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.

**Keywords:** Mitochondria, Ribosomal RNA, Hypovirulence, Group II intron, Senescence, *Castanea dentata*

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,
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 recipient virulents 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 (Solyomo 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™ Miniprep 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 [a\(^{33}\)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](image_url). 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 EcoRI digested genomic DNAs was hybridized with a probe generated from the wild-type 10.5-kb HindIII 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 EcoRI 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 EcoRI 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 HindIII–EcoRI 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.
searched for related sequences by means of BLAST (Altschul et al., 1997).

The secondary structure models of the putative rns group II intron was generated with the online program mfold (http://www.bioinfo.rpi.edu/~zukerm/rna/; Zuker, 2003). However, constraints were applied to the folds based on predictions by the online program RNAweasel (Lang et al., 2007; http://megasan.bch.umontreal.ca/RNAweasel/) and RNA secondary models previously published by Fedorova and Zingler (2007), Michel and Westhof (1990), Michel and Ferat (1995), Michel et al. (1982, 1989, 2000, 2009), Pyle et al. (2007), and Toor et al. (2001).

2.5. Reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed with AMV-RT (Seikagaku, Inc., Falmouth, Massachusetts) as described by Chiochia and Smith (1997). All RNA samples were treated with one unit DNase I for 15 min prior to first-strand synthesis. First-strand synthesis was performed at 45°C for 10 min. Controls were reactions that lacked RNA or where the RT step (first strand synthesis by reverse transcriptase) was omitted. The sequences of the primers that were used are as follows: P1 = 5'-GTTGATTTGGTTGATGG-3' (sense primer); P2 = 5'-CAGTCACCTGTCACCA-C3' (anti-sense primer); P3 = 5'-CTGACGGAGAAAGA-3' (sense primer); P4 = 5'-TACTCTTTAGGTTG-3' (anti-sense primer). The locations of these primers on the template RNA are shown in Fig. 1A.

2.6. Ribosome profiles

The mitochondria that were used for the analysis of the composition of the mitochondrial ribosomes were purified by the floatation gradient method of Lizardi and Luck (1971) as modified by Lembowitz (1979). The approach described by Collins et al. (1979) was used for the direct quantitative determination of ribosomal subunits in mitochondrial lysates. The sucrose gradients were loaded with 500 µl of mitochondrial lysates each containing 5 mg of protein. Triton X-100 was used at a concentration of 1% instead of Nonidet P40 and the centrifugation time was increased from 3 to 4 h. After centrifugation, the gradients were analyzed by upward displacement through a flow cell with monitoring of absorbance at 254 nm in an ISCO gradient fractionator.

3. Results

3.1. Mapping and characterization of the mtDNA mutation in KFC9

On the basis of asexual transmission experiments involving four different virulent recipient strains, the most likely region of the mtDNA bearing the mutation that causes hypovirulence in the KFC9 strain of C. parasitica was previously identified as an 11.5-kb HindIII restriction fragment (Baidyaroy et al., 2000). In the mtDNAs of the virulent strains, the corresponding fragment was found to be only 10.5 kb in size. Similarly, restriction mapping with EcoRI revealed that senescent mycelia harboring KFC9 cytoplasm had a 10.3-kb fragment, whereas the corresponding fragment from wild-type control strains was only 9.3 kb long (Fig. 1 A and B). The adjacent EcoRI fragment, immediately upstream of the fragment bearing the insertion that segregated with the hypovirulence trait, was only 7.9 kb in size in KFC9 but 8.3 kb in all of the other strains examined. However, this RFLP was not transmitted along with the 10.5-kb EcoRI piece when the hypovirulence syndrome was transferred from KFC9 (Fig. 1A) (Baidyaroy et al., 2000). Thus, it appeared that the hypovirulence determinant is located within the 5.8-kb HindIII-EcoRI fragment of the KFC9 mtDNA, which corresponds to a fragment that is only 4.8 kb long in the wild type strains.

The 11.5-kb HindIII segment of mtDNA was cloned from KFC9 as well as from derivatives of several aggressive strains that had been converted to hypovirulence by hyphal contact with this strain. The corresponding segment of wild-type mtDNA was also cloned from Ep155 and Ep289. In conjunction with the sequencing and characterization of the mitochondrial ribosomal RNA region of Ep155 wild-type and mutant strains (Monteiro-Vitorello et al., 2009; AF029891), the current study revealed that the mtDNAs from the senescence-prone, hypovirulent isolates harboring KFC9 cytoplasm had an insert of 971 nucleotides in the first exon of the rns gene. The inserted sequence starts within the rns gene at a point located 69 base pairs downstream of the putative 5' end of the mature mitochondrial small subunit rRNA. This insert is uniquely associated with all the strains that display the KFC9-type of mitochondrial hypovirulence (Baidyaroy et al., 2000), and has been named InC9 (Insert in KFC9).

The nucleotide sequence of InC9 (GenBank accession no. AF218209) showed no similarity to other DNA sequences within the NCBI databases, and its translation in all six reading frames by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/; genetic code 4) did not reveal any evidence of an open reading frame. However, upon closer examination of the InC9 sequence with RNAweasel (Lang et al., 2007; http://megasan.bch.umontreal.ca/RNAweasel/), the presence of a signature sequence and fold for group II introns was detected (Fig. 2). Near the 3' end of InC9, a segment could be folded into the domain V structure characteristic of group II introns (Jarrell et al., 1988; Michel et al., 1989; Toor et al., 2001). A BLASTn search using the putative domain V sequence as a query also recovered several group II intron domain V sequences from fungi and plant mtDNA group II introns. Next to domain V, a sequence was detected that folds into domain VI, a domain that harbors the bulging adenine that provides a 2' OH group for initiating a series of transesterification reactions that results in the splicing of the flanking exon sequences and the release of the intron RNA as a lariat (Costa et al., 2000; Jacquier and Michel, 1987; Kück et al., 1990; Lembowitz and Zimmerly, 2004; Michel et al., 1989, 2009; Schmelzer and Müller, 1987; Toor et al., 2001; van der Veen et al., 1986). The 5' end of the intron starts with the characteristic GUGYG sequence (reviewed in Bonen and Vogel, 2001) and features such as the IBS1 and 2 and the corresponding EBS1 and 2 motifs (Bonen and Vogel, 2001; Michel et al., 1989) were also detected (Fig. 2). Thus, this 971-bp InC9 insert is a group II intron. On the basis of the RNA fold, this intron can be assigned to the group II A1 family of group II intron secondary structures, which are commonly encountered in fungal mitochondrial genomes (Michel et al., 1989; Toor et al., 2001). Based on the nomenclature for introns proposed by Johansen and Haugen (2001), where the number following the abbreviated scientific name refers to the location of the intron with respect to the Escherichia coli 16 S rRNA gene (GeneBank accession no. J01695), InC9 can be referred to as Cpa. mS62.

3.2. InC9 is transcribed but not readily spliced

In order to determine whether or not the Cpa. mS62 sequence is transcribed from the rRNA precursor, total mitochondrial RNA (mtRNA) was extracted from flotation-gradient purified mitochondria. Unfortunately, significant amounts of mtRNA could be extracted only from relatively healthy young cultures of KFC9 as the strain degenerates rapidly in culture. When equal amounts of mtRNA, rather than the RNAs from equal amounts of mitochondria, were separated by electrophoresis through agarose gels, it appeared that KFC9 had the same proportion of small- to large-subunit rRNA as the Ep155 wild type (Fig. 3A). Based on related
observations on the role of mutant mtDNAs in the senescence process in Neurospora (Bertrand, 1995, 2000), it can be surmised that healthier cultures of KFC9 have more wild-type mtDNA molecules than degenerated cultures. This notion is further supported by the observation that diseased KFC9 mycelia that still grow at a relatively rapid rate frequently are heteroplasmoids that contain wild-type as well mutant forms of mtDNA (Baidyaroy et al., 2000). Since the RNA was extracted from the mitochondria of relatively healthy KFC9 cultures, the profiles shown in Fig. 3A possibly reflect predominantly rRNA molecules that were transcribed from the wild-type mtDNA fraction in the heteroplasmoids. Thus, the experiment did not eliminate the possibility that rRNA precursor molecules that were transcribed from mtDNA templates containing InC9 (Cpa. mS62) were not processed sufficiently to produce rRNA molecules that could be assembled into small ribosomal subunits and may have been degraded. Since equal amounts of mtRNA, rather than the total amount RNA extracted from equal amounts of mitochondria, were applied to the gels, it is important to note that these experiments were not designed to detect whether or not the mitochondria of the mutant contained less mtRNA than the mitochondria from the virulent control.

When the northern blots of mtRNA gels were hybridized against a probe which was derived by PCR from the InC9 sequence, several species of RNA that were detected in KFC9 were missing in Ep155 (Fig. 3B). One of these relatively abundant RNAs is approximately 1.0 kb larger than the 1.9-kb mature rns transcript and appears to be an rRNA transcript that retained the InC9 ribonucleotide sequence. It should be noted that the rns gene in the EP155 strain is about 9872 bp long and contains four introns (Monteiro-Vitorello et al., 2009), and the rns exons comprise an estimated 1875 nucleotides. In addition to the 2.9-kb RNA, three relatively small RNA species also hybridized with the InC9 probe. The largest of these RNAs is approximately 1-kb long and thus might represent InC9 (Cpa. mS62) sequences that were spliced from at least a fraction of the precursor transcripts of the rns gene. However, the other two RNAs in this group are significantly shorter than 971 bp. All of these smaller RNA species could be produced either through premature termination of transcription within the InC9 insert, or by splicing events that remove only part of the InC9 sequence from the primary transcript. The latter possibility appears less likely than the former because rRNA molecules that retain part of the insert are not apparent on the northern blot. Even the 1-kb RNA species could
be produced either by transcription termination events that occur near the downstream end of the InC9 DNA, or by cleavage of the RNA near the 3’ end of the insert due to a defective splicing process.

To determine whether or not the InC9 sequence (Cpa. mS62) might be spliced slowly from precursors of the rns transcripts in strains that have the KFC9 cytoplasm, a series of RT-PCR experiments were executed. The first experiment was performed with a pair of primers (P1 and P2; Fig. 1B) that were homologous to the exon sequences flanking the InC9 element and templates consisting of equal amounts of mitochondrial RNA from Ep155 and cultures of KFC9 that were in senescence stages more advanced than the culture that provided the RNA shown in Fig 3A. Primers P1 and P2 were expected to produce a 215-bp product if the InC9 sequence is spliced from the rns transcript and a 1.2-kb product if InC9 is retained in the transcript (Fig. 4). As shown in Fig. 4, only the expected 215-bp product was generated when RNA from Ep155 was the template. In contrast, the RT-PCR reaction with the KFC9 RNA template generated both the 1.2-kb and the 215-bp products (Fig. 4). This result indicates that the Cpa. mS62 intron either is spliced slowly or not at all from the predominant class of the rns transcripts. Since the senescing mycelia of the KFC9 strain usually are heteroplasmic for the mutant and wild-type forms of mtDNA (Baidyaroy et al., 2000), it is likely that the 215-bp RT-PCR product seen in the fourth lane of the gel originated from remnant normal rns transcripts that persist in the mutant.

3.3. KFC9 can efficiently splice group II introns but not the InC9 element

To determine whether the KFC9 strain has a defect that affects mitochondrial RNA splicing in general or merely cannot remove the InC9 RNA (Cpa. mS62) segment from the primary transcript of the rns gene, RT-PCR was performed with two pairs of primers, P1 and P2, flanking the putative Cpa. mS62 intron and P3 and P4 (see Fig. 1B) flanking a downstream group II intron (Cpa. mS785; Toor and Zimmerly, 2002; Monteiro-Vitorello et al., 2009). The design of the primers was based on the complete nucleotide sequence of the rns gene of C. parasitica (GenBank accession: AF029891). Primers P3 and P4 flanking the Cpa. mS785 intron were expected to produce a 140-bp RT-PCR product from mature rns transcripts and a 2340-bp product from unspliced precursor rns molecules. Whereas significant amounts of the 1.2-kb product representative of unprocessed precursor rRNA were produced with primers P1 and P2 when KFC9 RNA was used as the template, primers P3 and P4, that flank intron Cpa. mS785 produced detectable amounts of the 140-bp PCR product but no detectable amounts of the 2340-bp product from the KFC9 and Ep155 RNA templates (Fig. 5). Thus, the InC9 RNA segment (Cpa. mS62) is spliced very slowly or not at all from the existing pool of rRNA precursor molecules, whereas the intact Cpa. mS785 group II intron RNA is spliced as efficiently in the mitochondria of the KFC9 mutant as in the mitochondria of the Ep155 wild type.

3.4. KFC9 is deficient in mitochondrial ribosome assembly

To determine whether or not the mitochondrial rns transcript that is produced in the KFC9 strain affects the assemblage of ribosomes, mitochondria were purified and incubated with puromycin to dissociate small from large subunits. The mitochondria were then lysed with Triton X100 and the lysates were loaded onto continuous sucrose gradients for the separation of large and small subunits by centrifugation. The amounts and positions of nucleoprotein particles in the gradients were determined by UV-absorbance (Fig. 6). KFC9 was found to be deficient in small as well as large subunits of mitochondrial ribosomes, whereas the wild type Ep155, grown under similar conditions, had normal amounts of both. The experiment was repeated five times to confirm the virtual absence of ribosomes from the KFC9 mitochondria. The deficiency in small subunits was anticipated from the results indicating that most of the rns transcripts are abnormal in the mutant, but the deficiency of large subunits was unexpected. Since equal amounts of puromycin-treated extracts of purified mitochondria were loaded onto the sucrose gradients, it is apparent that the organelles from the mutant contained much less of both mature ribosomal subunits than the organelles from the virulent control.
3.5. Transmission of InC9

The InC9 sequence appears to move from the mtDNA of KFC9-type donor strains into the mtDNA of virulent recipients through hyphal anastomoses that are formed during transient periods of contact between the mutant and wild-type mycelia (Baidyaroy et al., 2000). The region of KFC9 mtDNA that invariably appears in derivatives of wild-type strains that were converted to hypovirulence by hyphal contact with KFC9 is the 5.8-kb \(\text{EcoRI-HindIII}\) fragment that contains InC9 (Fig. 1A). The unique \text{EcoRI} site, which is located upstream in the mtDNA of KFC9, but is absent from the mtDNAs of the virulent strains that were used as recipients in the transmission experiments, was not transmitted with InC9 (Fig. 1B). To explore if InC9 moves on its own or in conjunction with at least some of the flanking mtDNA, segments that are located upstream and downstream of the insertion point of this element were cloned from several strains as \text{EcoRI-HindIII} fragments and sequenced. The goal was to discover sequence polymorphisms that could serve as markers to resolve this question. Relative to the KFC9 mtDNA, a single base deletion was found in the mtDNA of the virulent J2.31 strain at a point that is located 82 bp upstream of the insertion site for InC9 (Fig. 7). This polymorphism appears to be stable and innocuous in the sense that it was absent from all the other virulent strains that were used in this study. Hence, it was used as a marker to track the occurrence and extent of co-conversion of flanking sequences during the transfer of InC9. In the converted hypovirulent form of J2.31, the pertinent base-pair was present and the sequence resembled that of KFC9. In contrast, the RFLPs that are characteristic of the rest of the mtDNA of the virulent form of J2.31 were retained by the converted strain (Baidyaroy et al., 2000). Thus, it appears that some of the KFC9 upstream mtDNA is transmitted at least sometimes into the mtDNA of recipient strains along with the InC9 element. The lack of an appropriate genetic marker has hampered the determination of whether or not downstream mtDNA sequences also are transferred with the InC9 element. However, the results suggest that the conversion of virulent strains to the hypovirulent state is caused by the suppressive accumulation of mtDNA molecules that retain distant RFLP markers of the recipient type, but have incorporated InC9 together with an undetermined length of flanking mtDNA by recombination with mtDNA molecules they had acquired from a KFC9 donor by transfer through hyphal anastomoses.

4. Discussion

4.1. The InC element is a group II intron that is implicated in hypovirulence and senescence

Molecular events that are associated with fungal hypovirulence are of interest for gaining a better understanding of the diverse genetic and physiological factors that are involved not only in the appearance of this phenotype, but also in its persistence in natural populations of \textit{C. parasitica} and other pathogenic fungi (Allen and Nuss, 2004; Bertrand and Baidyaroy, 2002; Dawe and Nuss, 2001; Hoeegger et al., 2003; Monteiro-Vitorello et al., 2000; Nuss, 2005). Previously it was shown that the infectious hypovirulence phenotype of the virus-free KFC9 strain of \textit{C. parasitica} might be
caused by a “suppressiv” mtDNA mutation (Baidyaroy et al., 2000). KFC9 was isolated from a healing canker on a tree in the Kellogg Forest in Michigan and showed a senescence phenotype that is associated with respiratory deficiencies. In this study, we have located the modification in the mtDNA that most likely represents this mutation, and have shown that it was generated by the insertion of a 971-bp group II intron (Cpa. mS62) within the first exon of the rns gene with reference to that of the Ep155 virulent strain as characterized by Monteiro-Vitorello et al. (2009). The evidence presented previously (Baidyaroy et al., 2000) and in this study suggests that integration of the InC9 sequence into the rns gene, either by intron transposition or by recombination, produces a “suppressiv” mitochondrial mutation that most likely debilitates the fungus by blocking mitochondrial protein synthesis through interference in the assembly of mitochondrial ribosomes.

As previously reported (Baidyaroy et al., 2000), the genetic element that causes hypovirulence in the isolates of C. parasitica at the Kellogg Forest site can be transmitted by hyphal contact from senescent and debilitated isolates to virulent strains. Such transfers result in the conversion of virulent recipients to the hypovirulent state, which is accompanied by the progressive degeneration of the mycelia characteristic of fungal senescence. These observations confirm that InC9 is a “suppressiv” mtDNA mutation. In the filamentous fungi, suppressiveness is attributed to the gradual displacement of normal mtDNA molecules by mutant forms resulting in the gradual disappearance of functional organelles and extinction of the wild-type phenotype (Bertrand, 1995, 2000). While the mechanism that causes the gradual displacement of wild-type mtDNA by deleterious mutant forms in growing mycelia, i.e. “suppresiveness”, is the subject of some controversy (Albert and Sellem, 2002; Jamet-Vierny et al., 1999), at least in Neurospora it has been shown that the phenomenon is manifested as senescence elicited by mtDNA deletions (Bertrand et al., 1980; de Vries et al., 1981; Gross et al., 1984) and insertions of plasmids (Akins et al., 1986; Bertrand, 2000; Bertrand et al., 1986; Cahan and Kennell, 2005; Court et al., 1991; Griffiths, 1992; Kennell and Cohen, 2004) that disrupt cytochrome-dependent electron-transport activity. In this context, it is likely that the mitochondria of the diseased strains from the Kellogg Forest site have a proliferative advantage over normal mitochondria because the KFC9 mitochondria are functionally crippled by the InC9 element. Thus, the “renegade” mutant mitochondria of the diseased strains can “infect” virulent strains through hyphal anastomoses and convert them to the hypovirulent state through the aggressive invasion of the mycelia by the mutant mitochondria or mtDNAs.

The InC9 sequence is an ORF-less group II intron that may have lost its ability to self-splice efficiently, possibly because it lacks an intron encoded maturase. Although group II introns are self-splicing in vitro, it is assumed that in vivo the process is expedited by intron-encoded maturases and/or host factors (Lambowitz and Zimmerly, 2004; Michel and Ferat, 1995; Saldanha et al., 1999). Degeneration of intron core sequences and structures is accompanied by an increased reliance on host factors for splicing (Hausner et al., 2006; Toor et al., 2001), but it is possible that host factors that can compensate for the missing Cpa. mS62 maturase either do not exist or cannot effectively process the defective intron in any of the C. parasitica strains that were used in this study.

Even though inefficient splicing of the Cpa. mS62 intron from the primary rns transcript readily explains the deficiency in the small ribosomal subunits, it does not provide a rationale for the near absence of the large ribosomal subunits in the mitochondria of mycelia of naturally occurring and converted KFC9 strains. One possible explanation would be that the mtDNA of C. parasitica contains a gene that specifies a protein (or maturase) that is required for the splicing of the rnl intron (mL2449) encoding the ribosomal protein RPS3 (Bullerwell et al., 2003; Hausner, 2003; Hauser et al., 1999; Sethuraman et al., 2009), thus the lack of properly assembled small ribosomal subunits might prevent the synthesis of proteins involved in the maturation of the rnl transcript or proper assembly of the large ribosomal subunits. Another possibility is that the stability of the large subunit somehow is linked to the assembly or presence of small subunits. Neither of these possibilities has been explored experimentally at this time.

4.2. Persistence of the InC9 element within populations

Repeated sampling of C. parasitica populations in cankers on chestnut tress at the Kellogg Forest site indicates not only that the InC9 mutation has been retained during the past two decades, but also that the proportion of isolates that have InC9 in their mtDNAs and have the mitochondrial hypovirulence phenotype has grown steadily and has surpassed the 90% mark (Baidyaroy et al., 2000). So far, the observations gathered from the Kellogg Forest strongly support the conclusion that InC9 provides a natural control over chestnut blight because the mtDNAs containing the defective intron are maintained and transmitted like infectious agents within the population of C. parasitica at that site.

As expected for a mutation that causes a particular effect, the InC9 element appeared in all the derivatives of virulent strains that had acquired hypovirulence by transient contact with mycelia that had the KFC9 cytoplasm. Moreover, the senescence traits emerged after short periods of vegetative propagation in virtually all of the single conidal isolates of the recipient type that were recovered from zones where virulent strains made contact with the mycelium of a vegetatively compatible KFC9-type donor. During this conversion process, the mycelia that were generated from individual conidia approached homoplasmy for mtDNA molecules of the recipient type into which the InC9 sequence was inserted. These effects could mean that InC9 is a site-specific mobile genetic element that actively invades the mtDNA of virulent recipients. However, the fact that at least some of the donor mtDNA that is immediately adjacent to InC9 also appeared in converted strains suggests that recombination is involved in the transfer of the InC9 sequence into the mtDNA of the recipients. The pattern of movement of InC9 involving the co-transfer of nearby upstream markers, possible via a recombination dependent pathway, is what one would expect for the movement of mobile introns (Bonen and Vogel, 2001; Hamari et al., 2001, 2002; Lambowitz and Belfort, 1993; Lambowitz and Zimmerly, 2004; Schafer, 2003; Sellem et al., 1996). Moran et al. (1995) showed that yeast group II introns lacking reverse transcriptase activity can move based on a DNA-level recombination mechanism. There is also the possibility that InC9 is mobilized by retrotransposition facilitated by transacting factors provided by a related reverse transcriptase-encoding group II intron. Moran et al. (1995) demonstrated that retrotransposition of some yeast group II introns is accompanied by efficient, but highly asymmetric, co-Conversion of nearby flanking exon sequences. The movement by retrotransposition would require at least a limited, albeit undetected, amount of splicing of the InC9 sequence from the primary rns transcript. Regardless of whether or not InC9 is mobile, the acquisition of even a very small amount of mtDNA that contains a suppressive mutation by a virulent mycelium can trigger its gradual conversion to the hypovirulent state. It is conceivable that the critical dosage of mutant mtDNA that can initiate the conversion process may be provided by a single mitochondrion, especially if the mutation is a defective intron that is still mobile and can spread into wild-type rns genes but is spliced rather inefficiently from the corresponding rRNA transcripts.

The ribosomal DNA that contains InC9 element probably is maintained in the population of C. parasitica in the Kellogg Forest because there is a flow of mitochondria through hyphal anastomo-
ses from the diseased clones into healthy clones and vice versa. It can be anticipated that a genetic equilibrium will be established eventually in local populations under natural conditions (Burt, 2003). Contributing to the establishment of this equilibrium might be factors such as the production of asexual spores, which are decidedly more abundant on healthy mycelia than the senescent mycelia of the Kellogg Forest strains. Sexual transmission of the lnC9 mitochondrial mutation so far has not been observed in the laboratory, mostly because senescent mycelia do not function as female parents in crosses. However, the maternal inheritance of C. parasitica mutant mtDNAs has been observed in the laboratory (Monteiro-Vitorello et al., 1995), and circumstantial evidence indicates that the KFC9 hypovirulence phenotype may be transmitted sexually within the genetically extremely heterogeneous population of the fungus at the Kellogg Forest site (Baidyaroy et al., 2000). From a practical perspective, maternal inheritance in a natural ecological setting would endow mitochondrial hypovirulence with an advantage as a biocontrol agent over virus-mediated hypovirulence because dsRNA hypoviruses usually are not transmitted through sexual spores (Anagnostakis, 1988; Nuss, 1992). Thus, mitochondrial hypovirulence potentially can be used as an alternative or a supplement to mycoviruses to control C. parasitica and other phytopathogenic fungi.

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