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# A 971-bp insertion in the *rns* gene is associated with mitochondrial hypovirulence in a strain of *Cryphonectria parasitica* isolated from nature

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ABSTRACT

In the chestnut-blight fungus *Cryphonectria parasitica*, cytoplasmically transmissible hypovirulence phenotypes frequently are elicited by double-stranded RNA (dsRNA) virus infections. However, some strains manifest cytoplasmically transmissible hypovirulence traits without containing any mycovirus. In this study, we describe an altered form of mtDNA that is associated with hypovirulence and senescence in a virus-free strain of *C. parasitica*, KFC9, which was obtained from nature and has an elevated level of cyanide-resistant respiration. In this strain, a 971-bp DNA element, named InC9, has been inserted into the first exon of the mitochondrial small-subunit ribosomal RNA (*rns*) gene. Sequence analysis indicates that InC9 is a type A1 group II intron that lacks a maturase-encoding ORF. RT-PCR analyses showed that the InC9 sequence is spliced inefficiently from the rRNA precursor. The KFC9 strain had very low amounts of mitochondrial ribosomes relative to virulent strains, thus most likely is deficient in mitochondrial protein synthesis and lacks at least some of the components of the cyanide-sensitive, cytochrome-mediated respiratory pathway. The attenuated-virulence trait and the splicing-defective intron are transferred asexually and concordantly by hyphal contact from hypovirulent donor strains to virulent recipients, confirming that InC9 causes hypovirulence.



## 1. Introduction

*Cryphonectria parasitica*, an ascomycetous fungus, is the pathogen responsible for chestnut blight, a disease that has virtually decimated the native chestnut tree (*Castanea dentata*) of North America. In addition to the common occurrence of virulent strains of this fungus in expanding cankers on trees, hypovirulent strains also have been recovered, most commonly from healing cankers (Grente, 1965; Grente and Berthelay-Sauret, 1969). These strains lacked the aggressiveness of the virulent types and have been causally implicated in the spontaneous regeneration of diseased trees. Most of the hypovirulent strains were found to contain attenuating, infectious, double-stranded RNA (dsRNA) viruses (Choi and Nuss, 1992; Dawe and Nuss, 2001; Enebak et al., 1994; Fulbright, 1999; Nuss, 1992, 2005; Tartaglia et al., 1986; Van Alfen et al., 1975). However, some hypovirulence also occurs naturally and can be induced by mutagens in strains that are completely devoid of viruses (Baidyaroy et al., 2000; Mahanti et al., 1993; Monteiro-Vitorello et al., 1995, 2000). Unlike the virulent wild-types and the dsRNA-containing attenuated strains, the virus-free hypovirulent isolates have high levels of mitochondrial alternative oxidase activity, which is manifested phenotypically in mycelia as cyanide-resistant and salicylhydroxamate-sensitive respiration (Baidyaroy et al., 2000; Mahanti et al., 1993; Monteiro-Vitorello et al., 1995). The hypovirulence trait of these strains also was found to be 'infectious' like that of the virus-containing diseased isolates because it can be transmitted by hyphal contact to virulent strains (Bertrand, 2000; Bertrand and Baidyaroy, 2002; Monteiro-Vitorello et al., 1995). Thus, whatever genetic determinant causes hypovirulence in virus-free strains of *C. parasitica* also is capable of modifying the functional state of mitochondria by eliciting a deficiency in cytochrome-mediated respiration (Baidyaroy et al., 2000; Monteiro-Vitorello et al., 1995). This type of hypovirulence has been called "mitochondrial hypovirulence" to distinguish it from those which are caused by mycoviruses (Baidyaroy et al., 2000; Bertrand, 2000; Monteiro-Vitorello et al., 1995).

In recent years, a virus-free strain of *C. parasitica*, KFC9, that clearly manifested the symptoms of mitochondrial hypovirulence,

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including high levels of cyanide-insensitive respiration (alternative oxidase activity), was isolated from a healing canker on an American chestnut tree located in the Kellogg Forest in Michigan (USA). The hypovirulence phenotype was found to be stably maintained in the KFC9 strain and infectious in the sense that it was transmitted readily to virulent strains by hyphal contacts (Baidyaroy et al., 2000). Moreover, the vegetative transmission of the hypovirulence phenotype to virulent recipients coincided with the transmission of a specific region of the mtDNA of the KFC9 isolate (Baidyaroy et al., 2000). We have analyzed this segment of the mtDNA and have concluded the hypovirulence and senescence traits that are characteristic of strains that have the KFC9 cytoplasm are due to disruption of the mitochondrial *rns* gene by a defective group II intron.

## 2. Materials and methods

### 2.1. Fungal strains and culturing conditions

*C. parasitica* was cultured in Endothia complete medium as described by Puhalla and Anagnostakis (1971). Methionine was added to the medium at a final concentration of 0.1 mg/ml, when required. The hypovirulent KFC9 strain was originally isolated from a healing canker on an American chestnut tree in the Kellogg Forest near Augusta, Michigan (Baidyaroy et al., 2000). Ep155, which commonly is used as a reference virulent strain (Allen and Nuss, 2004; Bell et al., 1996; Gobbi et al., 2003; Polashock et al., 1997), served as a wild-type control in all the experiments. A severely senescent single-conidial isolate derived from KFC9, KFC9-E6, was used in experiments involving the vegetative transmission of the hypovirulence phenotype from KFC9 to recipient virulent strains of *C. parasitica* identified by nuclear markers, namely Ep289 *met*, J2.31 *br* and F2.36 *br*. Ep289 *met* was originally obtained from S. Anagnostakis (Connecticut Agricultural Experiment Station), while J2.31 *br* and F2.36 *br* were generated in the D.W. Fulbright laboratory.

### 2.2. Preparation of DNA and RNA

Whole-cell DNA was isolated by the method described by Baidyaroy et al. (2000). Mitochondria were purified by the sucrose floatation-gradient procedure (Lambowitz, 1979) and mtDNA was prepared as described by Bell et al. (1996) with an added purification step using cetyltrimethylammonium bromide (Ausubel et al., 1987). RNA was isolated from purified mitochondria by the SDS-diethylpyrocarbonate procedure (Solymosy et al., 1968).

### 2.3. Molecular cloning and standard DNA and RNA manipulations

Digestion of DNAs with restriction endonucleases, agarose gel electrophoresis and molecular cloning were performed as recommended by Sambrook et al. (1989). Restriction fragments or PCR products were separated by gel electrophoresis through 0.7–1.0% agarose (Invitrogen, Carlsbad, CA, USA) gels in TBE buffer (Sambrook et al., 1989) and visualized under UV-light after staining with ethidium bromide. DNA fragments were sized using the 1-kb molecular weight ladder (Invitrogen) as a standard.

Southern and northern blot hybridizations were performed with dig-oxigenin-dUTP-labeled probes generated as directed by the manufacturer of the kit (Roche Diagnostics, Indianapolis, IN, USA). Binding of the probes was detected by chemiluminescence using an anti-dig-oxigenin Fab-alkaline phosphatase conjugate with CDP-star (Roche Diagnostics).

### 2.4. DNA sequencing and sequence analysis

Sequencing of DNA was carried out using plasmid DNA purified with the Wizard™ Minipreps DNA Purification System (Promega, Madison, WI) according to the manufacturer's recommended protocols. Dideoxy DNA sequencing was performed initially according to the protocols described by Sanger et al. (1977) with the modifications recommended by Zhang et al. (1991) and [$\alpha^{33}$P] dATP (Amersham Biosciences) in the labeling reaction, and later by automated sequencing with fluorescent dyes using Applied Biosystems 3100 Genetic Analyzers (Applied Biosystems, Foster City, CA, USA). For all templates, both strands were completely sequenced by the progressive extension of initial sequences obtained with the vector-based T7 and T3 primers (Stratagene) with appropriately designed, sequence-extending primers synthesized by the Research Technology Support Facility at Michigan State University.

Sequences were aligned and contigs were assembled through the use of the MicroGenie™ MG-IM-5.0 (Queen and Korn, 1984) and PCGene (IntelliGenetics Inc.) programs. NCBI databases were

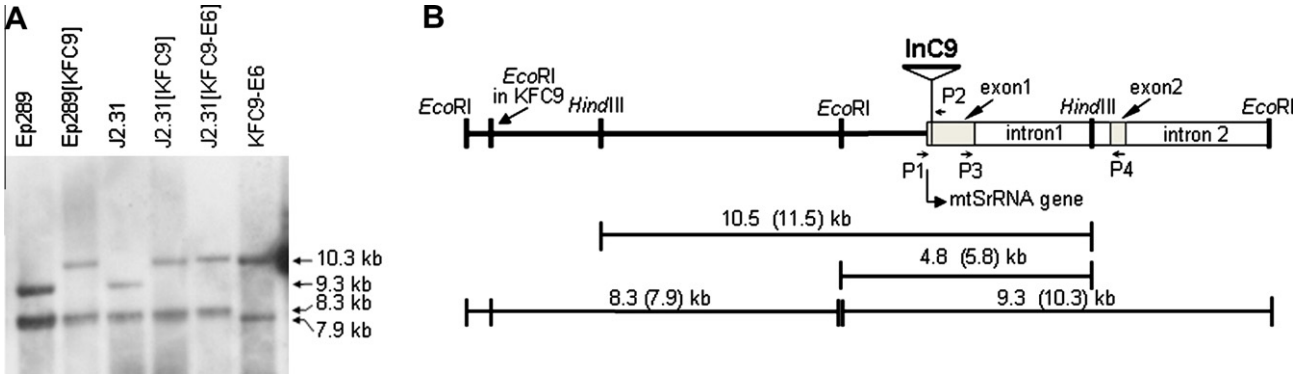


**Fig. 1.** Location and transmission of a mutation that causes hypovirulence in *C. parasitica* strains isolated from the Kellogg Forest in Michigan. (A) Southern blots illustrating the asexual co-transmission of mtDNA region bearing InC9 with hypovirulence from hypovirulent to virulent strains. A Southern blot of *Eco*RI digested genomic DNAs was hybridized with a probe generated from the wild-type 10.5-kb *Hind*III fragment (see Fig. 1B). The donor, KFC9 or KFC9-E6, of the cytoplasm in the converted Ep289 and J2.31 recipients is indicated in brackets. The 10.3-kb *Eco*RI piece that contains InC9 was co-transmitted with senescence and hypovirulence traits from both donors to the non-senescent, virulent recipients, but the adjacent 7.9-kb *Eco*RI fragment was not transferred. (B) Location of a 1-kb insert, InC9, on a segment of the physical and genetic map of the mtDNA of *C. parasitica*. The sizes of the wild-type restriction fragments are indicated in numerals above the corresponding pieces of DNA, whereas the sizes of the equivalent fragments from the hypovirulent mutant are indicated in parentheses. InC9 was cloned as a part of the 5.8-kb *Hind*III-*Eco*RI segment from KFC9. The locations of the different primers (P1–P5) that were used to characterize *rns* transcripts in mutant and wild-type strains by PCR are indicated by small arrows pointing in the direction of DNA synthesis.