetic transcript results in virulence attenuation of this

TABLE 2. Asexual sporulation and hypovirus transmission by five fungal species

Isolate[a]	Bench-top		76.2 μmol m^{-2} s^{-1}	
	Sporulation (spores/ml)	Transmission (%)[b]	Sporulation (spores/ml)	Transmission (%)
Cryphonectria parasitica	4.1×10^8	0	8.37×10^8	0
C. parasitica /Δp29	5×10^4	50	5.2×10^8	55
C. parasitica /wt.v	0	NA	6×10^4	100
C. radicalis	1.25×10^8	0	2.6×10^8	0
C. radicalis /Δp29	2×10^5	7	2.7×10^7	98
C. radicalis /wt.v	3×10^4	5	8.5×10^5	100
C. cubensis	4.5×10^8	0	5.7×10^8	0
C. cubensis /wt.v	2.7×10^7	0	4×10^7	0
C. havanensis	6.8×10^7	0	2.6×10^8	0
C. havanensis /Δp29	0	NA	4.3×10^5	41
C. havanensis /wt.v	1×10^4	86	1.1×10^6	66
Endothia gyrosa	5.6×10^6	0	1.6×10^8	0
E. gyrosa /Δp29	6.4×10^6	9	3×10^7	7
E. gyrosa /wt.v	0	NA	1.8×10^5	1

[a] Each isolate was tested at least three times for both sporulation and viral transmission rates. However, because of the labor involved in screening large numbers of conidial isolates, not all isolates were included in each trial. Values presented in this table are from a representative trial that did include each isolate grown under parallel conditions. As indicated in the text, only values reported in this table for *C. havanensis* differed appreciably from values obtained in other trials. Virus was found not to be transmitted to *C. cubensis* conidial isolates in five separate trials.

[b] Percent transmission values were derived by scoring 100 independent conidial isolates.

fungal pathogen on both pin oak and red oak (14). Thus, extension of hypovirus host range to a pathogenic fungus other than the natural host, *C. parasitica*, did result in virulence attenuation. An examination of the virulence levels for transfected *C. havanensis* and *C. cubensis* had been complicated by importation and quarantine restrictions of stem material from susceptible eucalyptus species. Apples have been shown to serve as a suitable substitute for dormant chestnut stems in determining the relative virulence of *C. parasitica* strains (18). Like *C. parasitica*, *C. havanensis* also formed sizable lesions when inoculated onto Granny Smith apples. As indicated in Table 3, transfection of *C. havanensis* with wild-type CHV1-713 resulted in an approximate 25-fold reduction in lesion size. This severe reduction in lesion size formation on apples contrasted sharply with the relative small (25%) reduction in growth rate observed for *C. havanensis* transfectants on the synthetic PDA medium (Table 1) and was consistent with virus-mediated virulence attenuation. Interestingly, neither *C. cubensis* nor *C. radicalis* produced sizable lesions in the apple assay.

Phylogenetic analysis. To obtain an indication of the phylogenetic relationship of the five fungal species successfully transfected with hypovirus transcripts, sequences for the 18S rDNA and ITS-1 were determined. For each species, 1,752 bases of the 18S rDNA sequence were determined, corresponding to all but 38 bases at the 5′-terminus of the gene (Fig. 4). Alignment of the *C. parasitica* sequence with the *N. crassa* 18S rDNA reference sequence revealed 91 base substitutions, one base deletion, one base addition, and the unexpected presence of a 547-bp group I intron inserted at map position 1,426. Surprisingly, comparisons of the sequences for *C. parasitica* and *C. radicalis* revealed the absence of variation, including the presence of the identical intron at the identical map position. The *C. cubensis* 18S rDNA sequence was also found to be identical to that of *C. parasitica*, with the exception that it lacked the group I intron. Also lacking the group I intron, *C. havanensis* 18S rDNA differed from *C. parasitica* at a single position and the *E. gyrosa* sequence differed at three positions, one of which corresponded to the single substitution observed for *C. havanensis*.

The 18S rDNA sequences were used to examine the evolutionary position of these species within the kingdom Fungi. Based on morphological considerations, *Cryphonectria* spp. have been placed in the class Pyrenomycetes, specifically the order Diaporthales (8), and, consequently, would be expected to be close relatives of the genus *Leucostoma*. To determine the position of these species within the pyrenomycetes, we included in our analysis 18S rDNA sequences from *Leucostoma persoonii*, as well as the additional pyrenomycete species *Neurospora crassa*, *Ophiostoma ulmi*, and *Pseudallescheria boydii*. We used the plectomycete fungal pathogens *Histoplasma capsulatum* (=*Ajellomyces capsulatus*) and *Coccidioides immitis* as outgroup species in order to root the tree. The branching order of species within the pyrenomycete class was the same as that found by Spatofora and Blackwell (44), except that the branching order of *O. ulmi* and *P. boydii* was not strongly determined in our analysis and is, therefore, shown as a trichotomy (Fig. 5). The *Cryphonectria* spp., along with *E. gyrosa*, were very strongly grouped within the tree. This group also clustered strongly with *L. persoonii*, which associates them with the order Diaporthales as expected. These data indicated a very recent divergence of the five fungal species successfully transfected with hypovirus transcripts.

Alignment of the ITS-1 sequences for the five species indicated only 10 differences between *C. parasitica* and *C. radicalis* (Fig. 6). This low level of divergence provided additional evidence that these two species most recently shared a common ancestor. The level of divergence within the ITS-1 region for the other isolates clearly defined them as separate species, but provided too few unambiguously alignable sequence positions for meaningful phylogenic analysis within the group.

TABLE 3. Effect of hypovirus transfection on lesion formation in apple assay (predictor of virulence) by *Cryphonectria havanensis*

	Lesion area (cm^2)[a]		
Apple number	*C. havanensis*	*C. havanensis*/Δp29	*C. havanensis*/wt.v
1	20.2	0.1	0.1
2	10.6	5.5	0.2
3	16.4	2.1	0.1
4	13.7	0.1	0.2
5	17.1	3.3	2.9
6	13.7	4.7	0.8
7	14.3	0.2	0.1
8	12.4	0.2	0.1
Mean ± SE	14.8 ± 3.0	2.0 ± 2.2	0.6 ± 1.0

[a] Data are presented as lesion area in square centimeters as measured on day 12 following inoculation. Values for mean lesion area ± SE are indicated at the bottom of each column.

DISCUSSION

Viruses of fungi are distinguished by genetic information composed of either single- or double-stranded RNA, with the latter predominating, and the universal absence of an extracellular route of infection (48). Transmission occurs vertically through asexual spores or via cytoplasmic mixing as transpires during mating or hyphal anastomosis. The fact that mycovirus dsRNAs have been associated with reduced virulence of certain pathogenic fungal hosts (2,6,9,10,25,29,37) has generated interest in the potential application of these viral agents for purposes of biological control. However, previous limitations in the range of genetic manipulations available for this group of viruses, coupled with the absence of an infectivity system, have hindered molecular characterizations required for the development of such applications. In this regard, the results presented in this and earlier reports (14,17) demonstrate the feasibility of generating infectious recombinant forms of mycovirus genomes and their use to further develop biological control potential.

Although hypovirus infections can be artificially initiated by either transformation or transfection, it is important to clearly distinguish the two processes and the properties of the resulting infected fungal strains. Plasmids used for the transformation method contain a functional cDNA copy of hypovirus RNA that gives rise to a cytoplasmically replicating viral RNA following its integration into the fungal chromosome (17). Since these plasmids also contain a gene that confers resistance to the antibiotic hygromycin, it is possible to select hypovirus-infected hyphae, derived from individual transformed spheroplasts, that contain a stably integrated viral cDNA copy in a homogenous nuclear genetic background. The presence of a chromosomally integrated, functionally infectious hypovirus viral cDNA allows transmission of virus to ascospore progeny of a sexual cross, a mode of transmission not observed for cytoplasmically replicating hypovirus RNA in natural hypovirulent strains. This trait should enhance biocontrol potential (13,17,36). In contrast, the RNA-based transfection system relies on the delivery of an intact synthetic hypovirus RNA coding strand transcript into the cytoplasm of fungal spheroplasts and the efficient migration of replicating viral RNA from individual transfected spheroplasts throughout regenerated mycelia without requirement for a selectable marker (14). Since the hypovirus-infected transfectants do not contain a chromosomally integrated viral cDNA, they lack the ability to transmit virus to ascospore progeny of a sexual cross.

Since two different forms of input hypovirus genetic information are used in the transformation and transfection systems, different intracellular processing pathways are utilized for the generation of replicating viral dsRNA. In both cases, 3′- and 5′-flanking nonviral vector sequences are precisely trimmed (15;

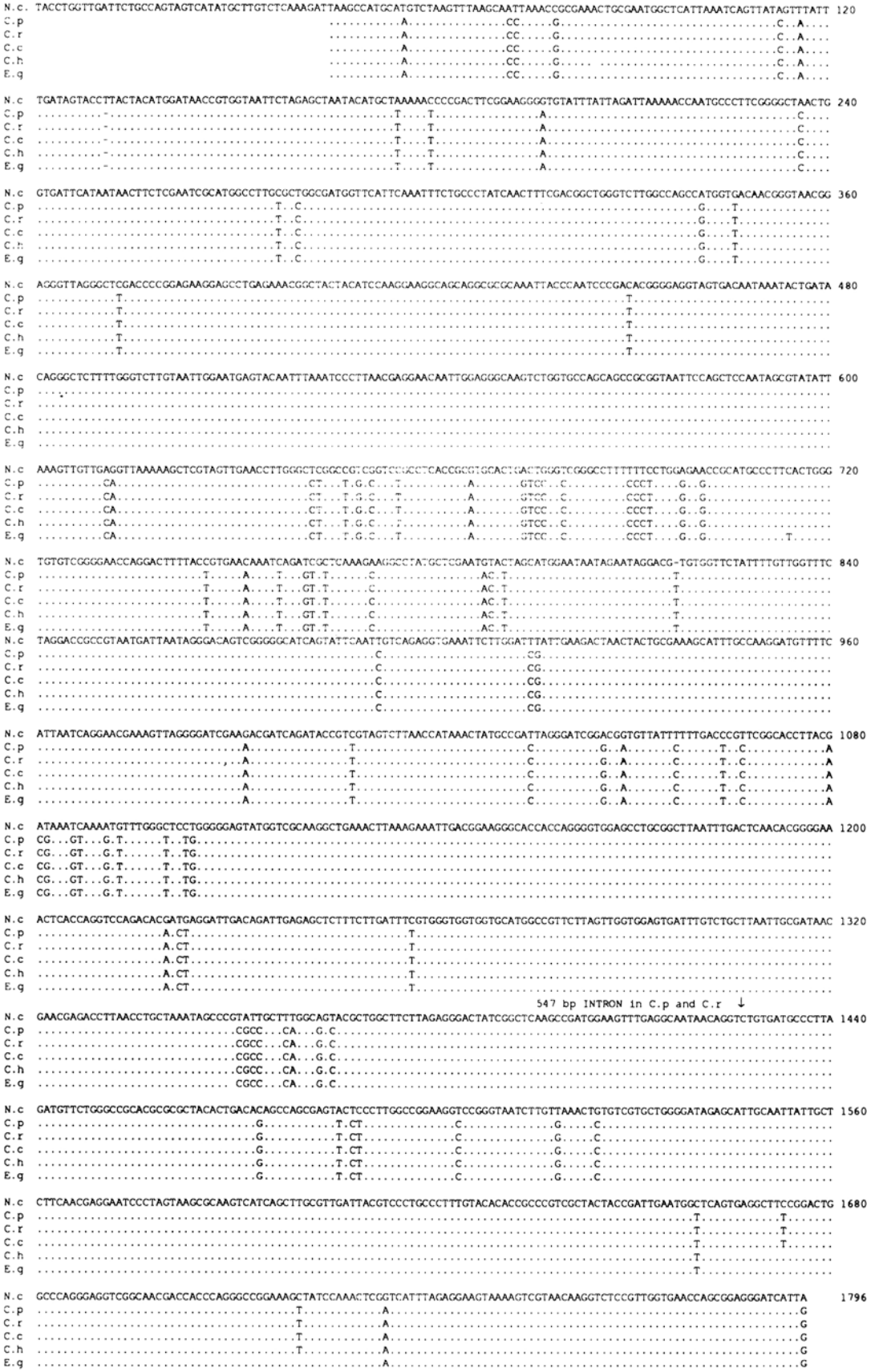

Fig. 4. Alignment of 18S rDNA sequences for *Neurospora crassa* (N.c.), *Cryphonectria parasitica* (C.p.), *C. radicalis* (C.r.), *C. cubensis* (C.c.), *C. havanensis* (C.h.), and *Endothia gyrosa* (E.g.). The location of a 547-bp group I intron between map positions 1,426 and 1,427 of both *C. parasitica* and *C. radicalis* 18S rDNA is indicated by the vertical arrow. The database accession numbers for this sequence information, including the group I intron, are as follows: C.p., L42441; C.r., L42442; C.c., L42439; C.h., L42440; and E.g., L42443.

data not shown). However, the nuclear cDNA-derived hypovirus dsRNA found in the transformed, "engineered" hypovirulent strains undergoes an additional, pre-mRNA splicing event at one of five potential cryptic splice sites to produce a 73-bp deletion located within the 5′-noncoding leader sequence (15). In this regard, two of the fungal species that readily supported hypovirus infection upon transfection with the synthetic viral transcript, *C. cubensis* and *E. gyrosa*, consistently failed to develop infections when transformed with the infectious hypovirus cDNA clone. It is possible that the differences in processing pathways described above may have some bearing on the relative utility of the two systems for expanding fungal host range. Analysis of the cDNA-derived transcripts in transformed *C. cubensis* and *E. gyrosa* is in progress to determine whether pre-mRNA splicing during export from the nucleus results in deletion events that interfere with subsequent viral RNA replication.

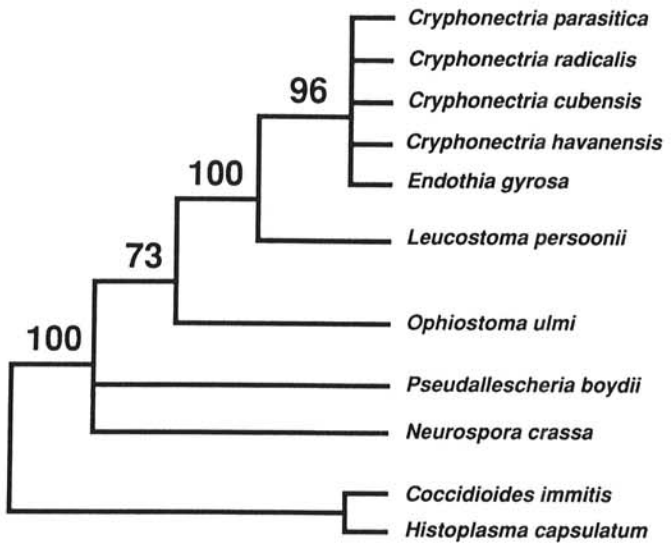

Fig. 5. Bootstrapped genetic distance tree showing the relationship of *Cryphonectria* and *Endothia* species to other fungi. This tree was created using the neighbor-joining algorithm based on 1,679 unambiguously aligned sequence positions in 18S ribosomal DNA. Bootstrap support for internal branches is shown. Branches supported in fewer than 70% of bootstrap replications have been collapsed. Branch lengths are arbitrary.

The pronounced phenotypic changes that often accompany hypovirus infection and virulence attenuation in the natural host *C. parasitica* have been described in considerable detail (2,27). It is also clear from the work of numerous investigators over the last several decades that there exists considerable diversity among natural hypovirus isolates (18,33,34,38) and that different hypovirus isolates can cause a different constellation of phenotypic changes in the same *C. parasitica* host (21). With development of the hypovirus transfection system, we can now examine the converse situation, as described in this report. We concluded that the same hypovirus genetic information can cause quite different phenotypic changes when introduced into different fungal species. Thus, by examining the changes caused by the same viral genome in different fungal host backgrounds, and vice versa, it should be possible to gain an indication of the relative contribution of the two genomes to the observed phenotypic changes. For example, the fact that hypovirus CHV1-713 caused reduced sporulation (Table 2) and laccase accumulation (data not shown) in all fungal species tested indicated that the viral genome was the primary determinant for these phenotypic changes. In contrast, the observations that CHV1-713 infection caused quite different changes in colony morphology, growth rate, and pigmentation in different fungal hosts (Fig. 2; Table 1) suggested that the host's genetic background significantly influenced the elaboration of these phenotypic changes.

The issue of relative contributions of viral and host genomes to specific phenotypic traits can have practical implications. For example, the efficiency of virus transmission through conidia is clearly dependent on the fungal host, ranging from near 100% for *C. parasitica* to 0% for *C. cubensis* (Table 2). It is clear from field studies with natural hypovirulent *C. parasitica* strains that biological control efficacy is significantly impacted by the efficiency of conidial and cytoplasmic hypovirus transmission (2,3,21,34). Thus, virulence attenuation of a fungal pathogenic species as a result of transfection-mediated hypovirus infection may be of limited practical utility for biological control if such strains are

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C.p  TCATTGCTGGAACTGTGGCCC-TCACGGGCGCAA-CCCCAGATACCCTTT-GTGAACTTATAACCATTTT--ATCGTTGCCTCGGCGCTG  90
C.r  TCATTGCTGGAACTGTGGCCC-TCACGGGCGCAA-CCCCAGATACCCTTT-GTGAACTTATAACCATTTT--ATCGTTGCCTCGGCGCTG
C.c  TCATTGCTGGAACTGCGCCCCCCCCACGGGCGCAAACCCCAGATACCCTTT-GTGAACTTATA-CCTTTTT--ATCGTTGCCTCGGCGCCG
C.h  TCATTGCTGGAATTTGTTGCCCTCACGGGCACAA-CCCCAGATACCCCTATGTGAACTTATT-CCTTTTTTAATCGTAGGCTCGGCGCTG
E.g  TCATTGCTGGAACT-GT-GCCCTCGCGGGCACAA-CCCCAGATACCCTTT-GTGAACTTATA-CCATTTT--ATCGTTGCCTCGGCGCTG

C.p  AGCCCGGGGGGGGGGTTGGCGAAGGAAGATTTTCTTCCTTCTCCCCTCCCTCCCCCCCCTCTTCCACCGTGA--AAACGGTT---GTTGGG 180
C.r  AGCCCGGGGGGGGGGTTGGCGAAGGTAGATTTTCTTCCTTCTCCCCTCCCTCCCCCCCCTCTTCCACCGTCA--AAACGGTT---GTTGGG
C.c  AGCC-GGG--------------AGTGCTC-TTCTGTG--CTCCCC----------------CACCGCGC--AAGCAGT---------G
C.h  AGCCCGGGGGG---AAGAGAAAGCTTGA TTTCC-----CTCC-CTCA---------CCCTT-CACGGGGGTGAAA-GGCCAGGGTT--G
E.g  AGCT-GGGGGCA-------------------------CTCTCCT-GTGCCCCCC-------CACCGTGC--AAGCGGT---------G

C.p  GAACAGGCCCGCCGGCGGCCCACTAAACTCTTTGTTTTTA-TAAC-CTATCTCTTCTGAGTA-CATAAACCAAAAA-AAATGAATTAAAA 270
C.r  GAGCAGGACCACCGGCGGCCCACTAAACTCCTTGTTTTAA-TAAC-CTATCTCTTCTGAGTA-CATAAACCAAAAT-AAATGAATCAAAA
C.c  GAGCAGGCCCGCCGGCGGCCCACCAAACTCTTTGTTTTTA-GAAC-GTATCTCTTCTGAGTGTTTATAA-CAAA--CAAATGAATCAAAA
C.h  GAGCAGGTCCGCCCGCGGCCCAGTTACGATCTAGTTATTAATAACCGTATCTCTTCTGAGTGGCATAAT-CAAAAG----TTAATCAAAA
E.g  GAGCAGGCCCGCCGGCGGCCCACCAAACTCTTTGTTTTTA-GA-CCGTATCTCCTCTGAGTGTTTA----CAAAAACAAATGAATCAAAA

C.p  CTTTCAACAACGGATCTCTTGGTTCTG
C.r  CTTTCAACAATGGATCTCTTGGTTCTG
C.c  CTTTCAACAACGGATCTCTTGGTTCTG
C.h  CTGGCAACAACGGATCTCTAGGTTCTG
E.g  CTTTCAACAACGGATCTCTTGGTTCTG
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Fig. 6. Alignment of internal transcribed spacer sequences for *Cryphonectria parasitica* (C.p.), *C. radicalis* (C.r.), *C. cubensis* (C.c.), *C. havanensis* (C.h.), and *Endothia gyrosa* (E.g.). Database accession numbers for this sequence information are as follows: C.p., L42446; C.r., L42447; C.c., L42444; C.h., L42445; and E.g., L42448.

deficient in conidial virus transmission, e.g., *C. cubensis* (Table 2). In this regard, engineered hypovirulent strains, because they contain a chromosomally integrated functional hypovirus cDNA copy, not only transmit viruses to ascospore progeny, but also transmit virus through asexual spores at a rate of 100% (13). These differences both illustrate the advantages provided by engineered hypovirulent strains for enhanced biocontrol potential and reinforce the need to determine why some fungal species that can support transfection-mediated hypovirus infection fail to launch replicating viral RNA after transformation with infectious hypovirus cDNA.

Several recent studies have demonstrated that hypovirus infection modifies certain cellular signal transduction pathways involved in fungal gene expression. For example, transcription of the *C. parasitica* gene *lac-1*, encoding the enzyme laccase, was shown to be suppressed in hypovirus-infected hyphae by a mechanism that involves perturbation of an IP_3/Ca^{2+}/calmodulin-dependent regulatory pathway that governs *lac-1* transcription in virus-free fungal strains (30,31). Choi et al. (16) recently showed that the accumulation of a heterotrimeric GTP-binding protein α subunit, CPG-1, is suppressed in hypovirus-infected *C. parasitica*. Significantly, transgenic suppression of CPG-1 accumulation, in the absence of virus infection, resulted in virulence attenuation (16). The latter study provides one of the first indications for the role of a G-protein linked signal transduction pathway in fungal pathogenesis and suggests a molecular basis for hypovirus-mediated attenuation of fungal virulence. It seems likely that similar modifications of signaling cascades underlie the variety of phenotypic changes displayed in the different hypovirus-infected fungal species examined in this study. Within this context, it is also conceivable that virus-mediated alterations of very similar regulatory or metabolic pathways in different fungal species could result in the elaboration of what appear on the surface to be quite different phenotypic changes. A candidate for such a manifestation is the apparent opposite effect of virus infection on pigment production in different fungal species (Fig. 2). We anticipate that the ability to install wild-type and mutant hypovirus into the genetic backgrounds of different fungal species will provide novel opportunities for examining and dissecting regulatory pathways that govern a variety of fungal biological processes ranging from sporulation to pigment production.

The available literature on the taxonomic placement of *C. parasitica* and related species is not straightforward. Following the first report of chestnut blight in North America in 1905 (35), the causative agent was described as *Diaporthe parasitica* Murr. (40). It was later placed in the genus *Endothia* and renamed *Endothia parasitica* (Murr.) P. J. & H. W. Anderson (43). In a 1978 monograph on the order Diaporthales (8), Barr altered the taxonomic status of the genus *Endothia* assigning *E. parasitica* and the related saprophytic species *E. radicalis* to the genus *Cryphonectria* within the tribe Valseae in the family Valsaceae. The oak pathogen *Endothia gyrosa* was retained within the genus *Endothia* and assigned to the tribe Endothieae in the family Gnomoniaceae. The placement of the two Eucalyptus pathogens examined in this study, *C. cubensis* and *C. havanensis*, is also a source of some confusion. Originally designated *Diaporthe cubensis* (Bruner), *C. cubensis* was transferred to *Cryphonectria* in 1980 by Hodges (28). Originally designated *Endothia havanensis* (Bruner), *C. havanensis* was assigned to *Cryphonectria* by Barr (8). There remains considerable disagreement regarding the relatedness of both *C. cubensis* and *C. havanensis* to morphologically similar isolates recovered from eucalyptus bark and canker samples (7).

Results of the phylogenetic analysis based on the 18S ribosomal sequences as presented in this report were consistent with the taxonomic placement of *Cryphonectria* spp. in the order Diaporthales. In addition, *E. gyrosa* grouped strongly with the *Cryphonectria* spp., and the number of substitutions between *E. gyrosa* and the *Cryphonectria* spp. within the 18S gene were essentially the same as those within the genus. Thus, the taxonomic decision to place *E. gyrosa* and *C. parasitica* into different genera within two separate families was not generally supported by the molecular data presented here. Although there were insufficient substitutions within the 18S genes to determine a branching order within the *Cryphonectria* group and the ITS sequences were too divergent to align for phylogenetic analysis, the small number of substitutions in the ITS between *C. parasitica* and *C. radicalis* did suggest that within the genus, these two species most recently shared a common ancestor. The presence of an identical group I intron at precisely the same position within the 18S rDNA of both *C. radicalis* and *C. parasitica* provided further evidence to support this conclusion.

The identification of an identical group I intron in two different *Cryphonectria* spp. is even more surprising when one considers the geographic origin of the *C. radicalis* and *C. parasitica* isolates analyzed: Greece and the United States, respectively. In this regard, rDNA sequence information and molecular markers such as the group I intron provide new tools for reconstructing the origin and development of the chestnut blight epidemics in North America and Europe. Anagnostakis (*personal communication*) has observed that *C. radicalis*, once readily isolated throughout the natural range of the American chestnut tree prior to the chestnut blight epidemic, can no longer be collected in areas where *C. parasitica* has became established. This observation suggests that *C. parasitica* either displaced or hybridized with *C. radicalis*. By analyzing available herbarium samples of *C. radicalis* strains isolated prior to the introduction of *C. parasitica* for the presence of the group I intron, it may be possible to provide clues that could distinguish between these two scenarios. Based on examinations of importation records, it has been proposed that *C. parasitica* was introduced into the United States from Japan on *Castanea crenata* (4,5). Again, it may be possible to clarify this issue by examining the rDNA, including the ITS sequences, for Asian and U.S. *C. parasitica* isolates. Additionally, it is anticipated that a clearer understanding of the correlation between fungal evolutionary relatedness and hypovirus extended host range will provide important insights into the origins and future practical utility of this group of viruses.

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