Attenuation of Fungal Virulence by Synthetic Infectious Hypovirus Transcripts

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Noninfectious, cytoplasmically transmissible viral double-stranded RNAs of the genus *Hypovirus* cause reduced virulence (hypovirulence) in the chestnut blight fungus *Cryphonectria parasitica*, providing the basis for virus-mediated biological control of a fungal disease. Synthetic transcripts corresponding to a full-length hypovirus RNA coding strand are infectious when introduced into fungal spheroplasts by electroporation. Hypovirus infections were readily established in *Cryphonectria parasitica* and in related fungal species not previously reported to harbor viruses. These results demonstrate the use of a synthetic mycovirus transcript to expand fungal host range, thereby broadening the potential application of virus-mediated hypovirulence to control fungal pathogenesis.

More than 100 fungal species have been reported to harbor viruses (mycoviruses) (*1*). Mycovirus infections are persistent, nontransmissible by an extracellular route, and generally nonsymptomatic (*1, 2*). However, members of the genus *Hypovirus* cause profound phenotypic alterations, including virulence attenuation (hypovirulence), in their host, the chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr. (*3*). Hypoviruses can be transmitted intercellularly to compatible virulent strains of the same species through fused hyphae (anastomoses) (*4, 5*). A previously virus-free strain can thus be converted to hypovirulence, providing the basis for biological control. The potential for effective biological control of chestnut blight is further enhanced by the availability of an infectious complementary DNA (cDNA) copy of hypovirus RNA (6). Strains of C. *parasitica* that are transformed with that cDNA (engineered hypovirulent strains) contain both a chromosomally integrated copy of viral cDNA and cytoplasmic-replicating, cDNA-derived viral RNA. In this configuration, the virus can be transmitted by a sexual cross with exchange of nuclear DNA as well as by the normal mode of cytoplasmic transmission (*7*). Virus transmission by nuclear inheritance evades barriers to cytoplasmic virus transmission imposed by the fungal vegetative incompatibility system.

The prototypic hypovirus, isolate CHV1-713 from hypovirulent C. *parasitica* strain EP713, is found predominantly as unencapsidated double-stranded RNA (L-dsRNA) with an organization similar to a replicative intermediate of a positive strand RNA virus (*8*). Attempts to transmit hypoviruses artificially by the introduction of viral dsRNA into virus-free fungal strains have been unsuccessful (*9*). The cDNA-derived L-dsRNA present in engineered hypovirulent C. *parasitica* strains was shown to be generated from a nuclear transcript that contains the viral RNA coding strand (*10*). This finding suggested that it should be possible to establish a hypovirus infection by the introduction of a synthetic copy of the L-dsRNA coding strand into fungal spheroplasts.

Transcripts corresponding to the viral RNA coding strand were synthesized from a full-length cDNA copy of CHV1-713 L-dsRNA in plasmid pLDST (Fig. 1A). Electroporation of spheroplasts derived from C. *parasitica* strain EP155, the same strain as the hypovirulent strain EP713 but lacking virus, with linearized pLDST DNA failed to yield virus-containing transfectants (Fig. 1B). In contrast, electroporation with a mixture of plasmid and viral transcripts, or deoxyribonuclease (DNase)-treated transcripts, yielded mycelia that contained cytoplasmic-replicating L-dsRNA (Fig. 1B). These results demonstrate that a synthetic transcript corresponding to the coding strand of a mycovirus dsRNA can initiate an infection when introduced into fungal spheroplasts.

To determine whether other taxonomically related fungal species could support hypovirus replication, we transfected three members of the genus *Cryphonectria* (family Valsaceae) and one member of a separate taxonomic family (Gnomoniaceae) in the genus *Endothia* (*11–13*). The fungi tested were C. *cubensis* (Bruner) Hodges, the causal agent of a canker disease of *Eucalyptus* spp. in many tropical areas of the world (*14*), C. *havanensis* (Bruner) Barr, also a pathogen of *Eucalyptus* (*13*), C. *radicalis* (Schw. ex Fries) Barr, a nonpathogenic saprophytic species (*13*), and *E. gyrosa* (Schw. ex Fries) Fries, a canker pathogen of *Quercus* spp., mainly pin oak (*15*). These species do not harbor natural hypoviruses,

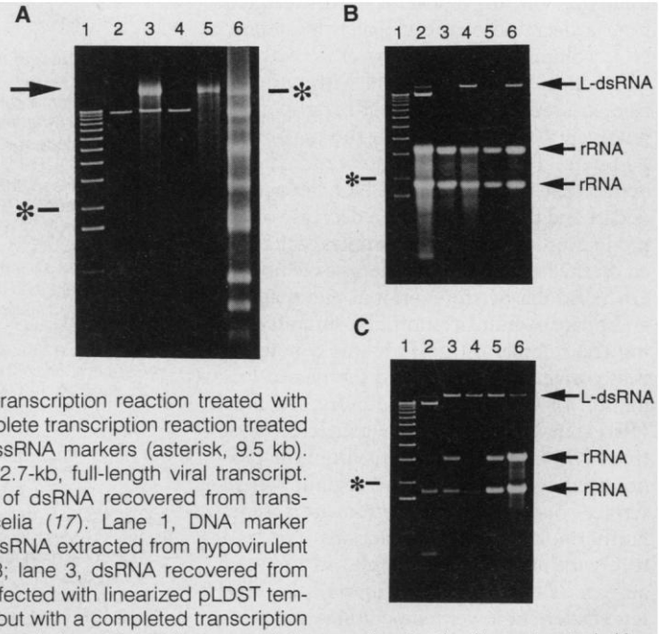

Fig. 1. Cell-free synthesis of full-length hypovirus transcripts and their use to transfect *C. parasitica* spheroplasts (*22*). (**A**) Agarose gel analysis of T7 polymerase–dependent cell-free transcription reactions with Spe I–linearized pLDST. Lane 1, 1–kilobase pair (kbp) DNA ladder (asterisk, 2 kbp); lane 2, linearized pLDST; lane 3, complete transcription reaction; lane 4, complete transcription reaction treated with ribonuclease; lane 5, complete transcription reaction treated with DNase; and lane 6, ssRNA markers (asterisk, 9.5 kb). The arrow indicates the 12.7-kb, full-length viral transcript. (**B**) Agarose gel analysis of dsRNA recovered from transfected *C. parasitica* mycelia (*17*). Lane 1, DNA marker (asterisk, 2 kbp); lane 2, dsRNA extracted from hypovirulent *C. parasitica* strain EP713; lane 3, dsRNA recovered from regenerated mycelia transfected with linearized pLDST template; lane 4, as in lane 3 but with a completed transcription reaction; and lanes 5 and 6, as in lane 3 but with a transcription reaction that had been treated either with DNase (lane 5) or with ribonuclease (lane 6) before transfection. The L-dsRNA and residual ribosomal RNAs are indicated at the right (*6*). Comparable transfection results were obtained whether or not $m^7G(5')ppp(5')G$ was added to the in vitro transcription reaction. Transfection with a modified synthetic transcript (introduced Not I site at L-dsRNA map position 12038) yielded transfectants with the corresponding modified L-dsRNA as determined by combined reverse transcription and polymerase chain reaction (*6*). (**C**) Expansion of hypovirus host range with infectious synthetic viral transcripts. Lane 1, 1-kb DNA ladder (asterisk, 2 kbp); lane 2, dsRNA recovered from *C. parasitica* strain EP713; lanes 3 to 6, dsRNAs recovered from transfected *C. radicalis*, *C. havanensis* (ATCC 56124), *E. gyrosa* (ATCC 48192), and *C. cubensis* (ATCC 64159), respectively.

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and hypovirus dsRNA was not transmissible from C. *parasitica* to these species by anastomosis (*16*). Each of these fungal species supported hypovirus replication following transfection with the infectious CHV1-713 L-dsRNA transcript (Fig. 1C).

Table 1. Virulence assay of uninfected and hypovirus-infected *E. gyrosa*, giving the mean canker area (in square centimeters) (*23*). For pin oak and red oak, the *t* values were 10.18 and 15.70, respectively and the probabilities were < 0.0005 for each.

Stem number	Pin oak		Red oak	
	Uninfected *E. gyrosa*	Hypovirus-infected *E. gyrosa*	Uninfected *E. gyrosa*	Hypovirus-infected *E. gyrosa*
1	6.89	0.60	22.28	2.96
2	6.01	0.94	19.82	2.81
3	5.67	0.55	24.92	2.47
4	7.94	0.84	20.43	2.01
5	4.58	0.61	26.33	2.40
Mean	6.22 ± 1.27	0.71 ± 0.17	22.76 ± 2.82	2.53 ± 0.37

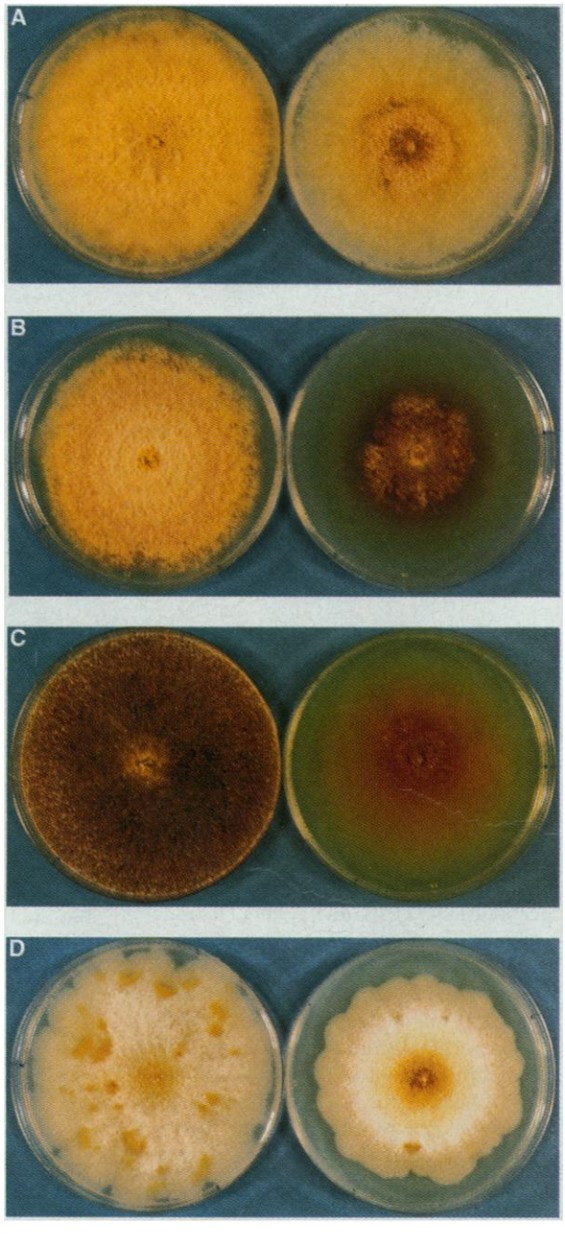

Fig. 2. Morphological changes associated with the replication of hypovirus RNA in transfected fungal species. Each pair compares the morphology of virus-free (left) and hypovirus-transfected (right) colonies on potato dextrose agar media. (**A**) Morphological changes observed for *C. radicalis* were similar to those observed for engineered hypovirulent *C. parasitica* strains (*6*), including a slight reduction in growth rate, the suppression of orange pigmentation, and reduced sporulation. Infection of (**B**) *E. gyrosa* and (**C**) *C. cubensis* resulted in more severe phenotypic changes, including a reduction in growth rate and increased production of a dark brown and a bright orange pigment, respectively. (**D**) Infection of *C. havanensis* resulted in mild morphological changes.

Hypoviruses cause a variety of profound phenotypic changes in the natural host C. *parasitica* (*3*, *17–19*). Hypovirus infection of additional fungal species was also accompanied by a variety of morphological changes (Fig. 2), as well as the attenuation of fungal virulence (Table 1). The expansion of hypovirus-mediated virulence attenuation to *E. gyrosa*, classified as a member of a separate taxonomic family (*11*), suggests that virus-mediated hypovirulence will find broader application in addition to the biological control of chestnut blight.

Two of the five fungal species that supported transfection-mediated hypovirus infection, C. *cubensis* and *E. gyrosa*, failed to develop infections when transformed with the infectious hypovirus cDNA. Thus, although the cDNA provides alternative transmission modes, the infectious RNA transcript appears to allow broader expansion of host range. The cDNA-derived hypovirus RNA in engineered hypovirulent C. *parasitica* strains undergoes posttranscriptional splicing, resulting in a 73-base deletion at one of several potential cryptic splice sites (*10*). The failure to launch viral dsRNA after transformation of some fungi may, therefore, be due to deficiencies in the processing of the cDNA-derived viral transcript.

Hypovirus infection of C. *parasitica* results in alterations of signal transduction pathways involved in fungal gene expression (*20*). It is likely that the variety of phenotypic changes observed in the different infected fungal species (Fig. 2) is also a manifestation of alterations in fungal signaling cascades. Comparative studies of these regulatory pathways and the interactions between pathway components in different hypovirus-infected fungal species will provide insights into the regulation of fungal gene expression and the basis of fungal virulence.

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16. All five hypovirus-transfected fungal species were found to convert virus-free strains of the same species efficiently after anastomosis, as described (*6*). No cytoplasmic virus transmission was observed from transfected *C. parasitica* to other fungal species and vice versa.
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22. Plasmid pLDST used as a template for cell-free synthesis of the viral coding strand transcript contained a full-length cDNA copy of CHV1-713 L-dsRNA (*6*) flanked at the 5′ terminus by a bacteriophage T7 promoter sequence (TAATACGACTCACTATAG) and at the 3′ terminus by 22 adenosine residues, corresponding to the natural heterogenous polyadenylate tail found in L-dsRNA (*8*), followed by an Spe I restriction site. Thus, the synthetic transcript generated from Spe I linearized pLDST contained a nonviral G residue at the 5′ terminus and 22 adenosine residues followed by the vector sequence CUAG at the 3′ terminus. Cell-free transcription was performed with Riboprobe transcription reagents (Promega, Madison, WI) according to the manufacturer's instructions. Where indicated, reactions were treated with 3 units of RQ1-DNase or 0.3 unit of ribonuclease A for 20 min at 37°C. All reactions were extracted with phenol:chloroform, and nucleic acids were recovered by ethanol precipitation. Fungal spheroplasts (2×10^7 per milliliter) (*21*) in 100 μl of 1 M sorbitol were transfected with nucleic acid preparations (in 20 μl of H_2O containing 200 units of RNasin) by electroporation at 1.5 kV, 200 ohms, and 25 μF. The spheroplast suspensions were diluted with 750 μl of ice-cold 1 M sorbitol and incubated on ice for 5 to 10 min. Aliquots (200 μl) were then placed in the center of 10-cm petri dishes, surrounded by 20 ml of regeneration medium (*21*), gently mixed, and incubated at 25°C. Mycelial plugs were transferred from the edge of the regenerated colony to potato dextrose agar. Because hyphal strands regenerated from spheroplasts fuse with neighboring strands, replicating hypovirus RNA from transfected spheroplast can spread throughout the colony. Consequently, virus-infected mycelia can be readily obtained even at low transfection efficiencies without the need for a selectable marker.
23. Virulence assays were performed for uninfected and hypovirus-infected *E. gyrosa* on dormant pin oak and red oak stems as described (*24*). Thirty-five days after inoculation, bark was removed around lesions and the extent of the necrotic tissue was measured. Data are presented as the mean lesion area (in square centimeters) on the basis of four inoculations each of uninfected *E. gyrosa* and hypovirus-infected *E. gyrosa* per stem. Differences between lesion formation by virus-free and virus-containing fungal strains were highly significant for both host tree stems as indicated by the *t* values listed. The *E. gyrosa* species was chosen for the virulence assay because the natural plant hosts were readily available, it is a serious pathogen of stressed pin oak in urban settings (*15*), and it is classified in a different taxanomic family from members of the genus *Cryphonectria* (*11*).
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Recovery of Visual Response of Injured Adult Rat Optic Nerves Treated with Transglutaminase

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Failure of axons of the central nervous system in adult mammals to regenerate spontaneously after injury is attributed in part to inhibitory molecules associated with oligodendrocytes. Regeneration of central nervous system axons in fish is correlated with the presence of a transglutaminase. This enzyme dimerizes interleukin-2, and the product is cytotoxic to oligodendrocytes in vitro. Application of this nerve-derived transglutaminase to rat optic nerves, in which the injury had caused the loss of visual evoked potential response to light, promoted the recovery of that response within 6 weeks after injury. Transmission electron microscopy analysis revealed the concomitant appearance of axons in the distal stump of the optic nerve.

Nerves of the adult mammalian central nervous system (CNS) do not regenerate successfully after axonal injury. Spontaneous growth of injured axons does occur, but ceases after a few hundred micrometers, without traversing the injury site and without elongating into the distal stump (*1–4*). The failure to regenerate has been attributed to the inhospitable nature of the axon's local environment (*5–7*). One of the components of this environment, myelin-associated molecules produced by mature oligodendrocytes, inhibits axonal growth (*6, 8*). Unlike mammals, fish CNS spontaneously regenerates its injured axons. We recently identified in regenerating fish optic nerves an interleukin-2 (IL-2)–like compound, which has an apparent molecular weight twice that of Il-2 and is cytotoxic to oligodendrocytes in vitro (*9*). From the regenerating fish optic nerves we purified a nerve-derived transglutaminase (TG_N), capable of dimerizing IL-2 and making it cytotoxic to oligodendrocytes (*10*) through an apoptotic mechanism (*11*). These results, taken together with observations that the nerve preparation (regenerating fish optic nerve) from which TG_N is purified induces axonal growth in mammalian CNS in vivo (*12*) and that oligodendrocytes inhibit nerve regeneration in vitro (6), suggested to us that dimerization of IL-2 after injury may be an effective way to facilitate nerve regeneration. In addition, because the enzyme belongs to the family of transglutaminases, known to participate in tissue healing processes, it was anticipated that the enzyme might have additional activities beneficial to nerve regeneration. Here we examined this possibility by injecting the enzyme locally into injured optic nerves of adult rats. The nerves were then analyzed electrophysiologically in terms of recovery of the visual evoked potential (VEP) response to light, previously shown to be a reliable measure of the integrity of the visual

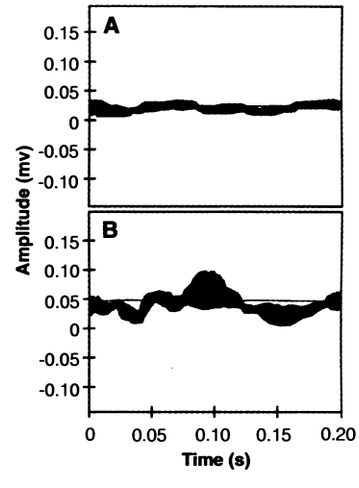

Fig. 1. Recordings of VEP response from a TG_N-treated injured nerve 1 week after injury. Nerves were injured and treated as described (*14*) and the VEP response was recorded (*15*). (**A**) Negative VEP response recorded from the left (injured) eye, while the right (uninjured) eye was covered during recording. (**B**) Positive VEP response recorded from the same animal while both eyes were open. Values are means obtained from three recordings (each involving 60 light flashes) (black line) ± SE (blue area). The baseline recording (black line) ± SE (yellow area) was made in the dark.

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