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Research Note

Detection of Mitochondrial DNA Transfer Between Strains After Vegetative Contact in *Cryphonectria parasitica*

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Field isolates of *Cryphonecteria parasitica* showing a transmissible hypovirulence phenotype not associated with dsRNA viruses have been recovered from surviving chestnut trees in North America. Mitochondrial dysfunction is thought to a play a primary role in the dsRNA-free hypovirulent strains since large amounts of respiration occur via the alternative oxidase pathway. To determine if mitochondrial exchange can occur in *C. parasitica*, vegetative pairings were established between genetically marked strains. mtDNA from the donor strain could be detected in the recipient strain providing laboratory evidence that field isolates of *C. parasitica* are capable of exchanging mtDNA with other strains.

Additional key words: hypovirulence, chestnut blight, hyphal anastomosis, alternative oxidase.

Transmissible hypovirulence in the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr, has been shown to be the result of infection (Choi and Nuss 1992) with transmissible double-stranded RNA (dsRNA) viruses of the family *Hypoviridae* (Hillman et al. 1994). Hypovirulent strains are reduced in virulence and are typically altered in growth rate, sporulation, pigmentation and/or culture morphology and are capable of transmitting these traits to virulent strains.

We have reported on strains of *C. parasitica* from recovering chestnut trees in Michigan that have all the characteristics of virus-associated hypovirulence but harbor no detectable dsRNA viruses (Mahanti et al. 1993). Initial studies on one particular isolate, CL25, showed that virus-free hypovirulence was cytoplasmically transmissible to virulent strains after hyphal contact with vegetatively compatible strains (Fulbright 1985). This finding when coupled with genetic (Mahanti and Fulbright 1991) and physiological studies (Mahanti et al. 1993) strongly suggested that hypovirulence in CL25 and other virus-free hypovirulent strains was caused by a nonnuclear, cytoplasmic genetic factor. More recently, it was determined that virus-free hypovirulent isolates may carry mitochondrial defects since most respiratory activity is cyanide-resistant and salicylhydroxamate sensitive, which is indicative of mitochondrial alternative oxidase induction (Mahanti et al. 1993). The cytoplasmic transmission of the respiratory defects and hypovirulence phenotype indicate that these virus-free hypovirulent isolates may carry mtDNA mutations similar to those in other fungi including *Neurospora* (Griffith 1992).

We initiated this study to determine if the cytoplasmic location of the factor that causes hypovirulence in virus-free isolates could be mitochondrial. If mitochondria are involved, then mtDNA, portions of mtDNA, or mitochondrial plasmids must be capable of cytoplasmic transfer in *C. parasitica*. Information regarding the movement of mitochondria during hyphal fusion of compatible as well as between incompatible strains is limited. Some reports indicate that mitochondria do not move during hyphal fusion in filamentous fungi (Brasier 1986; Casselton and Economou 1985). Gobbi et al. (1990) reported the absence of mitochondrial transfer in *C. parasitica*, but pointed out that low levels of mitochondrial transmission could have evaded their detection. These reports on the apparent lack of mitochondrial transfer during vegetative fusion contrast with the reports on mitochondrial transmission in other filamentous fungi (Baptista-Ferreira et al. 1983; Watrud and Ellingboe 1973). Collins and Seville (1990) have provided evidence for the transfer of the mitochondrial chromosome and plasmids during unstable vegetative fusion in *Neurospora*. In similar studies by Griffith et al. (1990), the transmission of a mitochondrial plasmid in a transient fusion between *N. intermedia* and *N. crassa* cells was reported.

Due to the conflicting reports on mtDNA transfer in fungi, we decided to reexamine the question of mitochondrial transmission in *C. parasitica*. Using mitochondrial and nuclear markers, we present evidence that mitochondria in *C. parasitica* can transfer between strains during hyphal anastomosis. Nuclear and mitochondrial markers were required to determine whether or not mitochondria could transfer during vegetative fusion. Pigmentation of *C. parasitica* has been described as a nuclear trait by Anagnostakis (1982) and, therefore, the light pigmentation phenotype of the dsRNA-free hypovirulent strain CL25 and the dark orange pigmentation phenotype of the vegetatively compatible virulent strain 4-C were used as nuclear markers.

A chloramphenicol-resistant (capR) strain of CL25 was isolated using the procedure of Brasier and Kirk (1986). The

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chloramphenicol-resistant isolate tolerated the prokaryotic-inhibiting antibiotic at relatively high concentrations (4 to 6 mg/ml) but a good distinction between capR and capS strains could be obtained at 8 mg of chloramphenicol per milliliter or greater. To determine that capR was cytoplasmic, reciprocal sexual crosses (Anagnostakis 1979) were made between CL25capR and 4-CcapS. Ascosporic cultures segregated 1:1 for the nuclear pigmentation gene, but only ascosporic cultures in which the maternal parent was chloramphenicol resistant were able to grow in the presence of chloramphenicol (8 mg/ml) demonstrating the cytoplasmic nature of the mutation.

To further help delineate mitochondria between the hypovirulent strain CL25capR and the virulent recipient strain 4-CcapS, the mtDNA of CL25 and 4-C were digested with restriction enzyme *Sau*3A. CL25 contained a unique fragment of about 2.5 kb, while 4-C contained two smaller bands not observed in CL25 (Fig. 1A). The presence of this fragment did not seem to correlate with the hypovirulence phenotype in CL25 since a virulent single-conidial isolate of strain CL25 also contained this band. The 2.5-kb *Sau*3A fragment was cloned into the *Bam*H1 site of the cloning vector pUC118. A clone of the 2.5-kb fragment of CL25 (pSK51) was used in Southern hybridizations (Maniatis et al. 1982) with CL25 mtDNA and other strains. Clone pSK51 hybridized to the 2.5-kb fragment in CL25 and to the two smaller bands in 4-C (Fig. 1B, lanes 1 and 4) and in other strains (data not shown). This restriction fragment length polymorphism pattern was subsequently used to detect CL25 and 4-C mtDNA after hyphal fusion.

To determine if mtDNA from CL25capR could transfer during vegetative contact to 4-CcapS, the two vegetatively compatible isolates were paired on solid culture medium. Cytoplasmic fusion was performed by placing inoculum plugs of the two isolates side-by-side approximately 5 mm apart on cellophane layered over potato-dextrose agar (PDA; Difco). The isolates were allowed to grow and fuse for 5 days. The cellophane and fusing cultures were lifted off the PDA and placed on PDA supplemented with 8 mg of chloramphenicol per milliliter. After pairing, subcultures from the 4-CcapS side of the pairing that grew on media amended with chloramphenicol were selected. Each subculture was divided and grown in Endothia complete broth (Puhalla and Anagnostakis 1971) with and without the addition of chloramphenicol (2 mg/ml). The mtDNA from these subcultures was extracted to determine the presence or absence of the RFLP associated with CL25 mtDNA. The mtDNA of subculture 4-C(H) had the mtDNA RFLP of both CL25 and 4-C when grown in the continuous presence of chloramphenicol (Fig. 1B, lane 3). When grown in broth without chloramphenicol, it appeared to have only the RFLP pattern of 4-C (Fig. 1B, lane 2). However, upon longer exposure of the same blot, the mtDNA RFLP of CL25 was also present in 4-C(H) (Fig. 1C, lane 2) indicating that the CL25 mtDNA RFLP was present in the 4-C(H) thallus even when selection pressure was eliminated. These results strongly suggest that mitochondria have moved from CL25 to 4-C via hyphal fusion. The increased intensity of the mitochondrial RFLP unique to CL25 when 4-C(H) was grown continuously in the presence of chloram-

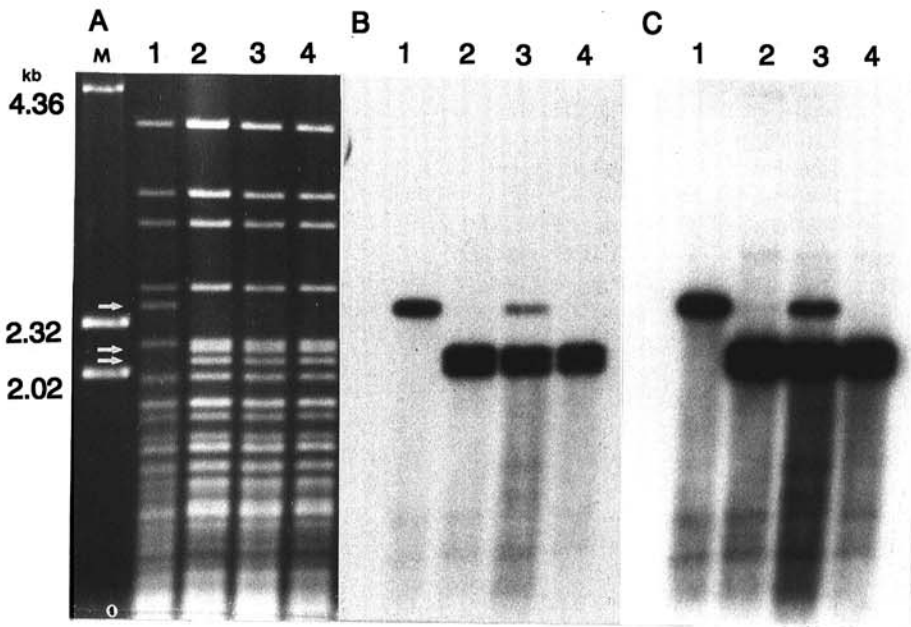

Fig. 1. *Sau*3A digestion pattern of mtDNA of different isolates of *Cryphonectria parasitica*. Lane 1, CL25 (donor). Lane 2, 4-C(H) grown in broth (without chloramphenicol). Lane 3, 4-C(H) grown in broth supplemented with chloramphenicol. Lane 4, 4-C (recipient). **A,** Ethidium bromide stained gel, M= λ DNA digested with *Hin*dIII used as molecular size marker. **B,** Southern blot of gel in A hybridized with 2.5-kb fragment clone of CL25 mtDNA ^{32}P-labeled using the random primer method (Feinberg and Volgestein 1983). Polymorphic bands in the gel are indicated with arrows. **C,** A longer exposure of the hybridized blot B. mtDNA was isolated following the procedure of Mackenzie et al. (1988). Each lane represents 25 g of fresh mycelium. The mitochondria were treated with DNase, pelleted by centrifuging, lysed, and digested. The mtDNA restriction fragments were separated by electrophoresis in 0.7% agarose gels using TBE buffer (Maniatis et al. 1982). Gels were electrophoresed at room temperature for 18 h at 35 to 40 V. The gels were ethidium bromide (0.5 μg/ml) stained and photographed. The restriction fragments on the gel were transferred to nylon hybridization membranes (MSI, Westboro, Mass.) as described by Maniatis et al. (1982).

phenicol is clearly due to the fact that chloramphenicol enhances the selection of the capR mitochondrial population within the 4-C(H) thallus.

To eliminate the possibility that the light-pigmented CL25 hyphae might be contaminating the pigmented strain, single-conidial isolates of a 4-C(H) subculture growing on chloramphenicol were selected. A small amount of mycelia was removed from the 4-C(H) subculture and placed in a tube of sterile distilled water. The tube was vortexed and a 10-fold serial dilution of the conidia was performed. Each dilution was plated onto PDA and incubated until individual colonies were observed, at which time 80 colonies were subcultured to separate plates containing PDA. All 80 single-conidial isolates were dark orange, indicating they had the 4-C nuclear type. Twelve of the dark-orange isolates were screened for their mtDNA RFLP pattern. Ten single-conidial isolates showed the mitochondrial RFLP banding pattern of 4-C and two showed the mitochondrial RFLP pattern of CL25, thus eliminating the possibility of hyphal contamination.

As previously reported (Mahanti et al. 1993), subculture 4-C(H) showed high levels of alternative oxidase and was hypovirulent in virulence tests on apple fruit (Fulbright 1984). Virulence analysis of the 12 single-conidial isolates of 4-C(H) demonstrated that five of these isolates were hypovirulent and seven of them were virulent. Thus, the mitochondrial genome and the factor responsible for the hypovirulence phenotype are capable of cotransfer during hyphal fusion.

The segregation of mtDNA RFLP patterns and the virulence phenotype in the single conidial isolates of 4-C(H) suggest that strain 4-C(H) is a heteroplasmon. The single-conidial isolates may be the result of nonreciprocal recombination between DNAs of the two different types of mitochondria in 4-C(H) and their subsequent vegetative segregation. Recently, Polashock and Hillman (1994) reported evidence for mitochondrial recombination in *C. parasitica*. Genetic studies of *Neurospora* and yeast have shown that recombination between mtDNA occur frequently in heteroplasmons. Ultimately a single mitochondrial type predominates, be it the either a parental type or recombinant type. When a recombinant type predominates, it can result in unidirectional gene conversion (Manella and Lambowitz 1978, 1979; Dujon 1980). Whether these or other genetic mechanisms are responsible for the dsRNA-free hypovirulent phenotype and the recently observed senescence-like phenotype (Huber et al. 1994) in this pathogen remains to be determined.

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