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dissertation entitled

Genetic Elements Involved in Mitochondrial Hypovirulence in the Chestnut Blight Pathogen Cryphonectria parasitica

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany

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Date January 5, 2000

GENETIC ELEMENTS INVOLVED IN MITOCHONDRIAL HYPOVIRULENCE IN THE CHESTNUT BLIGHT PATHOGEN *CRYPHONECTRIA PARASITICA*

By

Dipnath Baidyaroy

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

2000

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ABSTRACT

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By

Dipnath Baidyaroy

Cryphonectria parasitica, an ascomycetous fungus, is the causal organism of the chestnut blight disease that has resulted in the virtual decimation of American chestnut trees in the north-eastern United States over the past century. Apart from virulent strains of the fungus, hypovirulent strains that are incapable of causing disease also have been isolated in nature. The hypovirulence syndrome of these strains is cytoplasmically transmissible and can be caused either by the presence of double-stranded RNA viruses, or by debilitating mitochondrial factors. This study presents an analysis of two types of genetic factors, namely mutant forms of mitochondrial DNA and a mitochondrial plasmid, that give rise to mitochondrial hypovirulence and are responsible for the spread and persistence of such syndromes in nature. To elucidate the role of the mtDNA in the natural occurrence of mitochondrial hypovirulence, abnormalities in a virus-free, hypovirulent strain, KFC9, were examined. The debilitating mutation in this strain was found to be an insertion of a 973-bp nucleotide sequence of unknown function and origin, located in the first exon of the mitochondrial small subunit ribosomal RNA gene, only 67-bp downstream of its 5' terminus. This element, named InC9, was cytoplasmically transmitted among strains in perfect correlation with the hypovirulence phenotype.

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Molecular analyses revealed that the segment of RNA corresponding to InC9 was not spliced efficiently or accurately from the precursor transcript of the mt-SrRNA, but true introns were spliced effectively. This abnormality resulted in a defect in the assembly of mitochondrial ribosomes. Apart from InC9, the mutant strains sometimes also contained high amounts of a small, circular, mitochondrial plasmid-like element, named pleC9. Only a relatively small portion of the pleC9 DNA was homologous to the mitochondrial DNA, and the 1.3-kb element did not have a sizable open reading frame. However, it was determined that pleC9, despite negatively affecting the growth of the strains on synthetic medium, did not by itself have an effect on virulence, because isolates that had this element, but lacked InC9, remained virulent. The *bona fide* mitochondrial plasmid pCRY1 was also found to incite hypovirulence in *C. parasitica*, but only in a strain-specific manner. The plasmid, and probably other infectious mitochondrial elements, was found to be cytoplasmically transmitted across vegetative incompatibility barriers. The plasmid does not appear to cause a mitochondrial dysfunction, and the mechanism by which it elicits hypovirulence remains unclear. In an effort to understand the mechanism by which pCRY1 causes hypovirulence, as well as to establish the requirements of DNAs that might be used as vectors in future mitochondrial transformation experiments, the replication mechanisms of mitochondrial plasmids were analyzed by two-dimensional gel electrophoresis and electron microscopy. It was determined that mitochondrial plasmids that encode a DNA polymerase normally replicate through a rolling circle mechanism initiating from multiple origins, whereas plasmids that encode reverse transcriptases can replicate by reverse transcription, or by a rolling circle mechanism from fixed origins.

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ACKNOWLEDGEMENTS

I am indebted to several individuals who have had an impact on me and my research. First of all, I thank my advisor Dr. Helmut Bertrand for his guidance and encouragement throughout this work, as well as for his generous patience and material support. Dr. D.W. Fulbright provided valuable scientific counsel and helped immensely in collection and sampling of fungal strains from nature. I would also like to thank Dr. L. Snyder and Dr. J.D. Walton for serving on my committee, participating in many helpful discussions and critically reviewing manuscripts that helped to substantially improve this dissertation. I am extremely grateful to Dr. Georg Hausner for his assistance in every aspect of this work. Thanks are due to the members of the Bertrand laboratory, namely Claudia B. Monteiro-Vitorello, Katherine Nummy, David Huber, Denise Searles, Jonathan Glynn and Tak Ko. Finally, I wish to express my heart-felt gratitude to my family, and especially to my wife Monica, without whom this would have never been accomplished.

Primary financial support for this work was provided by Unites States Department of Agriculture and the Michigan Agricultural Experiment Station. Funding from the Sigma Xi organization and the A.L. Rogers Fellowship is greatly appreciated. The Department of Botany and Plant Pathology also contributed financial support through several teaching assistantships.

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CHAPTER 1

Literature Review

Introduction. *Cryphonectria parasitica* (Murr.) Barr, a facultative saprophyte, is a filamentous ascomycetous fungus in the order Diaporthales. The fungus was initially named *Diaportha parasitica* Murrill, and described as a fungus causing a disease on chestnut trees by Murrill (1906). Subsequently, Anderson and Anderson (1912) transferred the species to the genus *Endothia*. Barr (1978) afterward transferred *Endothia parasitica* and four other species of *Endothia* to the genus *Cryphonectria*. However, some studies (Griffin *et al.*, 1986) do not agree to this transfer of the *Endothia* species to *Cryphonectria*, whereas others (Micales and Stipes, 1987) are in agreement. According to Barr's classification (1978), the genus *Endothia* is in the family Gnomoniaceae (subfamily Mamianioideae, tribe Endothieae) while *Cryphonectria* is placed in the family Valsaceae (subfamily Valsoideae, tribe Diaportheae). The account of the fungus, as proposed by Barr (1978), has been the popular preference among researchers in recent times when describing the fungus.

Cryphonectria parasitica reproduces asexually through uni-nucleate conidia (Puhalla and Anagnostakis, 1971) produced in pycnidia developed in the stroma. Sexual reproduction results in formation of ascospores that are produced within perithecia. Dispersal of conidia, which are extruded as sticky masses on tendrils, has been

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documented on insects, birds and mammals (Anagnostakis, 1987), whereas dispersal of conidia by wind is not thought to be significant. However ascospores, which are ejected from perithecia, may be wind dispersed. The mating system of the fungus includes both outcrossing and self-fertilization, and this unusual biological phenomenon of multiple paternity also has been demonstrated in the laboratory (Anagnostakis, 1982a). A study by Milgroom *et al.* (1993), of the outcrossing rate found that natural populations did exhibit a mixed mating system (both outcrossing and self-fertilization). A comparison of the genetic diversity of *C. parasitica* in China with the North American population using restriction fragment length polymorphisms showed greater genetic diversity in China than in North America, as would be expected due to founder effects in the North American population (Milgroom *et al.,* 1992). The genetic diversity of populations has also been examined using vegetative incompatibility groups. In this case, diversity has been found to be high in some North American populations (Anagnostakis and Kranz, 1987; Milgroom *et al.*, 1991).

Cryphonectria parasitica is a wound-infecting pathogen of chestnut trees. Despite its devastating effects on American (*Castanea dentata*) and European (*C. sativa*) chestnut trees, the pathogen has only mild effects on the oriental trees (*C. mollissima, C. crenata).* Hence, it has been suggested that the pathogen originated in Asia since endemic areas usually contain host populations that coexist with native pathogens. It was hypothesized that the fungus made its way to North America as a weak pathogen of the Chinese chestnut . However, Milgroom *et al.* (1996) recently demonstrated that, while the pathogenic species may have originated in China, the predominant strains that have invaded North America were from Japan or another Asian country where pathogen

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Most infections caused by *C. parasitica* occur either at the base of the stems or on stems at natural branch scars where the scars lead to the vascular cambium (Fulbright, 1999). However, not much is known about the nature and volume of inoculum necessary to cause disease. The finding that trees with more than one canker generally harbor clonal populations of the pathogen suggests that these infections occur through conidia rather than by outcross-produced ascospores (Milgroom *et al.,* 1991). However, some studies (Baidyaroy *et al.*, 2000) have shown that strains from multiple canker-bearing trees can be genetically different. This observation indicates that ascospores probably also are instrumental in the spread of the disease.

There are no known toxins or secondary metabolites produced by the fungus which are directly responsible for the death of the stem. Previous studies suggesting toxin involvement have not been corroborated. The disease is generally thought to be a disease of the phloem, however the actual cause of the death of the plant tissue and of the stem is still not known. Once infection has been initiated, it appears that mycelial fan formation, a dense mat-like growth of the fungus, is essential for enlargement of the canker (Hebard *et al.,* 1984). Most studies have focussed on the colonization of the outer bark, vascular phloem, and outer xylem. Yet, the initial symptom is chlorosis and wilting of the leaves, suggesting the involvement of the xylem. Ewers *et al.* (1989) found a dramatic reduction in fluid conductance around artificially inoculated and natural infections with a strong correlation to the death of leaves distal to the infection. They also found evidence that

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death of the vascular cambium is involved in the death of leaves and concluded that any survival of the vascular cambium may result in the survival of the leaves. McManus *et al.* (1989) reported extensive colonization of the xylem rings associated with natural and artificial infections. This finding indicates that the pathogen is not restricted to the vascular cambium and outer xylem.

As for any fungal plant pathogen, several cell wall degrading enzymes have been isolated from *C. parasitica* and their individual roles in pathogenesis evaluated. Targeted disruption of *enpg-1*, the gene that encodes a major extra-cellular endopolygalactouronase, did not decrease the levels of polygalactouronases in the mutant, indicating that this enzyme might not play an important role in pathogenesis (Gao *et al.,* 1996). However, the identification of two other acidic polygalactouronase activities expressed predominantly, if not exclusively, *in planta* provides new opportunities for examining the importance of polygalactouronases in *C. parasitica* pathogenesis. Another enzyme that might be important in pathogenesis is an acid proteinase, named endothiapepsin. So far, four different genes encoding different forms of this enzyme have been identified (Razanamparany *et al.*, 1992; Choi *et al.,* 1993; Jara *et al.* 1996). However their roles in pathogenesis remains to be ascertained. In addition, extracellular cellulase activity has been implicated in contributing to the pathogenic potential of the fungus. A gene *chb-1*, which encodes a cellobiohydrolase (exoglucanase) has been characterized and was found to be down-regulated in strains that are incapable of causing disease (Wang and Nuss, 1995). Similarly, a cutinase gene, which has been associated with pathogenicity in other plant pathogenic fungi, was found to be down-regulated in strains that did not cause disease (Varley *et al.*, 1992).

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Several compounds produced by the fungus, namely oxalic acid, laccase and a hydrophobin called cryparin, have been implicated in pathogenesis and disease resistance. Oxalic acid binds with cations and is thought to affect host tissues by lowering the pH of the host-cell environment. Oxalic acid may also act in combination with pectic enzymes in the digestion of calcium-pectate of the middle lamella (Vannini *et al.*, 1993). The fungus produces large amounts of oxalic acid in culture, but conflicting reports of its concentration in host tissues leave the role of this compound in doubt. The enzyme laccase was found to be down-regulated in *C. parasitica* strains that do not cause disease (Choi *et al.*, 1992) and hence, has been implicated in pathogenicity. In other fungi, laccase has been associated with fungal sporulation (Leatham and Stahmaan, 1981), pigmentation (Clutterbuck, 1972), lignin degradation (Kirk and Shimada, 1985), and pathogenesis (Marbach *et al.*, 1985). However, mutations that caused reduced laccase activity had no significant effect on fungal pigmentation, sporulation, or virulence (Rigling, 1995). The compound cryparin also is thought to be concerned with pathogenicity because it is down-regulated in strains that are incapable of causing disease (Carpenter *et al.*, 1992; Zhang *et al.*, 1994). Cryparin belongs to a group of fungal hydrophobic cell surface proteins and has sequence similarity to cerato-ulmin, a phytotoxin produced by *Ophiostoma ulmi*, the cause of the Dutch elm disease.

History of chestnut blight. The demise of the American chestnut as a result of blight caused by *Cryphonectria parasitica* early in this century is familiar to plant pathologists as a classic example of a plant disease epidemic caused by the introduction of an exotic organism. Detailed accounts of the origin, progression, and consequences of the blight

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epidemic have been described in several reviews (Anagnostakis, 1982b; Anagnostakis, 1987; MacDonald and Fulbright, 1991; Fulbright, 1999). The first report of chestnut blight in North America appeared in 1904 and described symptoms on American chestnut trees located within the New York Zoological Gardens (Merkel, 1906). The causal fungus moved rapidly through the New York and Pennsylvania countryside destroying most of the trees in the eastern parts of these and other nearby states by 1912 (Hepting, 1974). The causative fungus, *C. parasitica*, was apparently introduced into North America on nursery stock of resistant oriental chestnut species. Gradually the blight spread throughout the natural range of the American chestnut, which extended from Maine to Alabama and west to the Mississippi river, destroying several billion mature trees within a 50-year period (Anagnostakis, 1982b).

Chestnut was the dominant forest tree in the Appalachian mountain states including Pennsylvania, West Virginia, Kentucky, Tennessee and North Carolina. In these states, the American chestnut was in its greatest numbers and largest size, growing to heights of 120 feet with basal diameters of 12 feet or greater (Kuhlman, 1978). At the end of the epidemic, a host population estimated at between 3-4 billion mature trees was devastated, dead, or surviving only as understory stump sprouts that harbored the pathogen until the sprout died and again resprouted (Hepting, 1974). This is because the root systems of the diseased trees often remain unaffected and continue to generate new shoots. However, since the shoots are susceptible, they rarely reach maturity. Consequently, the American chestnut now survives as an understory shrub where once it was the dominant species.

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Since the American chestnut was the major component of the eastern hardwood forest and served as an important source of timber, tannin, and food for human and animal consumption, the ecological, economic and even social consequences of the blight disease were understandably severe (Paillet, 1982, 1984). Its value to the southern Appalachian region was incalculable as in some regions 25% of the trees harvested in the forest were chestnut.

A brief account of the history of the chestnut blight disease in Europe is also necessary to gain a complete understanding of this epidemic. The blight disease was first reported on European chestnut trees (*Castanea sativa* Mill.) in 1938 near Genoa, Italy (Woodruff, 1946). The European chestnut, which is similar to the North American chestnut trees in size and appearance, proved to be equally susceptible to the blight. Consequently, European chestnut farmers also suffered great losses as the disease spread (Pavari, 1949).

Transmissible hypovirulence in the chestnut blight pathogen. Biraghi (1953) first recorded the observations that eventually led to the discovery of transmissible hypovirulence by describing several examples of trees that were surviving in spite of being blighted. Rather than the deeply indented cankers normally found on dying, blight-infested trees, he noted superficial cankers that appeared to be in the process of healing. However, it was thought at that time that it was the trees that were resistant to the fungus. Later, French pathologists discovered that the unusual symptoms described by Biraghi were not due to a resistant variety of chestnut, but were a consequence of infection by altered forms of *C. parasitica* (Grente, 1965; Grente and Sauret, 1969; Grente and Berthelay-Sauret, 1978). These investigators found that the fungal cultures obtained from

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the surviving trees consisted of two different morphs: one that appeared normal in culture, i.e., produced an orange pigmentation, sporulated abundantly, and were virulent, and a new form that was significantly reduced in pigmentation (white) and sporulation. When re-inoculated onto chestnut, the white isolates produced small, superficial cankers that were incapable of killing trees. The margin of the resulting cankers callused and the infection site became swollen from wound periderm. The canker ultimately closed resulting in the appearance of abnormal cankers (Fulbright *et al.,* 1983). It was this observation that led to the introduction of the term 'hypovirulence' as a descriptive phrase for these new isolates (Grente, 1965).

A significant observation regarding these strains was that the hypovirulent strains had a curative effect when inoculated on to existing cankers (Grente and Sauret, 1969). The application of hypovirulent strains resulted in the conversion of resident virulent strains into hypovirulent forms (Grente and Berthelay-Sauret, 1978). The observation that virulent strains could be converted to hypovirulence in the laboratory following hyphal anastomosis with a hypovirulent strain indicated that the hypovirulence phenotype was transmissible and suggested the involvement of a cytoplasmic genetic determinant. Subsequently, natural spread of hypovirulence resulted in a corresponding improvement of chestnut trees in Italy to a point where the disease was no longer a problem in the cultivation of chestnut in that country (Mittempergher, 1978).

Reports of the successful control of chestnut blight in Europe as a result of natural dissemination (Italy) or artificial application of hypovirulent *C. parasitica* strains (France) (Grente and Berthelay-Sauret, 1978) stimulated efforts to examine whether transmissible hypovirulence might be effective in controlling blight in North America.

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Initial reports indicated that the European hypovirulent strains indeed could cure cankers incited by virulent North American strains of the pathogen (Anagnostakis and Jaynes, 1973; Van Alfen *et al.*, 1975). Starting with an isolate from surviving chestnut trees in Michigan (Anagnostakis, 1982b; MacDonald and Fulbright, 1991), researchers subsequently isolated native North American hypovirulent strains were from several sites, and collected evidence for ongoing biological control in these locations (Fulbright *et al.*, 1983; Garrod *et al.*, 1985).

However, initial attempts to control chestnut blight in North America by the artificial introduction of hypovirulent strains did not result in successful attenuation of the disease progress (Anagnostakis, 1982b; Griffin, 1986; MacDonald and Fulbright, 1991). Although clear evidence for the conversion of virulent strains to hypovirulent forms on contact was obtained, biological control by the introduced hypovirulent strains was not sustainable. Among numerous reasons, one clear factor that contributed to this failure was the vegetative incompatibility system that controls the ability of different *C. parasitica* strains to undergo anastomosis. So far, seven nuclear genes (*vic* genes) have been implicated in the control of vegetative incompatibility in this fungus (Huber, 1996; Cortesi and Milgroom, 1998). Two strains are vegetatively compatible and freely undergo stable anastomoses and exchange cytoplasm if they have the same alleles at each of these loci. The ability to form viable anastomoses decrease as the number of dissimilar alleles increase. Since the hypovirulence phenotype does not appear to be transmitted to virulent strains during mating (Anagnostakis, 1982b, 1988), but only during anastomosis with strains of closely related vegetative compatibility groups, the complexity of vegetative compatibility structure within the fungal population is expected to impact the

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dissemination and persistence of the introduced hypovirulence. Consistent with this prediction, diversity in vegetative compatibility groups was found to be significantly higher in North American populations of the fungus than in the European populations (Anagnostakis *et al.*, 1986). Moreover, reduced fitness and reduced sporulation in many of the hypovirulent strains could negatively affect the spread of hypovirulence in a natural environment (Elliston, 1985).

The first clear indication of the physical nature of the cytoplasmic determinant of hypovirulence in *C. parasitica* was provided by Day *et al.* (1977). Several hypovirulent strains were shown to contain double-stranded RNA (dsRNA) elements which were absent in the virulent strains. It was subsequently shown that the conversion of a virulent strain to its corresponding hypovirulent form was concomitant with the transmission of dsRNA elements (Anagnostakis and Day, 1979). Elimination of dsRNA molecules from a hypovirulent strain following treatment with cycloheximide is accompanied by restoration of the virulence phenotype (Fulbright, 1984). These studies, as well as numerous other findings, have provided correlative evidence to suggest that the cytoplasmic genetic elements responsible for the induction of the hypovirulence phenotype in *C. parasitica* consist of cytoplasmically replicating dsRNA molecules.

Apart from dsRNA-mediated hypovirulence in *C. parasitica*, another form of hypovirulence in this fungus has been reported (Fulbright, 1985, 1990). In this case, the hypovirulent strains do not seem to contain any dsRNA elements. However, these dsRNA-free hypovirulent isolates demonstrate high levels of cyanide-resistant respiration indicating that the bulk of their respiratory activity is mediated by the nuclear-encoded mitochondrial alternative oxidase rather than the cytochrome-based electron transport

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system (Mahanti *et al.*, 1993). The fact that the alternative oxidase mediated pathway is induced in the dsRNA-free hypovirulent strains implies that whatever deficiency is present in these isolates must be related specifically to a deficiency in cytochrome-mediated respiration and to the mitochondria in general. The hypovirulence syndrome, along with the altered respiratory phenotype, can be transferred from dsRNA-free hypovirulent strains to virulent strains following hyphal anastomosis. In addition, transmission studies in *C. parasitica* have shown that mitochondrial DNA (mtDNA) can be transferred from one strain to another by hyphal contact (Mahanti and Fulbright, 1995; Polashock *et al.*, 1997). These findings suggest that a cytoplasmic genetic factor other than dsRNA, and probably located in the mitochondria, might be involved in generation of the hypovirulence phenotype. Mechanisms of induction of hypovirulence mediated by dsRNA molecules and by mitochondrial elements are discussed in the following sections.

Hypovirulence mediated by dsRNA elements. Since most mycoviruses have genomes composed of dsRNA (Buck, 1986; Ghabrial, 1994), several studies were initiated to isolate virus particles from hypovirulent *C. parasitica* strains. However, the hypovirulence-associated dsRNA elements are unencapsidated and instead are associated with membranous vesicles (Dodds, 1980; Newhouse *et al.*, 1983; Hansen *et al.*, 1985) which contain an RNA-dependent RNA polymerase activity that probably functions in replication of the dsRNA (Fahima *et al.*, 1993). Like mycoviruses, hypovirulence-associated dsRNAs are not infectious in the classical sense, i.e., cell-free preparations are not infectious when applied to fungal hyphae or spheroplasts and new infection can only occur by hyphal anastomoses (Nuss, 1992). Surveys of the dsRNAs that are associated

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with different hypovirulent strains revealed considerable variations in the concentration, copy number, size and genetic composition of their components (Anagnostakis and Day, 1979; Dodds, 1980; Elliston, 1985; Tartaglia *et al.*, 1986; Shapira *et al.*, 1991b; Hillman *et al.*, 1992). Even, a single strain can contain more than one type of dsRNA (Smart and Fulbright, 1995). The most common types of dsRNAs have recently been assigned to a new virus family, the Hypoviridae (Hillman *et al.*, 1994). Members of the Hypoviridae are cytoplasmic and contain a single dsRNA element of 10-13 kb that is phylogenetically most closely related to single-stranded RNAs of the plant virus family Potyviridae. However, dsRNA elements have been also identified in *C. parasitica* (Polashock and Hillman, 1994) and in *Ophiostoma novo-ulmi* (Hong *et al.*, 1999) which are, unlike the others, mitochondrial in location and closely related to the yeast cytoplasmic T and W dsRNAs (Polashock and Hillman, 1994). Many strains, apart from containing the full-length dsRNA elements, contain smaller dsRNA elements, which are related to the largest dsRNA in their respective cytoplasms. These smaller elements appear to be the result of internal deletions in the large dsRNA elements (Shapira *et al.*, 1991a).

Mainly three different types of cytoplasmic dsRNA viruses have been found so far in *C. parasitica* and are designated as CHV1, CHV2 and CHV3 (Hillman *et al.*, 1994) because they all share some common characteristics (Fulbright, 1999). All are large linear molecules without structural proteins and are polyadenylated at their 3' termini (Hillman *et al.*, 1992; Tartaglia *et al.*, 1986; Hiremath *et al.*, 1986). CHV1-Ep713 was the first member to be characterized. It is 12,712 base pairs (bp) in length with two open reading frames (ORFs) designated as ORF A and ORF B, which overlap by a single base pair (Nuss, 1992). ORF A encodes two proteins, named p29 and p40, with p29 being a

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protease which is auto-catalytically released from the ORF A polypeptide during translation (Choi *et al.*, 1991). Transformation of *C. parasitica* with ORF A and subsequent expression of p29 and p40 therein resulted in the transformants showing some of the phenotypes associated with hypovirulence, including loss of pigmentation, reduced sporulation, and a reduction in laccase activity without loss of virulence (Craven *et al.*, 1993). Apart from encoding another protease (p48) which is similar to the ones encoded by ORF A, the polyprotein encoded by ORF B contains motifs for an RNA-dependent RNA polymerase and an RNA helicase (Nuss, 1992). Transformation of virus-free, virulent *C. parasitica* strains with a cDNA copy of the CHV1 virus resulted in the manifestation of the transmissible hypovirulence phenotype (Choi and Nuss, 1992) which was now meiotically stable (Chen *et al.*, 1993). The CHV2 virus is almost identical to the CHV1 type with the only major difference being that the ORF A protein in CHV2 does not undergo autoproteolysis and does not produce an active protease (Hillman *et al.*, 1992; Hillman *et al.*, 1994). On the other hand, the CHV3 type, which is 9 kb in length, is distinctly different with no sequence similarity to either of the CHV1 or the CHV2 dsRNAs. It consists of only one ORF with polymerase and helicase domains (Durbahn, 1992).

The effect of the dsRNA viruses on *C. parasitica* was first analyzed by Powell and Van Alfen (1987a, 1987b), and later by Kazmierczak *et al.*, (1996). They showed that, as a result of viral infection, many fungal transcripts and their protein products were down-regulated. Subsequently, down-regulation in virus-containing strains of several elements thought to be necessary for pathogenesis was reported. These include potential virulence factors like polygalactouronases (Gao *et al.*, 1996), cutinase (Varley *et al.*,

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1992), laccase (Rigling *et al.*, 1989; Rigling and Van Alfen, 1991) and oxalic acid (Havir and Anagnostakis, 1983; Vannini *et al.*, 1993). However, some of these putative virulence factors were later found not to be of specific importance, as the disruption of these genes did not lead to a reduced virulence phenotype (Gao *et al.*, 1996). Some other compounds that have been found to be down-regulated by the virus include the cell surface hydrophobin cryparin (Carpenter *et al.*, 1992; Zhang *et al.*, 1994) and a pheromone precursor (Zhang *et al.*, 1998) that is thought to be essential for sexual reproduction (Zhang *et al.*, 1993).

Studies on transcriptional repression of the laccase (*lac-1*) gene due to the presence of dsRNA viruses resulted in interesting discoveries. It was found that lac-1 was regulated by two different and opposing regulatory pathways: a negative control pathway limiting transcript accumulation that requires ongoing protein synthesis, and a stimulatory pathway that is dependent on the inositol triphosphate and calcium second messenger systems (Larson *et al.*, 1992). The finding that the hypovirulence-associated virus interferes with the transduction of an inositol triphosphate-calcium-dependent signal, thereby repressing *lac-1* expression, led to the conclusion that the dsRNA elements interferes with cellular processes by actively perturbing signal transduction pathways (Larson *et al.*, 1992). Consequently, studies on GTP-binding proteins (G-proteins) in *C. parasitica* were initiated since they are a family of regulatory proteins that play an essential role in the response of eukaryotic cells to many environmental stimuli (Gilman, 1987). It was found that viral infection of *C. parasitica* results in reduced accumulation of the G-protein α subunit CPG-1 (Choi *et al.*, 1995). Transgenic co-suppression (Choi *et al.*, 1995) and targeted disruption of *cpg-1* (Gao and Nuss, 1996)

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also led to the hypovirulence phenotype as seen in virus-containing strains. Unlike *cpg-1*, disruption of another gene encoding a G-protein α subunit, named *cpg-2*, did not affect virulence (Gao and Nuss, 1996). However, targeted disruption of the G-protein β subunit gene (*cpgb-1*) also resulted in significant reduction in pigmentation, sporulation and virulence (Kasahara and Nuss, 1997). Thus, these studies suggested that at least one mechanism for hypovirus interference with virulence is by disruption of one of the key components of fungal signaling (Nuss, 1996).

Some hypovirulent strains contain a dsRNA element that is very different from the dsRNA molecules of the family Hypoviridae. In fact, upon sequencing of this dsRNA element, a sizable ORF was obtained only when the mitochondrial codon usage was applied (Polashock and Hillman, 1994). Molecular analyses revealed that this particular element was localized in the mitochondria and more related to the T and W dsRNAs of yeast than to the known hypovirulence-associated dsRNA viruses. That this mitochondrial element can be potentially more persistent than its cytoplasmic counterparts because it is maternally inherited in sexual crosses (Polashock *et al.*, 1997). In contrast, the fungus is usually cured of cytoplasmic mycoviruses through sexual crosses (Nuss, 1992). In addition, whereas vertical transmission of the cytoplasmic dsRNAs through conidia can be very inefficient (Russin and Shain, 1985), the mitochondrial element is transmitted in conidia with 100 % efficiency (Polashock *et al.*, 1997). However, the molecular mechanisms responsible for elicitation of the hypovirulence phenotype by the presence of the mitochondrial dsRNA remains to be investigated.

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Senescence and hypovirulence mediated by mitochondrial elements. Degenerative mycelial symptoms, accompanied by slow growth and hypovirulence phenotypes resulting from dysfunctional mitochondria has been found in several filamentous fungi. The reasons of mitochondrial malfunction has been attributed to a variety of causes ranging from mtDNA mutations and accumulation of plasmid-like elements, to disruption of essential mitochondrial genes by insertion of extra-genomic mitochondrial plasmids (reviewed in Wolf and Del Guidice, 1988; Dujon and Belcour, 1989; Kück, 1989; Griffiths, 1992). That these phenotypes are essentially transmissible by hyphal anastomosis has been shown in *Neurospora* (Pittenger, 1958; Manella and Lambowitz, 1978), *Podospora* (Marcou and Schecroun, 1959), *Ophiostoma* (Brasier, 1983) and *C. parasitica* (Monteiro-Vitorello, 1995).

Most mitochondrial mutations are known to be suppressive to wild type. This means that mutant mitochondrial genomes gradually replace wild-type molecules in heteroplasmons. This phenomenon provides for the unique opportunity to employ mitochondrial mutations in bio-control of fungal populations given the infectious properties of mutant mitochondria. Jinks (1966) pointed out that suppressiveness was to be expected in mitochondrial mutants of filamentous fungi because a new mutation will affect only a single mtDNA molecule. Hence, the mutation will pass unnoticed unless it is dominant to wild type. Several modes have been suggested to explain this unusual phenomenon of suppressiveness (Bertrand, 1995). One model suggests that mutant mitochondrial chromosomes are able to replicate more efficiently than wild-type molecules because they have more or more efficient origins of replication and smaller genomic size, which is often the case (Blanc and Dujon, 1980; Almasan and Mishra,

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1988). However, this model does not adequately account for the induction of suppressiveness by point mutations and insertions (Bertrand *et al.,* 1986). In this respect, a model proposed by Bertrand (1995) more cogently explains this discrepancy. Bertrand notes that suppressiveness is associated with only those mitochondrial mutations that inhibit electron transport and thereby adversely affect oxidative phosphorylation. Thus, mutant mitochondria will accumulate in a strain provided the mutation inhibits the electron transport system. This notion gains credence from the fact that even wild-type mitochondria can be induced to multiply at a faster rate by chemical inhibition of oxidative phosphorylation (Bertrand, 1995).

Cytoplasmically-transmissible degenerative syndromes have been observed and well analyzed in filamentous fungi, namely *Podospora*, *Aspergillus*, *Ophiostoma* and *Neurospora*, and are known as 'senescence'. In most cases, mitochondria have been implicated. To better understand the processes involved in the induction of these phenotypes, a brief discussion of these systems is presented in the following section.

Senescence in *Podospora anserina*. *Podospora anserina* strains degenerate after continuous growth on solid medium and the life span of a culture is known to be a strain-specific trait (Marcou, 1961; Esser and Tudzynski, 1980). This phenomenon has been known as 'senescence' and has been shown to be a maternally inherited trait (Marcou, 1961; Smith and Rubenstein, 1973). However, several other factors, which can be nuclear, cytoplasmic or even environmental, seem to play a role in the process of senescence in this fungus. For example, genes that influence translational accuracy (Silar *et al.,* 1999), a copper-modulated transcription factor (Borghouts *et al.,* 1997), a

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mitochondrial plasmid (Hermanns and Osiewacz, 1996), the allele of the mating-type locus (Marcou, 1961), several other nuclear genes (Tudzynski and Esser, 1979), and even growth conditions (Marcou, 1961) can alter the life-span of a strain.

Often the same *Podospora* strains can have different life spans based on culture conditions. Cultures that are maintained on media with certain carbon sources such as acetate have prolonged life spans (Tudzynski and Esser, 1979). Strains that are maintained in liquid medium do not senescence and, when transferred to solid medium, often have longer life spans than strains maintained entirely on solid media (Turker and Cummings, 1987). Degenerating cultures of *Podospora* can be rejuvenated by storage at 4°C, or by sexual crosses (Marcou, 1961). Several compounds are known to prolong life spans in this fungus. Mycelial growth in the presence of sub-lethal levels of inhibitors, such as the DNA-intercalating drug ethidium bromide (Tudzynski and Esser, 1979; Belcour and Begel, 1980; Koll *et al.*, 1984), the mitochondrial protein synthesis inhibitor streptomycin, respiratory chain inhibitor mucidermin (Tudzynski and Esser, 1979), and antioxidants like reduced glutathione (Munkres and Rana, 1978) can increase the life-span of a culture.

The molecular biology of the senescence mechanisms in *Podospora* has been investigated in detail (for reviews, see Wolf and Del Guidice, 1988; Dujon and Belcour, 1989; Griffiths, 1992). Upon comparison of mtDNA between juvenile and senescent strains, it was found that the senescent mycelia contained numerous small, circular molecules (Dujon and Belcour, 1989; Griffiths, 1992). The circular molecules comprised a multimeric series in which a reiterated sequence is arranged in head-to-tail fashion and were named 'senDNA'. These senDNA molecules can be of heterogeneous sizes, and the

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different types are named α, β, γ, δ, ε or ω according to the region of the mitochondrial genome from which they are derived (Dujon and Belcour, 1989; Griffiths, 1992). However, senDNA α, which corresponds exactly to the first intron of the *cox1* gene (Osiewacz and Esser, 1984), is involved in most cases of senescence in *Podospora*. In the senescent cultures, this group II intron is excised, amplified and circularized. The long ORF on this senDNA encodes a protein that has homologies with retroviral and retrotransposon reverse transcriptases, as is true of the ORFs of other class II fungal mitochondrial introns (Michel and Lang, 1985). Transcripts corresponding to the senDNA α are observed (Wright and Cummings, 1983; Kück *et al.*, 1985) and an RNA-dependent DNA polymerase activity has been also reported from senescent, but not juvenile *Podospora* cultures (Steinhilber and Cummings, 1986). Incidentally, senDNA β is also not thought to be replicated by reverse transcriptase-mediated mechanisms and has been shown to be equally responsible for the senescence phenotype as senDNA α (Jamet-Vierny *et al.*, 1997a). However, contradictory findings regarding the role of senDNA β has been also reported (Jamet-Vierny *et al.*, 1999). The complicity of the senDNA α is evidenced by the entire or partial loss of the first intron of the *cox1* gene in the longevity mutants of *P. anserina*, which are believed to have increased life spans due to a lack of the senDNA α (Belcour and Vierny, 1986; Schulte *et al.*, 1988, 1989). In other longevity mutants, namely *mex3* and *mex6*, the regions of mtDNA that give rise to the other types of senDNAs are rearranged (Koll *et al.*, 1987). In addition, nuclear double mutants that interfere with the excision of senDNA α (*gr viv*) or its amplification (*i viv*) are also long-lived (Tudzynski *et al.*, 1982). In summary, senescence in *Podospora anserina* is

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However, contrary views exist regarding the role of senDNAs in the induction of senescence in *Podospora*. Although the loss of the first intron of the *cox1* gene in the longevity mutants may be responsible for the long-life phenomenon, their increased life-spans could be due to a lack of cytochrome aa_3 (Belcour and Vierny, 1986; Sainsard-Chanet and Begel, 1990) with the mutants respiring by the alternative oxidase pathway (Schulte *et al.*, 1988). Moreover, there are several senescing *Podospora* strains that do not accumulate any senDNAs. For example, cytosolic ribosomal mutations cause senescence without the concomitant appearance of senDNAs (Silar *et al.*, 1997), propagation of a senescence phenotype is controlled by translational accuracy (Silar *et al.*, 1999), and senescent strains of *Podospora curvicolla* do not have either the first intron of the *cox1* gene or the autonomously replicating senDNA α (Böckelmann and Esser, 1986). In addition, the observation that senDNA molecules could be transferred to a young culture without the co-transmission of the senescence phenotype and that the determinant of senescence did not segregate as a mtDNA mutation suggested that the senDNAs are probably only a consequence of senescence rather than being the causal agent (Jamet-Vierny *et al.*, 1999). Mutations in nuclear DNA resulted in either escape from senescence (Borghouts *et al.*, 1997; Contamine and Picard, 1998) or elicitation of senescence with accompanying amplification of senDNA molecules (Jamet-Vierny *et al.*, 1997b). By analyzing mutants generated by insertional mutagenesis of nuclear genes, Rossignol and Silar (1996) found that a large number of nuclear genes can actually modulate life span in this fungus. Thus, it appears that senescence is triggered by an

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event, e.g., a mutation, but is manifested probably because of the accumulation of mutant mtDNAs. Collectively, these particular observations implicate nuclear factors over senDNAs in the induction of senescence in *Podospora anserina.*

Senescence in *Aspergillus amstelodami.* Vegetative death (*vgd*) mutants of *Aspergillus amstelodami* have been described that demonstrate many of the features of senescent strains of *Podospora* and *Neurospora*, including slow growth-rates, conidial death, and suppressive cytoplasmic inheritance (Caten, 1972). Unfortunately the *vgd* mutants have not been examined at a molecular level, but a less severe version, named ragged (*rgd*) has been studied at a molecular level in *A. amstelodami* (Lazarus *et al.,* 1980; Lazarus and Küntzel, 1981). The *rgd* mutants were found to contain small, circular, amplified elements derived from two specific regions of the mtDNA (Lazarus and Küntzel, 1981). The amplified elements are usually present in a concatameric series comprised of circular head-to-tail oligomers of a unit length of DNA. The size of the unit length DNA was variable in each mutant, but the few molecules that were analyzed shared a common region. This situation is reminiscent of the senDNA β amplifications of *Podospora.* However, mechanisms by which these small plasmid-like elements mediate senescence in *Aspergillus* remains to be investigated.

Mitochondrial disease in *Ophiostoma.* *Ophiostoma novo-ulmi* is the causal pathogen of the Dutch elm disease that was first noticed in Holland as a wilt disease on elm trees in the early part of this century. It spread rapidly from Europe to Great Britain (1927), the United States (1930) and Canada (1945) and killed millions of elm trees in the process

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(Brasier, 1991; Hubbes, 1999). However, it was noticed that instead of one, there have been two pandemics of the disease in this century which were mediated by two different forms of fungal pathogen (Gibbs and Brasier, 1973). The two forms were subsequently characterized and the aggressive group was renamed *Ophiostoma novo-ulmi* whereas the non-aggressive form was retained as *O. ulmi* (Brasier, 1991).

A disease syndrome, very much like the hypovirulence phenotype observed in *C. parasitica*, was observed in *O. novo-ulmi* (Brasier, 1983). Diseased isolates have slow-growing, unstable colonies with an abnormal 'amoeboid' morphology and produce few viable conidia (Brasier, 1983, 1986). The disease phenotype was cytoplasmically transmissible. Experiments in which the nuclei of diseased and healthy isolates were genetically marked have shown that the diseases are transmitted by cytoplasmic genetic elements, which were termed 'd-factor' (Brasier, 1983, 1986). Subsequently, it was found that the d-factors were comprised of dsRNA elements (Rogers *et al.*, 1986) which are faithfully transmitted with the disease to healthy isolates. Moreover, loss of dsRNA elements results in the reversion to the healthy phenotype. However, out of the 10 different dsRNA molecules discovered initially in a diseased isolate, only three molecules correlated with the disease phenotype indicating that some of the dsRNAs might not have any effect on the fungus (Rogers *et al.*, 1986). A later study (Sutherland and Brasier, 1997) analyzed the potential of thirteen different dsRNA elements in causing disease and has reported that the effects varied considerably from mild to moderate to severe.

The dsRNA elements co-purified with mitochondria (Rogers *et al.*, 1987) and sequence analyses of the dsRNA molecules revealed a similarity to the mitochondrial dsRNA virus of *C. parasitica* (Hong *et al.*, 1999). Characterization of the different

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dsRNA elements and cross-hybridization studies revealed that out of the initial 12, only four different dsRNA genomes could be identified with the others probably being satellites (Cole *et al.*, 1998; Hong *et al.*, 1998b, 1999). These four complete viruses replicate independently of each other and all of them contain conserved motifs characteristic of RNA-dependent RNA polymerases (Hong *et al.*, 1998a; Hong *et al.*, 1999).

However, Rogers *et al.*, (1987), while initially investigating the localization of the dsRNA elements, had found that the mitochondria of the diseased isolates were deficient in cytochrome aa_3. This is a phenomenon that has been associated with mitochondrial dysfunction as witnessed in *Podospora* (Belcour and Vierny, 1986; Sainsard-Chanet and Begel, 1990) as well as in *C. parasitica* (Monteiro-Vitorello *et al.*, 1995). Subsequently, Charter *et al.*, (1993) reported the presence of small, circular plasmid-like DNA elements in the mitochondria of the diseased strains. These elements were derived from different regions of the mtDNA. Nucleotide sequence analysis of one of the plasmid-like elements indicated that it is derived by recombination between two long repeat sequences in the mitochondrial large subunit ribosomal RNA gene (Abu-Amero *et al.*, 1995). The plasmids were not transmitted to sexual progeny. Asexual transmission of the disease phenotype did not accompany transmission of the plasmid-like elements from the donor to the recipient isolate. Instead, it resulted in de-novo generation of a novel set of plasmids derived from the recipient's mtDNA (Charter *et al.*, 1993). Thus, it appears that the mitochondrial plasmid-like elements are a consequence of stress on the mitochondria (as a result of the presence of dsRNA elements) rather than the causal agent of the disease phenomenon. This observation is in conformity with the notion that the senDNAs of

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Podospora, or the plasmid-like elements in the mitochondrially hypovirulent mutants of *C. parasitica*, are not the causal agents of senescence phenotypes but are an outcome of mitochondrial stress.

Senescence in *Neurospora*. Senescence in *Neurospora* can be broadly categorized into two classes: one that is caused by mtDNA mutations resulting from integration of plasmids into the mitochondrial genome and one that is not plasmid-induced. Induction of senescence phenotypes by mitochondrial plasmids has been characterized extensively and will be discussed in a separate section. Apart from that, nuclear mutations that affect mitochondria and result in mitochondrial dysfunction are also known in this fungus. The nuclear natural death (*nd*) mutant appears to destabilize the mitochondrial genome through hyperactive recombination in the mtDNA (Seidel-Rogol *et al.*, 1989; Bertrand *et al.*, 1993). This recessive, pleiotropic mutant has phenotypic and molecular defects similar to those seen in senescence, including deficiencies in cytochromes aa_3 and *b*, and accumulation of gross rearrangements, and large deletions in the mitochondrial genomes of vegetatively propagated mycelia.

Several different mtDNA mutations have been reported in *Neurospora*. While some of them were reported to be non-suppressive, like the [*mi-3*] mutant which resulted as a missense mutation in the *cox1* gene (Lemire and Nargang, 1986; Hawse *et al.*, 1990), most of the mutations were dominant (suppressive) over wild-type. For instance, in data combined from two separate studies, 38 of 42 heterokaryons constructed between the wild-type and the mutant [*poky*] acquired the mutant phenotype (Manella and Lambowitz, 1978, 1979). However, the [*poky*] mutant cannot be described as senescent

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as the strain, despite having a slow-growth phenotype, does not senesce and die. Thus, it appears that not all mtDNA mutations result in senescence irrespective of whether the mutation is suppressive or not. The mutation in the [*poky*] strain was detected as a 4 bp deletion in a conserved region of the 19SrRNA promoter that reduces promoter activity drastically (Kennell and Lambowitz, 1989). Consequently, aberrant 5' ends were detected in residual [*poky*] 19S rRNA transcripts (Akins and Lambowitz, 1984) which are probably initiated from an upstream promoter or promoters (Kennell and Lambowitz, 1989). Akins and Lambowitz (1984) observed this same deletion in several other mitochondrial mutants, namely the [*exn*] and the [*SG*] strains, that do not complement the [*poky*] mutation in a heterokaryon (Bertrand and Pittenger, 1972). Lemire *et al.*, (1991) found an alteration in the *cox2* gene in the [*exn-5*] mutant that results in a deficiency of cytochrome c oxidase subunit 2.

Secondary effects on the mitochondrial genome often complicate the phenotypes of these mutants. The most common effect is manifested as amplification of derivatives of mtDNA. Thus, even though the causal mutation of the senescence phenotype in a few [*poky*] remains the 4 bp deletion, variant forms of mtDNAs have been reported in [*poky*] strains (Manella *et al.*, 1979). The aberrant forms are maintained as circles that vary in size. The smaller molecule is usually present as head-to-tail concatamers. A similar situation exists for the [*SG-3*]-*551* mutant that has the 4 bp deletion and also contains an amplified region of the mtDNA that is approximately 20 kb in size (Bertrand *et al.*, 1980). These observations indicate that the accumulation of these aberrant derivatives of mtDNA is probably a consequence of the mutation-induced stress on mitochondrial function.

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The 'stopper' mutants of *Neurospora*, which have a characteristic stop-start growth phenotype, are also known to contain populations of defective mtDNA molecules that vary in concentration depending on whether the mutant is senescing or not. In most cases, the defective mtDNA populations of stopper mutants consist of large circular deletion derivatives (Almasan and Mishra, 1988, 1990; Bertrand *et al.*, 1980; DeVries *et al.*, 1981; Gross *et al.*, 1984). However, stopper mutants can also contain abnormal mtDNAs resulting from inversions as seen in the mutant [*SG-1*] (Srb, 1958; Infanger and Bertrand, 1986). Similar to the senDNAs of *Podospora anserina* and the plasmid-like elements in *Aspergillus amstelodami* and *Ophiostoma novo-ulmi*, the circular stopper DNAs can also vary in length and can be arranged as head-to-tail concatamers. However, the stopper DNAs are usually very large in comparison to the abnormal molecules of *Podospora*, *Aspergillus* and *Ophiostoma*, and generally comprise of a 20-kb core region that includes the ribosomal RNA genes, the *coIII* gene, the *nd6* gene and most of the mitochondrial tRNA genes. Smaller mtDNA-derived plasmid-like elements that vary in length from approximately 140 to 400 base pairs have also been reported in stopper mutants (Gross *et al.*, 1989b).

Some stopper mutants, similar to the [*poky*] mutants, can have more than one aberrant form of mtDNA, as seen in the mutant [*E35*] (DeVries *et al.*, 1985, 1986a,b) where the larger and the smaller molecules contain 5-kb and 40-kb deletions, respectively. The regions of deletions in the two populations of molecules are mutually overlapping and include the *ndh2* and *ndh3* genes. Consequently, the strain is deficient in NADH dehydrogenase activity. The deletion breakpoints of the two subgenomic mtDNA molecules of stopper [*E35*] are located at the base of stem-loop structures formed by

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different GC-rich palindromes, which are common in intergenic spaces (Yin *et al.*, 1981), indicating that the ligation of ends arising from single or double-stranded breaks at these points produced these aberrant molecules (Bertrand, 2000). Another method of generation of aberrant mtDNA forms by intramolecular recombination involving two directly repeated $tRNA^{Met}$ sequences was surmised by Gross *et al.*, (1984, 1989a,b) in yet another stopper mutant. Almasan and Mishra (1991) found a 9-bp repeat element that was associated with hair-pin structures and was involved in intramolecular recombination events thereby generating suppressive or residual mtDNA molecules. Thus, the molecular characterization of the stopper mutants suggest that, in contrast to the accumulation of full-sized mutant mtDNA in the [*poky*] mutant, preferential increases of subgenomic molecules in the mitochondria can contribute to senescence.

Plasmid-mediated senescence in *Neurospora*. Although mitochondrial plasmids are found in many genera of filamentous fungi (reviewed in Meinhardt *et al.*, 1990; Fecikova, 1992; Griffiths, 1995; Kempken, 1995), they are probably best studied in the genus *Neurospora*. Both linear and circular plasmids have been detected in *Neurospora* (Griffiths, 1995). Whereas linear plasmids generally contain genes that encode DNA and RNA polymerases (Chan *et al.*, 1991; Court and Bertrand, 1993) and in some cases reverse transcriptases (Walther and Kennell, 1999), circular plasmids contain genes that either encode a DNA polymerase (Li and Nargang, 1993) or a reverse transcriptase (Nargang *et al.*, 1984; Akins *et al.*, 1988). Despite the apparent ubiquity of mitochondrial plasmids in fungi (Griffiths, 1995), there exists a lack of understanding regarding their function. In many cases where plasmids have been detected in pathogenic fungi, the

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hypothesis has been entertained that these elements are responsible for specific pathogenic properties of plasmid-containing strains in comparison with strains carrying no plasmid. This idea has been invalidated in several fungi, namely *Fusarium oxysporum* (Hirota *et al.*, 1992; Momol and Kistler, 1992) and *Rhizoctonia solani* (Miyashita *et al.*, 1990; Jabaji-Hare *et al.*, 1994). However, a reverse-transcriptase encoding circular plasmid from *Alternaria alternata* (Kaneko *et al.*, 1997) has been linked with toxin production (Katsuya *et al.*, 1997) despite the lack of understanding of the mechanism by which this association is manifested.

The first fungal phenotype shown to be produced by an extragenomic plasmid is senescence in *Neurospora* spp. (reviewed in Dujon and Belcour, 1989; Griffiths, 1992). The linear plasmids of *Neurospora*, namely the Kalilo and the Maranhar plasmids are invertrons (Sakaguchi, 1990) with long terminal inverted repeats and contain two genes that encode a DNA and an RNA polymerase (Bertrand *et al.*, 1985, 1986; Myers *et al.*, 1989; Vierula *et al.*, 1990; Chan *et al.*, 1991; Court *et al.*, 1991; Court and Bertrand, 1992, 1993). Both plasmids trigger the process of senescence by integration into the mitochondrial genome, thereby disrupting essential genes (Bertrand *et al.*, 1985, 1986; Myers *et al.*, 1989, Chan *et al.*, 1991; Court *et al.*, 1991). Integration of the entire plasmid occurs at various points in the mitochondrial genome and generates very large inverted repeats of mtDNA flanking the insert (Dasgupta *et al.*, 1988). The Kalilo plasmid integrates with a loss of no more than 20 bp from each end by matching 5 bp from anywhere within the last 20 bp or so at its terminus with an identical quintet in the mtDNA (Chan *et al.*, 1991). The Maranhar plasmid also probably integrates by a similar mechanism (Court *et al.*, 1991). The integration event often leads to a suppressive

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mtDNA mutation and during subsequent growth, the mutant mtDNA accumulates at the expense of the wild-type molecule in the culture. Direct confirmation of the suppressive nature of such integration events comes from the demonstration that the plasmid-mediated senescence phenotype is readily transmitted in heterokaryons with normal strains of *N. crassa* and *N. intermedia* (Griffiths *et al.*, 1990).

Circular plasmids of *N. crassa* (Collins *et al.*, 1981; Nargang *et al.*, 1984) and *N. intermedia* (Akins *et al.*, 1988) that encode reverse transcriptases also are known to cause senescence by ectopic integration into the mitochondrial genome (Dujon and Belcour, 1989; Griffiths, 1992). In plasmid-containing senescent strains, defective mtDNAs were detected, some showing deletions and some containing insertions of a portion of the plasmid DNA into the mtDNA where the mtDNA-plasmid junction always corresponded exactly to the 5' end of the plasmid transcript (Akins *et al.*, 1986; Akins and Lambowitz, 1990). In addition, the free plasmids in these senescent strains were found to be altered in the sense that they had acquired mtDNA additions, most commonly a mitochondrial tRNA at or near their 5' ends (Akins *et al.*, 1989). Furthermore, a plasmid reverse transcriptase activity was detected in these strains and the protein was subsequently purified (Kuiper and Lambowitz, 1988; Kuiper *et al.*, 1990). These findings suggested that the insertion process, as well as the generation of the free mutant plasmids, probably occur through an RNA intermediate.

The circular plasmids that encode DNA polymerases, namely the LaBelle (Stohl *et al.*, 1982; Pande *et al.*, 1989) and the Fiji plasmids (Stohl *et al.*, 1982; Li and Nargang, 1993) have so far not been found to be associated with any senescence phenotypes in these fungi. However, a substantial part of the LaBelle plasmid has significant sequence

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homology with the *Neurospora* mtDNA (Nargang *et al.,* 1992). This suggests that part of the LaBelle plasmid was inserted into the mtDNA, a process reminiscent of induction of senescence events by plasmid integration as seen for other plasmids. However, since the homologous region in the mtDNA was interrupted by insertions, and some strains that contained this region did not contain the free plasmid, it can be argued that the integration event is probably an ancient one with no deleterious consequences (Kempken, 1995). The observation of this homology of mtDNA with the LaBelle plasmid, nevertheless, points out to the fact that probably the DNA polymerase-containing mitochondrial plasmids are also biologically capable of eliciting senescence phenotypes upon integration.

Mitochondrial hypovirulence in *Cryphonectria parasitica*. The term 'mitochondrial hypovirulence' has been used to describe the hypovirulence syndromes associated with mitochondrial dysfunctions owing to reasons other than dsRNA elements. In some strains of *C. parasitica* from Michigan, the hypovirulence syndrome was demonstrated to occur despite the conspicuous absence of any dsRNA element (Fulbright, 1985; Mahanti *et al.,* 1993; Huber *et al.*, 1994). All of these strains showed high levels of alternative oxidase activity (Mahanti *et al.*, 1993; Huber, 1996), which is symptomatic of blockages in the mitochondrial cytochrome-mediated respiratory pathway (Lambowitz and Slayman, 1971; Lambowitz and Zannoni, 1978; Vanlerberghe and McIntosh, 1997). In addition, since the hypovirulence syndrome as well as the altered respiratory phenotype were transmissible through hyphal anastomosis (Huber, 1996; Monteiro-Vitorello *et al.,* 1995), the causative element for the hypovirulence phenomenon in these strains were putatively assigned to be located in the mitochondria.

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The discovery that alternative oxidase is induced in the virus-free hypovirulent strains suggested that the attenuation in virulence was associated with a respiratory deficiency which could be caused by a mutation in the mtDNA. Similar syndromes have been observed in other filamentous fungi and have been described as 'senescence' (Griffiths, 1992; Bertrand, 1995). To assess whether mtDNA mutations can actually incite the transmissible hypovirulence phenotype in *C. parasitica*, Monteiro-Vitorello *et al.*, (1995) generated mtDNA mutations in the standard virulent wild type Ep155 strain. The mutants exhibited slow-growth symptoms, were hypovirulent on apples, chestnut bark and trees, and had elevated levels of alternative oxidase activity. Moreover, the hypovirulence and the altered respiratory trait were cytoplasmically transmissible and maternally inherited in sexual crosses. Investigation of the mtDNA of these mutants revealed structural abnormalities represented by highly amplified, plasmid-like DNAs. In addition, the mitochondria of the hypovirulent mutants were deficient in cytochrome *a*. These results suggested that mtDNA mutations can indeed result in the transmissible hypovirulence syndrome as seen in some strains from nature (Fulbright, 1985; Mahanti *et al.*, 1993; Huber, 1996). Therefore, the virus-free type of hypovirulence in the chestnut blight pathogen, which is accompanied by elevated levels of alternative oxidase activity, has been termed 'mitochondrial hypovirulence' (Monteiro-Vitorello *et al.*, 1995).

In an effort to better comprehend the nature of mtDNA mutations that can lead to the infectious hypovirulence syndromes, Bell *et al.*, (1996) generated a physical map of the *C. parasitica* mtDNA. In addition, a mitochondrial plasmid, named pCRY1, was also detected in several strains of *C. parasitica* (Bell *et al.*, 1996) that appeared to be unstable in culture. Mitochondrially hypovirulent strains of *C. parasitica* have been isolated from

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recovering American chestnut trees in Michigan and Ontario (Mahanti *et al.*, 1993; Huber, 1996; Fulbright, 1999) where mitochondrial hypovirulence appears to be as effective a control of chestnut blight as hypoviruses. A virus-free strain, named KFC9, isolated from a healing canker on an American chestnut tree in the Kellogg Forest, Augusta, Michigan, demonstrated cytoplasmically transmissible hypovirulence phenotype (Huber, 1996). Strains from that location also showed high levels of alternative oxidase activity (Mahanti *et al.*, 1993). The contribution of the mitochondrial plasmid or the natures of the mtDNA mutations that elicit hypovirulence phenotypes in these strains remain to be characterized.

Dissertation content. Several aspects regarding the nature of the causal agent and the viability of mitochondrial hypovirulence remain to be investigated. The overall goal of this project was to determine the nature of mtDNA mutations that can give rise to transmissible hypovirulence syndromes in *Cryphonectria parasitica*, and the feasibility of using these mutations as biological control agents for this fungus in nature. Specifically, the aims of this study were:

1. to determine and characterize the nature of mtDNA mutations and abnormalities associated with the virus-free, mitochondrially hypovirulent strain KFC9 (Huber, 1996),
2. to characterize the spread of this mutation, and hence mitochondrial hypovirulence, in the Kellogg Forest area from which the KFC9 strain was

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isolated, to gain an understanding of the nature of spread and persistence of similar syndromes in nature,

3. to analyze the horizontal movement of mitochondrial elements, namely mitochondrial plasmids and mtDNA, across vegetative incompatibility barriers through hyphal anastomoses, in order to comprehend the dynamics and biological capability of transfer and spread of mitochondrial elements in natural populations.

4. to analyze the mechanisms of replication of circular mitochondrial plasmids of filamentous fungi, and of mtDNA, in an effort to better comprehend the mechanisms by which some plasmids cause senescence without integrating into the mtDNA, and to gain an understanding of the requirements essential in the development of a mitochondrial shuttle vector that can deliver mtDNA mutations into wild-type strains.

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CHAPTER 2

Transmissible Mitochondrial Hypovirulence in a Natural Population of *Cryphonectria parasitica*

ABSTRACT

A cytoplasmically-transmissible hypovirulence syndrome has been identified in virus-free strains of the chestnut blight fungus *Cryphonectria parasitica* isolated from healing cankers on American chestnut trees in southwestern Michigan. The syndrome is associated with symptoms of fungal senescence, including a progressive decline in the growth potential and abundance of conidia, and elevated levels of respiration through the cyanide-insensitive alternative oxidase pathway. Conidia from senescing mycelia exhibited varying degrees of senescence ranging from normal growth to death soon after germination. Cytoplasmic transmission of hypovirulence between mycelia occurred by hyphal contact and coincided with the transfer of a specific RFLP from the mitochondrial

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Note. The content of this chapter has been published as Baidyaroy, D., Huber, D.H., Fulbright, D.W., and Bertrand, H. 2000. Transmissible mitochondrial hypovirulence in a natural population of *Cryphonectria parasitica*. Mol. Plant-Microbe Interact. 13: 88-95.

DNA (mtDNA) of the donor strains into the mtDNA of virulent recipients. The transmission of the senescence phenotype was observed not only among vegetatively compatible strains but also among incompatible strains. Hypovirulence was present in isolates from the same location with different nuclear genotypes as identified by DNA fingerprinting. This study confirms that mitochondrial hypovirulence can occur spontaneously and spread within a natural population of a phytopathogenic fungus.

INTRODUCTION

Several different infectious and debilitating, cytoplasmic factors can effectively reduce the aggressiveness of plant pathogenic filamentous fungi (Buck, 1986). In particular, infection by mycoviruses of *Cryphonectria parasitica* (Nuss, 1992), *Helminthosporium victoriae* (Ghabrial, 1988) and *Rhizoctonia solani* (Lakshman and Tavantzis, 1994) have been shown to cause reduced virulence. In *Ophiostoma ulmi*, the cytoplasmic d-factor has been shown to be associated with reduced virulence (Brasier, 1983; Rogers *et al.*, 1986).

Cytoplasmically-transmissible reduced aggressiveness phenotypes in the chestnut blight fungus *Cryphonectria parasitica* have been termed as 'hypovirulence' and have resulted in the recovery of chestnut trees in nature. These hypovirulence phenotypes are primarily caused by infection of the fungus by double-stranded RNA (dsRNA) viruses (hypoviruses) (reviewed in Nuss, 1992), which have specific effects on fungal

pathogenicity by interfering with G-protein signaling pathways (Nuss, 1996). However, some hypovirulent strains isolated from healing cankers do not contain any virus (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995). Most of the virus-free hypovirulent strains of *C. parasitica*, when tested for respiration, show high levels of alternative oxidase activity (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995) which normally is not exhibited by either wild-type strains or hypovirulent strains containing dsRNA viruses. This unique phenotype is singularly important in differentiating between hypovirulent strains that lack viruses from those that contain viruses. The fact that the alternative oxidase-mediated pathway of respiration is induced in the dsRNA-free hypovirulent strains and that the attenuated state is transmitted cytoplasmically (Monteiro-Vitorello *et al.*, 1995) suggests that this type of hypovirulence is associated with genetic alterations in the mitochondria that cause deficiencies in cytochrome-mediated respiration. The phenotypic characteristics of this dsRNA-free type of hypovirulence (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995) suggest that it is comparable to debilitating phenotypes caused by mtDNA mutations in *Neurospora*, *Podospora* and *Aspergillus* (reviewed in Griffiths, 1992).

To test whether mtDNA mutations can indeed cause a cytoplasmically-transmissible, hypovirulence phenotype in *Cryphonectria*, Monteiro-Vitorello *et al.* (1995) artificially induced mutations in the mitochondrial chromosome of the virulent, wild-type strain Ep155. The mutant strains were found to have elevated levels of alternative-oxidase activity, which is symptomatic of blockages in the cytochrome-mediated respiration pathway (Lambowitz and Slayman, 1972; Lambowitz and Zannoni, 1978; Vanlerberghe and McIntosh, 1997) and were significantly reduced in virulence.

That the alternative-oxidase activity in these strains was induced because of a defect in the mitochondrial electron-transport system was confirmed by the finding that the mutants were deficient in cytochromes *a* and *b*. The mutant (hypovirulence) phenotype was also cytoplasmically-transmissible among vegetatively compatible strains of *C. parasitica*; hence it appears to have infectious properties similar to those associated with viral hypovirulence syndromes. Thus, it was established that mtDNA mutations could cause a debilitating disease similar to that encountered in virus-free, hypovirulent strains of *Cryphonectria* isolated from healing cankers on trees. Hence the syndrome has been called 'mitochondrial hypovirulence' to distinguish it from virus-mediated hypovirulence (Monteiro-Vitorello *et al.*, 1995). However, the genetic basis of the naturally occurring mitochondrial hypovirulence in virus-free *C. parasitica* strains remains to be elucidated.

Hypovirulent strains of *Cryphonectria* that do not contain detectable levels of dsRNA virus have been isolated from healing cankers of American chestnut trees in the Kellogg Forest, Augusta, Michigan (Mahanti *et al.*, 1993; Huber, 1996). In this study, one isolate (KFC9) from that location has been characterized to determine the genetic basis of the hypovirulence trait that has arisen spontaneously in nature. The results indicate that this novel type of hypovirulence not only is associated with senescence and elevated levels of cyanide-resistant respiration, but also with a characteristic modification in the mitochondrial chromosome that is transmitted concordantly with the attenuated state to virulent strains by hyphal contact.

MATERIALS AND METHODS

Fungal strains, culturing conditions, respiration assays and virulence tests. The strains of *C. parasitica* used in this study are listed in Table 2.1. Cultures were grown in Endothia complete medium (ECM) as described (Puhalla and Anagnostakis, 1971) or on Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, Michigan). Tests for alternative oxidase activity was performed as described by Monteiro-Vitorello *et al.* (1995). Virulence tests were performed either on apples (Fulbright, 1984) or on live chestnut tissue (Lee *et al.*, 1992).

Transmission of hypovirulence phenotype through hyphal contact. Mycelial plugs of the donor and recipient strains were placed side-by-side, about 0.5 cm apart, on ECM near the walls of Petri-dishes and were allowed to grow until the cultures reached the opposite sides of the plates. Small mycelial plugs from the periphery of cultures of the recipient strain were then taken and subcultured on fresh plates. The subculturing was repeated until the recipient demonstrated the senescence phenotype, which normally occurred between 2-5 transfers. Virulent strains that have acquired hypovirulence by hyphal contact are mentioned by their strain name followed by '[KFC9]' to denote that they have a cytoplasmic factor derived from the strain KFC9.

Table 2.1. Strains of *Cryphonectria parasitica* used in this study.

Strain	Senescence	Source
Ep155	none	ATCC 38755
KFC9	present	Kellogg Forest, MI
KFC9-E6 (subculture of KFC9)	present	Kellogg Forest, MI
KFD9	present	Kellogg Forest, MI
KFD10	present	Kellogg Forest, MI
KFD18	present	Kellogg Forest, MI
KFD19.1	present	Kellogg Forest, MI
KFD19.2	none	Kellogg Forest, MI
KFD27.1	present	Kellogg Forest, MI
KFC27.1	present	Kellogg Forest, MI
KFD27.2	present	Kellogg Forest, MI
KFC27.2	present	Kellogg Forest, MI
KFC27.3	present	Kellogg Forest, MI
KFD27.4	present	Kellogg Forest, MI
KFC27.4	present	Kellogg Forest, MI
KFD27.10	present	Kellogg Forest, MI
Ep289	none	Conn. Agri. Exp.[a]
Ep289[KFC9]	present	DWF collection
J2.31	none	DWF collection[b]
J2.31[KFC9]	present	This study
F2.36	none	DWF collection
F2.36[KFC9]	present	This study

[a] Sandra Anagnostakis, Connecticut Agricultural Experiment Station.
[b] Dennis W. Fulbright collection, Michigan State University.

Isolation of genomic and mitochondrial DNA. For genomic DNA preparations, mycelia were grown overnight in 500 ml of ECM broth while shaking at 200 rpm. Approximately 3-5 g of each mycelium was collected by filtration and then disrupted by grinding with acid-washed sand (Fisher Scientific, Pittsburgh, Pennsylvania) in the cold. The disrupted hyphae were suspended in 8-10 ml of a solution containing 0.2 % sodium-dodecyl sulphate and 0.1 M EDTA and the homogenate was incubated at 70°C for 15 min. Then, 2 ml of 5 M potassium acetate were added to the tubes, the solutions mixed gently, and incubated for one hour on ice. The mixture was centrifuged at 14,000 rpm for 10 min in a Sorvall SS-34 rotor. The supernatant was collected carefully and DNA was precipitated with an equal volume of isopropanol. The DNA was redissolved in 0.5 ml of TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.6) supplemented with RNaseA (10 μg/ml) and incubated at 37°C for 30 to 45 min. The DNA was further purified by successive phenol, phenol:chloroform and chloroform extractions. In some cases, the DNA was also purified with cetyltrimethylammoniumbromide (CTAB; USB Corporation, Cleveland, Ohio) (Ausubel *et al.*, 1987). Mitochondrial DNA was purified as described by Bell *et al.*, (1995) with an added purification step using CTAB.

DNA manipulations and Southern blot hybridization. Restriction enzymes were obtained from Gibco BRL (Gaithersburg, Maryland). Enzymatic digestions of DNAs, agarose gel electrophoresis and Southern blotting were done as described by Sambrook *et al.* (1989). Southern blot hybridizations were performed with chemiluminescent probes as directed by the manufacturer (Boehringer Mannheim, Indianapolis, Indiana). For nuclear

DNA fingerprinting, Southern blots were hybridized with a plasmid (pMS5.1) containing a cloned moderately repetitive nuclear DNA fragment (Milgroom *et al.*, 1992).

Polymerase chain reaction (PCR). PCR was done as suggested by the manufacturer (Promega Inc., Madison, Wisconsin). Template DNAs were boiled for 5 min before being added to the reaction tubes. The DNA was denatured at 93°C for 3 min and subjected to PCR for 30 cycles, each consisting of the following successive steps: 93°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The last cycle consisted of an extension reaction at 72°C for 10 min. The sequence of the primers used in these reactions were designed on the basis of the nucleotide sequence of the mitochondrial small subunit ribosomal RNA (mtS-rRNA) gene of *Cryphonectria parasitica* (GenBank accession no. AF029891) and are as follows: 5'- GGTTGGTGATTCTTTCATGG-3' (forward primer) and 5'-TACACTCACCTGTACAC-3' (reverse primer).

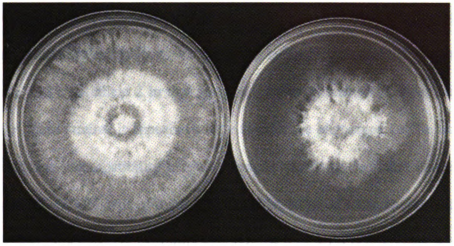
RESULTS

Phenotypic characteristics of KFC9. Relative to the wild-type strains, the growth-rate of KFC9 was significantly reduced with progressive degeneration of the mycelium upon vegetative growth. The strain generally formed flat, highly pigmented cultures with irregular edges (Figure 2.1). Conidiation was restricted to the center of the culture while the edges produced almost no aerial hyphae. Subcultures generated from mycelium taken

from the center of degenerating cultures produced normal-appearing mycelia as they began to grow. However, as the hyphae advanced, growth became progressively thinner and the mycelia grew only within, rather than on the surface of the agar medium. Growth always ceased before the edge of the Petri-dishes was reached. Because of the similarity of this process to the cytoplasmically-transmitted degenerative process described as 'senescence' in other fungi, particularly *Neurospora* and *Podospora*, this term also has been adopted to describe this process in *Cryphonectria*. Subcultures from degenerating mycelia were found to resume growth according to the stage of senescence present in the sampled region of the mycelium. That is, subcultures taken from the center of the senescing culture, where the mycelium appeared normal, exhibited the same pattern of growth and degeneracy as the parental culture, while subcultures started from plugs of mycelia taken progressively closer to the growth front showed increasingly advanced stages of senescence.

Cultures started from individual conidia from KFC9 also showed different degrees of senescence depending on the distance of their origin from the dying front of the parental culture. Conidia collected from the fast-growing center of a senescent culture generally demonstrated similar growth and debilitation patterns as the parental culture. However, conidia collected progressively from the center towards the edges formed colonies that showed increasing levels of degeneration. Some conidia produced only small colonies in which the mycelia grew very slowly before growth ceased.

Serial transmission of the senescence phenotype through hyphal contact. To determine if nuclear or cytoplasmic factors were responsible for the senescence phenotype of KFC9, transmission studies were performed where genetically-marked recipient virulent strains were grown side-by-side with a donor hypovirulent strain. The leading edge of the recipient culture became very debilitated, and formed appressed thin mycelia that were morphologically similar to the degenerative phenotype of the leading edge of the donor mycelium. This degeneration then spread laterally through the leading edge of the recipient mycelium. Transmission of the senescence phenotype was accomplished serially, thereby demonstrating that infected recipients can also act as donors. The serial transmission of the senescence agent was conducted in this order by Huber (1996): from KFC9 to Ep289 to generate the hypovirulent strain Ep289[KFC9], from Ep289[KFC9] to A1.13 producing A1.13[KFC9], and then from A1.13[KFC9] to J2.31 generating the corresponding hypovirulent J2.31[KFC9] (Table 2.2) showing that the phenotype was indeed cytoplasmically transmissible. KFC9-E6, a subculture of KFC9, was subsequently used as donor with several other virulent recipient strains (Table 2.2). Conidia collected from Ep289[KFC9], a strain converted by KFC9 (Huber, 1996), also showed the symptoms of senescence in some of the resulting colonies, thereby demonstrating that the effect of the senescence agent upon conidia is also transmitted between strains. Multiple attempts at extracting dsRNA failed to reveal the presence of any mycoviruses in any of the senescent strains (data not shown).



Ep155 **KFC9**

Figure 2.1. Phenotypic and growth characteristics of the mutant KFC9-E6 and the wild type Ep155. Both cultures are 7 days old.

The senescence phenotype previously was found to be transmitted with high efficiency among compatible strains by Huber (1996). Our data suggests that the phenotype is also transmitted among incompatible strains that do not allow transmission of viruses among themselves. For example, the senescent KFC9-E6 strain was able to convert two strains, F2.36 and J2.31, which are mutually incompatible because they differ at the strongest incompatibility locus, *vic* 2 (Huber, 1996). The strain F2.36 has allele 1 at locus 2 while J2.31 has allele 2 in that position. However, since KFC9-E6 was able to infect both of these strains, it appears that the senescence phenotype can be transmitted even across strong incompatibility barriers. Nonetheless, it is clear that barriers to the transmission of the KFC9-type of hypovirulence exist between some strains as described by Huber (1996). Since the compatibility genotype of KFC9-E6 is unknown, it is unclear whether or not the lack of transfer was caused by allelic differences at *vic* loci or by other yet undetermined factors that affect hyphal fusions. Collectively, the observations suggest that this novel senescence phenotype is not only cytoplasmically transmissible like dsRNA viruses, but also can be transmitted across barriers that prevent virus transmission between some highly incompatible strains.

Respiration, senescence and reduced virulence. Since the senescence and hypovirulence traits of KFC9 were transmitted asexually and independently of nuclei, experiments were conducted to determine the intracellular localization of the causative genetic-factor(s) for senescence. In filamentous fungi like *Neurospora, Podospora anserina* and *Aspergillus amstelodami* (reviewed in Griffiths, 1992), *Ophiostoma ulmi* (Charter *et al.,* 1993) and *Cryphonectria parasitica* (Monteiro-Vitorello *et al.,* 1995),

cytoplasmically-transmissible phenotypes that induce slow growth and/or reduced virulence traits are typically associated with mitochondrial dysfunctions caused by mutant forms of mtDNA. Based on these observations, an attempt was made to establish whether or not the genetic agent that produces senescence in KFC9 is associated with mitochondria and might cause respiratory defects. Respiration was assayed in the senescent strains to determine if a defect in cytochrome-mediated respiration might be indicated by the presence of high levels of cyanide-resistant respiration (alternative oxidase activity).

The field-collected strains KFC9 and a subculture thereof, KFC9-E6, were found to have high levels of alternative oxidase activity (Table 2.3), whereas the activity in the wild-type strains of Ep289, C1.20, J2.31 and F2.36 was low and in the range of values for other virulent strains reported by Mahanti *et al.* (1993) and Monteiro-Vitorello *et al.* (1995). However, the converted senescent forms that were generated by hyphal contact of these virulent strains with KFC9 and KFC9-E6 exhibited high levels of cyanide-resistant respiration (61-88.5%) (Table 2.3). Thus, it was evident that the senescence phenotype correlates with the transmission of a cytoplasmic factor capable of eliciting a mitochondrial dysfunction that induces the alternative respiratory pathway.

Virulence of the wild-type and senescent strains was measured by the size of lesions produced on apples as described by Fulbright (1984) and/or on live chestnut tissue by the method of Lee *et al.* (1992). The virulence level of KFC9-E6, a subculture of the

Table 2.2. Transmission of the 'senescence agent' by vegetative contact.

Donor	Recipient[a]	Senescent Strain
KFC9	Ep289 *met*	Ep289[KFC9] *met*
Ep289[KFC9] *met*	C1.20 *cre*	C1.20[KFC9] *cre*
Ep289[KFC9] *met*	A1.13 *cre*	A1.13[KFC9] *cre*
A1.13[KFC9] *cre*	J2.31 *br*	J2.31[KFC9] *br*
KFC9-E6	J2.31 *br*	J2.31[KFC9-E6] *br*
KFC9-E6	F2.36 *br*	F2.36[KFC9-E6] *br*

[a] Recipients always differed from the donor by a nuclear marker as indicated: *met* indicates methionine deficiency, whereas *br* and *cre* stand for brown and cream color of mycelia, respectively. The wild type pigmentation is orange.

wild-collected KFC9 strain was found to be very low (Table 2.3). The virulence of all of the wild-type strains was found to be drastically reduced when they were infected with the senescence agent. In fact, the strains Ep289 [KFC9], J2.31[KFC9] and F2.36[KFC9] often failed to grow when inoculated into apples or chestnut bark. Hence, these strains are afflicted with a disease syndrome which can be referred to as 'mitochondrial hypovirulence' as defined by Monteiro-Vitorello *et al.* (1995).

Identification of the segment of mtDNA bearing the senescence agent. The mtDNAs isolated from purified mitochondria of KFC9-E6, Ep289 and Ep289[KFC9] were digested with *Hind*III and the resulting fragments were separated by agarose gel electrophoresis. The restriction digestion patterns of the mtDNAs of KFC9-E6 and Ep289 differed by multiple restriction fragment length polymorphisms (Figure. 2.2A). In contrast, Ep289 differed from Ep289[KFC9] in the size of only one fragment; i.e., a 10.5-kb band in Ep289 was replaced by an 11.5-kb band in Ep289[KFC9]. This 11.5-kb fragment appeared to have originated from the mtDNA of KFC9, which produces it and not the 10.5-kb fragment when digested with *Hind*III. That the nuclei of the recipient remained unaltered during its infection by KFC9 was evident because the nuclear DNA fingerprints of Ep289 and Ep289[KFC9] were found to be identical to each other and different from that of KFC9-E6 (Figure. 2.3). The 11.5-kb *HindIII* fragment of mtDNA also appeared in the converted J2.31[KFC9], whereas restriction of the mtDNA for the virulent J2.31 strain produced only the 10.5-kb *Hind*III fragment (Figure 2.2B). The

Table 2.3. Alternative oxidase activity as percent of total respiration and virulence of wild-type and corresponding senescent strains cytoplasmically infected with the senescence-inducing agent from KFC9 and KFC9-E6.

Strain	Phenotype	Virulence on chestnut tissue[a]	Virulence on apple[a]	Alt. Oxidase as % of total resp.
Ep155	N[b]	NT[c]	21.1 ± 2.0	7.5 ± 0.3
KFC9-E6	S	NT	1.0 ± 1.1	38.6 ± 7.8
Ep289	N	4.2 ± 0.2	NT	21.3 ± 2.7
Ep289[KFC9]	S	0.2 ± 0.1	NT	81.7 ± 1.1
J2.31	N	6.4 ± 0.4	17.3 ± 2.0	13.1 ± 3.3
J2.31[KFC9]	S	0.3 ± 0.2	< 1.0	80.5 ± 4.2
J2.31[KFC9-E6]	S	NT	< 1.0	88.5 ± 3.1
F2.36	N	NT	13.2 ± 1.4	10.5 ± 6.0
F2.36[KFC9-E6]	S	NT	< 1.0	68.4 ± 9.6

[a] Virulence measured as area of lesion in cm^2.
[b] Non-senescent strain.
[c] Not tested.
[d] Senescent strain.
[e] The strain was lost to senescence before virulence tests could be performed.

displacement of the 10.5-kb *Hind*III fragment by an 11.5-kb fragment also was associated with the transfer of the hypovirulence trait from KFC9-E6 to the wild-types F2.36 and J2.31 (data not shown). Since this was the only DNA fragment that correlated with the transmission of the senescence phenotype, the 11.5-kb fragment was hypothesized to contain the KFC9 senescence-agent. The fact that the converted hypovirulent recipients retained most of the mtDNA of their virulent progenitors further supports the argument that the 11.5-kb fragment includes the genetic factor that causes the senescence phenotype and hypovirulence traits found in KFC9. The putative mutation associated with this segment of the mtDNA was mapped and identified to be a 973-bp insert, which is located in the mitochondrial small subunit ribosomal RNA (mtS-rRNA) gene and lacks features that would identify it either as an intron or a mobile genetic element. The nucleotide sequence of this insert and its effect on the processing of the precursor of the mitochondrial mtS-rRNA are described in the following chapters. All of the converted hypovirulent strains were found to contain the mutant form of the mtS-rRNA gene. The corresponding segment of their mtDNA was amplified in a PCR reaction with primers that generate a 700-bp fragment from wild-type virulent strains and a 1.7-kb fragment in strains that have the KFC9 senescence phenotype (Figure 2.4). However, some of the converted-hypovirulent strains were occasionally found to be heteroplasmic, as indicated by amplification of both the wild-type and the mutant forms of the mtDNA in PCR reactions (Figure 2.4, lane 3). Contamination of the PCR reaction mixtures was ruled out because similar products were always obtained from reactions using the same preparation of DNA as the template.

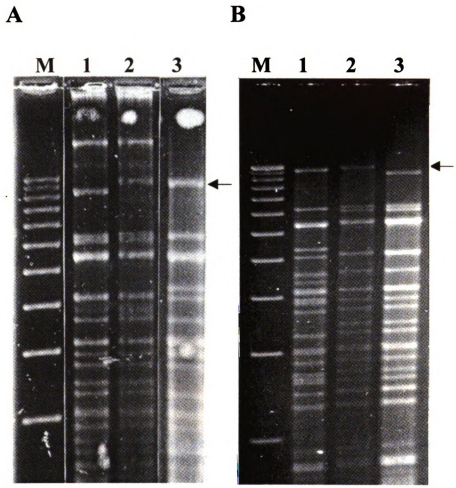


Figure 2.2. Identification of the region of mtDNA bearing the putative mutation in KFC9-E6. *Hind*III digested mtDNA was fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining. The invasive *Hind*III fragment bearing the senescence-inducing mutation is indicated by arrows. **A**, Lane 1, Ep289. Lane 2, Ep289[KFC9]. Lane 3, KFC9-E6. **B**, Lane 1, KFC9-E6. Lane 2, J2.31[KFC9-E6]. Lane 3, J2.31. Lane M in both panels represents molecular weight markers.

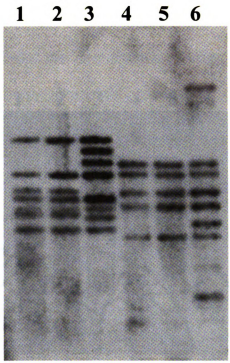


Figure 2.3. Comparison of nuclear genotypes of the wild-type strain Ep289, converted hypovirulent strain Ep289[KFC9] and the hypovirulent strain KFC9. Genomic DNA isolated from the strains was digested with *Hinc*II (Lanes 1-3) and *Hind*III (Lanes 4-6) and fractionated on an agarose gel. DNA from the gel was transferred to a nylon membrane and hybridized with the fingerprinting probe pMS5.1. Lanes 1 and 4, Ep289. Lanes 2 and 5, Ep289[KFC9]. Lanes 3 and 6, KFC9.

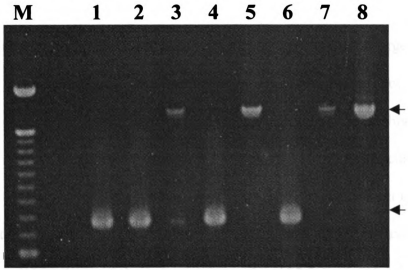


Figure 2.4. Conversion of wild-type mtDNA into mutant form in the converted hypovirulent strains as detected by PCR. Products generated from the PCR reaction were separated by agarose gel electrophoresis and observed by ethidium bromide staining. Lane 1, Ep155. Lane 2, Ep289. Lane 3, Ep289[KFC9]. Lane 4, J2.31. Lane 5, J2.31[KFC9]. Lane 6, F2.36. Lane 7, F2.36[KFC-E6]. Lane 8, KFC9-E6. Lane M represents the molecular weight marker. The 0.7-kb and 1.7-kb fragments are indicated by arrows. DNA bearing the putative mutation is approximately 1-kb larger than the wild-type fragment.

Dissemination of mitochondrial hypovirulence in nature. Several strains were collected from trees located in the area of origin of KFC9, and genomic DNA was prepared from each isolate. These DNAs were used as templates for the amplification of the mtS-rRNA region that is affected in KFC9 by PCR with primers that flank the putative insertion. In the strains that were included in this experiment, only one was found that did not contain the putative senescence agent (Figure 2.5). This implies that the agent that causes hypovirulence and senescence in KFC9 occurs in a large proportion of the *C. parasitica* population at that location. Only the strain that did not contain the senescence agent (KFD19.2) was found to be virulent and did not show high levels of cyanide-resistant respiration (data not shown). Among 13 strains collected in 1997, twelve were moderately to severely hypovirulent (Table 2.4). In contrast, out of 19 strains collected from this site in 1990, only six demonstrated any degree of hypovirulence. None of these strains were found to contain any dsRNA virus. The data in Table 2.4 indicates that there has been a marked shift in the ratio of hypovirulent to virulent strains from 1990 to 1997, suggesting that the mtDNA that causes this trait is probably invading the population of *C. parasitica* at the Kellogg Forest site. This notion gains support from the observation that hypovirulence was not observed in this site before 1990 (Likins, 1990).

To explore whether or not the strains isolated from the Kellogg Forest site represent a genetically homogenous population, nuclear genotypes were determined using a probe for repetitive DNA, as described by Milgroom *et al.,* (1992). Out of the 12 strains included in this experiment, eight unique patterns of hybridization were identified (Figure 2.6). Strains isolated from the same tree were often found to have different nuclear genotypes. For example, strains KFD19.1 and KFD19.2 originated from the same tree

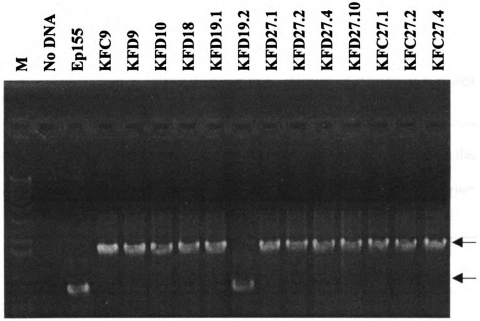


Figure 2.5. Dissemination of the mutation of KFC9 in nature as detected by PCR. PCR products were separated by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide staining. The 0.7-kb and 1.7-kb fragments are indicated by arrows. Lane M represents the molecular weight marker.

Table 2.4. Progression of hypovirulence in *C. parasitica* strains at Kellogg Forest in Augusta, Michigan.

Virulence status	Area of lesion produced on apples in cm^2	Strains collected in 1990	Strains collected in 1997
Virulent	>15	13	1
Moderate	8-15	2	3
Hypovirulent	< 8	4	9

(number 19), but had different DNA fingerprint patterns. The same was true for strains KFC9 and KFD9 from tree 9 and strains KFD27.1, KFD27.2, KFD27.4 and KFD27.10 from tree 27. In contrast, some of the strains from different trees were found to have similar nuclear genotypes, i.e. KFC9 (tree 9), KFD18 (tree 18) and KFD27.2 (tree 27). Although relatively few strains were included in this study, different strains from the same canker were always found to be similar, for example KFD27.2 and KFC27.2 (tree 27, canker 2) and KFD27.4 and KFC27.4 (tree 27, canker 4). Several highly debilitated strains could not be included in this analysis because they died before DNA could be extracted from their mycelia. None of these strains contained a detectable amount of any dsRNA virus. Collectively, these observations suggest that the hypovirulence factor may be transmitted asexually through hyphal anastomoses between mycelia of different genotypes or disseminated sexually by maternal inheritance in crosses between different strains.

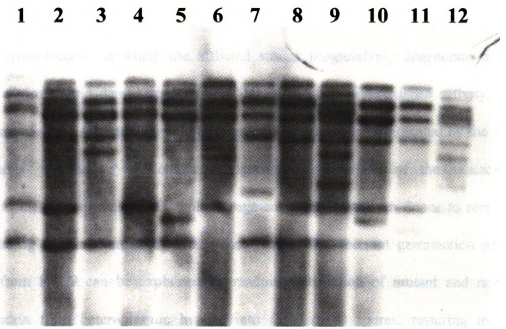


Figure 2.6. Nuclear genotypes of strains obtained from the Kellogg Forest area. Genomic DNA isolated from the strains was digested with *Pst*I and separated on an agarose gel. DNA from the gel was transferred to a nylon membrane and hybridized with the fingerprinting probe pMS5.1. Lane1, KFC9. Lane 2, KFD9, Lane 3, KFD10, Lane 4, KFD18. Lane 5, KFD19.1. Lane 6, KFD19.2. Lane 7, KFD27.1. Lane 8, KFD27.2. Lane 9, KFD27.4. Lane 10, KFD27.10. Lane 11, KFC27.2. Lane 12, KFC27.4.

DISCUSSION

In this study, we provide a characterization and a brief analysis of the dispersal of a novel mitochondrial hypovirulence and senescence syndrome that has appeared spontaneously in *C. parasitica* on American chestnut trees. The study describes a novel type of hypovirulence in which the afflicted strains progressively degenerate during vegetative growth and demonstrate elevated levels of alternative oxidase activity. The association of mtDNA mutations with this senescence syndrome is indicated by the non-synchronous germination of conidia, cytoplasmic transmission of the senescence phenotype and the co-transfer of a unique segment of mtDNA from donor to recipient strains during transmission of the senescence factor. The aberrant germination of the conidia from KFC9 can be explained by random distribution of mutant and normal mitochondria from heteroplasmic hyphae into the asexual spores, resulting in fast germination of conidia containing few mutant mitochondria and slower germination for the ones that contain high amounts of dysfunctional mitochondria. These characteristics are similar to the transmissible senescence syndromes of *Neurospora* which are caused by suppressive mtDNA mutations producing dysfunctional mitochondria that proliferate more rapidly than normal mitochondria and thus invade the coenocytic mycelia of this organism (Bertrand *et al.*, 1986; Bertrand, 1995).

In many filamentous fungi, senescence has been shown to be associated with respiratory defects that are caused by a variety of mtDNA mutations. Excision, circularization and subsequent amplification of small segments of the mitochondrial

chromosome are observed in senescent strains of *Podospora anserina* (Jamet-Vierney *et al.*, 1980), *Aspergillus amstelodami* (Lazarus *et al.*, 1980) and *Ophiostoma ulmi* (Abu Amero *et al.*, 1995). Amplification of segments of the mitochondrial chromosome also has been observed in the mitochondria of mitochondrially-hypovirulent *C. parasitica* strains (Monteiro-Vitorello *et al.*, 1995). Large deletions in the mtDNA have been found in senescent stopper mutants of *N. crassa* (Bertrand *et al.*, 1980) as well as in the ragged mutants of *Aspergillus amstelodami* (Lazarus and Küntzel, 1981) and in senescent cultures of *Podospora curvicolla* (Böckelmann and Esser, 1986) as well as *P. anserina* (Contamine *et al.*, 1996). Small deletions (Manella and Lambowitz, 1978) and integration of plasmids into the mtDNA (Bertrand *et al.*, 1986; Akins *et al.*, 1986) can also lead to senescence in *Neurospora*. Therefore, in the case of the KFC9-type of senescence in *C. parasitica*, it is plausible that the 973-bp DNA insert in the mtS-rRNA gene causes the debilitation of the fungus. However, the possibility that the phenotype is caused by a point mutation that is closely linked to this insert or exists elsewhere in a non-polymorphic region of the mtDNA cannot be excluded at this time. Since a variety of mtDNA mutations can cause senescence or suppressive slow-growth phenotypes, it seems that it is the final physiological effect of these mutations, namely respiratory deficiency, that causes suppressiveness, rather than the individual mutations themselves (Bertrand, 1995).

The concomitant transfer of an insertion in the mtDNA of KFC9 with the senescence and hypovirulence phenotypes potentially could represent any of the following three situations. First, the insertion could be a stable, deleterious mutation in the mtS-rRNA that causes the suppressive accumulation of defective mtDNA molecules

upon its transfer into unaffected strains, thereby causing senescence. In this case, the mechanism for the transfer of the insert into the mtDNA of recipient strains and the disappearance of the mtDNA from the donors remains unexplained. Secondly, the insertion could be a site-specific mobile genetic element that spreads by inserting into mtS-rRNA gene, thus converting normal mtDNA into the mutant form. In this case, senescence would be a reflection of the conversion process, but the insert lacks features identifying it as a transposable element. Finally, the insertion could be a homing intron that moves into intron-less copies of the mtS-rRNA gene. However, senescence would not be expected in this case because the intronic sequence most likely would be spliced from the rRNA primary transcript. On the basis of information available about senescence in other fungi, the first and the third options are the most and the least likely, respectively, of the mechanisms that could account for senescence in strains that have the KFC9 cytoplasm. The precise identification of the molecular and physiological processes that are involved in the progressive attenuation of the population of *C. parasitica* at the Kellogg Forest site requires further experimentation.

Since the presence of the 11.5-kb *Hind*III fragment in the mtDNA of different strains correlates with the appearance of the hypovirulence phenotype (Figure. 2.2), the screening of a sample of isolates from trees in the Kellogg Forest for this DNA segment by PCR can be used to assess the extent and progress of the dissemination of the KFC9 type of hypovirulence at that location. Among all of the isolates recovered from the Kellogg Forest site in 1997, only one strain did not contain the mutant mtDNA (Figure 2.5). This strain was found to be virulent and does not demonstrate elevated levels of alternative-oxidase activity. Due to the nuclear heterogeneity present among these strains

(Figure 2.6), it can be assumed that not all of them are vegetatively compatible with each other. Hence, the KFC9-senescence phenotype might be transmitted not only vegetatively through hyphal anastomoses, but also may be sexually inherited. In *Cryphonectria*, it has been shown previously that some debilitating mtDNA mutations indeed can be sexually inherited (Monteiro-Vitorello *et al.*, 1995). This particular feature of mitochondrial hypovirulence is relevant because *Cryphonectria* strains are known to be cured of dsRNA hypoviruses during the sexual cycle (Nuss, 1992). Asexual transmission of the phenotype among incompatible strains demonstrates that, like viruses, mitochondrial hypovirulence can be transmitted efficiently through hyphal contacts. It has been found that vegetative incompatibility loci sometimes may reduce the rate of, and even block, the asexual transmission of mitochondrial mutations (Caten, 1972). However, the observations presented in Figure 2.5 suggest that the dynamics of the dissemination of the KFC9 factor in a genetically heterogeneous population of *C. parasitica* that exists in a natural environment may be more liberal than that which may be deduced from laboratory studies.

The presence of the senescent factor in most of the strains from Kellogg Forest also implies that the KFC9 type of hypovirulence is not only highly transmissible, but also is viable and stably maintained in nature and can infect a substantial proportion of virulent strains in a population of the fungus. This observation is consistent with the finding that most of the infected trees in the Kellogg Forest are now recovering. Collectively, these results indicate that mitochondrial hypovirulence in *Cryphonectria* might be used effectively instead of, or in addition to, dsRNA viruses in the biological control of this fungus. Since a majority of plant diseases are caused by fungi, the

discovery and analyses of mtDNA mutations and other deleterious mitochondrial genetic elements could significantly impact the development of effective biological control methods. In this context, the findings of this study suggest that the dissemination of hypovirulent strains containing mtDNA mutations might be useful as a remedial measure against fungal diseases and potentially can be applied to control virulent populations of a variety of phytopathogenic fungi.

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CHAPTER 3

Molecular basis of mitochondrial hypovirulence in KFC9, a *Cryphonectria parasitica* strain isolated from nature

ABSTRACT

In the chestnut-blight fungus *Cryphonectria parasitica*, cytoplasmically transmissible hypovirulence phenotypes frequently are elicited by double-stranded RNA (dsRNA) virus infections. However, some of the strains that have been isolated from nature and manifest cytoplasmically transmissible hypovirulence traits do not contain any mycoviruses. In this study, we describe a mtDNA mutation that is implicated as the causative factor of hypovirulence in a virus-free strain of *C. parasitica* obtained from nature. The mutation consists of a 973-bp DNA element, named 'InC9', of unknown origin and function, that is inserted into the first exon of the mitochondrial small-subunit ribosomal RNA (mtS-rRNA) gene. The InC9 element lacks features that identify it either as an intron or a mobile genetic element. *In vivo* and relative to *bona fide* introns, the segment of RNA corresponding to InC9 was found to be spliced very slowly, if at all, from the precursor transcript of the mtS-rRNA. As expected, the hypovirulent strain was found to be deficient in mitochondrial ribosomes. Hence, the mutant is defective in

mitochondrial protein synthesis and deprived of some of the essential components of the cytochrome-mediated respiratory pathway.

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*) in 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 Sauret 1969; Mahanti *et al.*, 1993; reviewed in Fulbright, 1999). These strains lacked the aggressiveness of the virulent types and have been causally implicated in spontaneous regeneration of diseased trees. Most of the hypovirulent strains were found to contain infectious double stranded RNA (dsRNA) viruses, which were shown to attenuate aggressive strains of the fungus (Van Alfen *et al.*, 1975; Tartaglia *et al.*, 1986; Choi and Nuss 1992; Nuss 1992; Chen *et al.*, 1994; Fulbright, 1999). However, some hypovirulent strains were found to be completely devoid of known viruses. Unlike the virulent wild-types and the dsRNA-containing attenuated strains, the virus-free hypovirulent isolates manifested high levels of mitochondrial alternative oxidase activity, which is manifested phenotypically in mycelia as cyanide-resistant and salicylhydroxamate-sensitive respiration (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000).

The hypovirulence trait of these strains also was found to be 'infectious' like that of the virus-containing diseased isolates because it can be easily transmitted by hyphal contact to virulent strains (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000). Collectively, these observations suggest that whatever genetic determinant causes hypovirulence in virus-free strains *C. parasitica* also is capable of modifying the functional state of mitochondria by eliciting a deficiency in cytochrome-mediated respiration (Monteiro-Vitorello *et al.*, 1995). The fact that the phenotypic and genetic characteristics of these strains mimic those of most of the well-characterized mitochondrial mutants of other filamentous fungi (reviewed in Griffiths, 1992; Bertrand, 2000), particularly *Neurospora* (Bertrand, 1983; Bertrand and Griffiths, 1989), *Podospora* (Böckelmann and Esser, 1986), *Aspergillus* (Lazarus and Küntzel, 1981) and *Ophiostoma* (Rogers *et al.*, 1986; Abu-Amero *et al.*, 1995), supports the notion that this type of hypovirulence is caused by mitochondrial DNA (mtDNA) mutations. Hence, it has been called 'mitochondrial hypovirulence' to distinguish it from that which is caused by mycoviruses (Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000).

Direct evidence for the association of the attenuated state in *C. parasitica* with mtDNA mutations was established through the induction in the virulent, virus-free Ep155 standard wild-type strain of mutants that exhibited increased levels of cyanide-resistant respiration and hypovirulence phenotypes (Monteiro-Vitorello *et al.*, 1995). The genetic determinants of the induced hypovirulence trait not only were transmitted asexually to virulent strains by hyphal contact, but also were maternally inherited in sexual crosses. Aberrant forms of mtDNA also were detected in these mutants. This set of observations

led to the conclusion that the genetic determinants of this type of hypovirulence are mutant forms of genes that are located on the mitochondrial chromosome.

The above-described observations prompted the investigation of the cause of hypovirulence in a virus-free strain of *C. parasitica*, KFC9, which was isolated from a healing canker on an American chestnut tree located in the Kellogg Forest in Michigan (Baidyaroy *et al.*, 2000). The hypovirulence phenotype of KFC9 was found to be stably maintained and infectious in nature. Vegetative transmission of the hypovirulence phenotype from attenuated to virulent strains coincided with the transmission of a specific region of the KFC9 mtDNA (Baidyaroy *et al.*, 2000). In this study, we have identified the mtDNA mutation that most likely is responsible for the hypovirulence trait as well as a progressive debilitation syndrome that characteristically appear in strains that have KFC9 cytoplasm. The results show that mitochondrial mutations indeed can cause cytoplasmically transmissible mitochondrial-hypovirulence in *C. parasitica* in nature and potentially can be used to control at least some of the diseases that are caused by phytopathogenic filamentous fungi.

MATERIALS AND METHODS

Fungal strains, culturing conditions and respiration assays. *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 mutant strain KFC9-E6 of unknown mating type was isolated from a healing canker of an American chestnut tree in the Kellogg Forest near Augusta, Michigan. Ep155 served as a wild-type control in all the experiments. Three virulent strains of *C. parasitica* with nuclear markers, Ep289 *met*, J2.31 *br* and F2.36 *br*, were used as recipients in experiments involving the vegetative transmission of the hypovirulence phenotype from KFC9-E6. Ep289 *met* was originally obtained from S. Anagnostakis (Connecticut Agricultural Experiment Station), while J2.31 *br* and F2.36 *br* were generated by David H. Huber in D. W. Fulbright's laboratory. Tests for alternative oxidase activity were performed as described by Monteiro-Vitorello *et al.* (1995).

Preparation of genomic DNA, mitochondrial DNA and RNA. Genomic 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 cetyltrimethylammoniumbromide (Ausubel *et al.*, 1987). RNA was isolated from purified mitochondria by the SDS-diethylpyrocarbonate procedure (Solymosy *et al.*, 1968).

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). Southern and Northern blot hybridizations were performed with probes labelled with dig-oxigenin-dUTP as directed by the manufacturer (Boehringer Mannheim). Binding of the probes were detected by

chemiluminescence using a anti-dig-oxigenin Fab-alkaline phosphatase conjugate with CDP-star (Boehringer-Mannheim).

DNA sequencing. Sequencing was performed manually by the di-deoxy chain-termination method (Sanger *et al.*, 1977) with αP^{33}-labelled deoxy-adenosine triphosphates (dATP) followed by resolution of the reaction products in polyacrylamide gels and visualization through autoradiography. Automated fluorescent sequencing of DNA was performed using the ABI Catalyst 800 kit for Taq cycle sequencing and an ABI 373 Sequencer. Initial sequences of restriction fragments of mtDNA that were cloned as inserts in the BluescriptKS+ vector (Stratagene) were obtained by using vector-specific forward and reverse primers. These initial sequences were subsequently extended with sequence-specific primers synthesized by the Michigan State University Macromolecular Synthesis Facility. Both strands were completely sequenced in all cases. Sequences were aligned through the use of the MicroGenie™ MG-IM-5.0 programs (Queen and Korn 1984) and current databases were searched by means of BLAST (Altschul *et al.*, 1997) computer software through the internet.

Primer extension. Primer extensions were performed on templates consisting of whole mitochondrial RNA with αP^{33}-labelled ATP and resolution of the products in polyacrylamide gels as described by Court and Bertrand (1993), with the following modifications: the RNA and primer mixture was heated to 90°C in 50 mM KCl, 20 mM Tris–HCl, pH 8.5, 0.5 mM EDTA and 8 mM $MgCl_2$ and then cooled to 45°C over a period of 20 min in a thermocycler. The primer was extended at 45°C for 45 min with 0.25 units

AMV reverse transcriptase (AMV-RT; Seikagaku Inc.) in the presence of 100 mM DTT and 4 units of RNase Inhibitor (Boehringer Mannheim). The sequence of the primer that was used for this purpose is as follows: 5'- CCATGTATTTAAGTTCTGGG-3'.

Reverse transcriptase-mediated polymerase chain reaction (RT-PCR). RT-PCR reactions were performed with AMV-RT (Seikagaku, Inc.) as described by Chiocchia and Smith (1997). All RNA samples were treated with 1 unit DNaseI for 15 min prior to first-strand synthesis. First-strand synthesis was performed at 45°C for 10 min. The sequences of the primers that were used are as follows: P1 = 5'-GGTGAGTTTGGTGATGG-3' (sense primer); P2 = 5'-CACTCACCTGTACACCAC-3' (anti-sense primer); P3 = 5'-CTGACGTTGAGGAACGA-3' (sense primer); P4 = 5'-CATTACTCTTGAGGTGG-3' (anti-sense primer).

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 Lambowitz (1979). Ribosome subunits were separated on sucrose gradients and analyzed as described by Collins and Bertrand (1978) with the following changes: Triton X-100 was used at a concentration of 1% instead of Nonidet P40 and centrifugation time was increased from 3 to 4 hr.

RESULTS

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 *Hind*III 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 *Eco*RI revealed that the senescent strains had the putative mutation in a 10.3-kb fragment, whereas the corresponding wild type fragment was only 9.3 kb long (see Figure 3.1A, Figure 3.1B). The adjacent *Eco*RI fragment, immediately upstream of the fragment bearing the mutation, 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 *Eco*RI piece when the hypovirulence syndrome was transferred from KFC9 (Figure 3.1B). Thus, it appears that the region of mtDNA that bears the mutation probably is contained within the 5.8 kb *Hind*III-*Eco*RI fragment of KFC9, which is only 4.8 kb long in the wild type strains.

The 11.5-kb *Hind*III 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 cloned from Ep155 and Ep289. Sequence analysis revealed that the mtDNAs from the senescence-prone, hypovirulent cultures contained a block of 973 nucleotides that is not

present in the mtDNAs of the corresponding wild types. Since this insert appears to be a distinguishing feature of strains that display the KFC9-type of mitochondrial hypovirulence (Baidyaroy *et al.*, 2000), it has been named InC9 (Insert in KFC9). The InC9 element is located within the mtS-rRNA gene and begins 67 base pairs downstream from the point on the mitochondrial chromosome that corresponds to the beginning of the mature transcript (Figure 3.1A). The nucleotide sequence (GenBank accession no. AF218209; Appendix A) of this element was not homologous to any known DNA sequence, and its translation in all six reading frames by means of the appropriate mitochondrial genetic code did not reveal any open reading frames (ORFs) of significant size. The element was unusually GC-rich (41.1%) relative to known sequences of the mtDNA of *C. parasitica* (21%; Hausner *et al.*, 1999; Monteiro-Vitorello *et al.*, 2000). However, the InC9 element contained four very short regions that were homologous to previously known sequences (see Appendix A). Unfortunately no function has been attributed to these conserved regions so far. The 973-bp sequence does not appear to be an intron, for it lacks consensus sequences and structural features necessary for RNA splicing (Waring and Davies, 1984; Burke *et al.*, 1987; Burke, 1988; Cech, 1990, 1988; Lambowitz and Belfort, 1993; Sellem and Belcour, 1994). It also does not have any features that identify it as a transposable element (Calos and Miller, 1980; Kleckner, 1989, 1990; Scott and Churchwood, 1995).

InC9 is transcribed but not readily spliced. Mitochondria were isolated and total mitochondrial RNA was prepared using standard procedures. When equal amounts of mitochondrial RNA were applied to slots in agarose gels and separated by

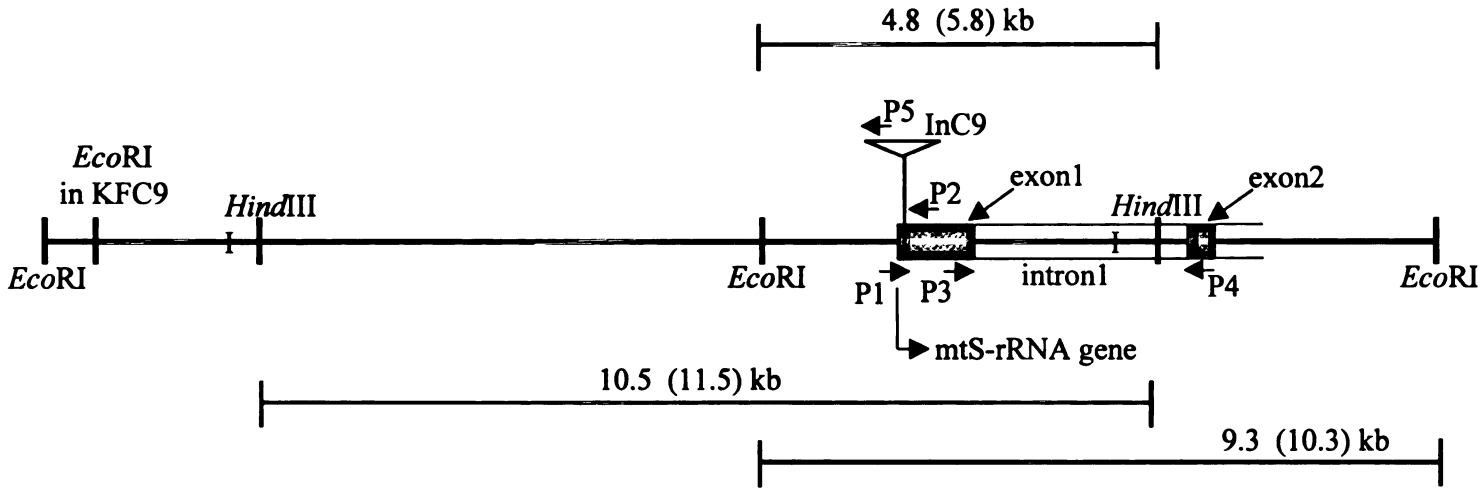

Figure 3.1A. Physical map of the region of mtDNA bearing the mutation that causes hypovirulence in KFC9. The position of InC9 on the 11.5-kb *Hind*III fragment of KFC9 mtDNA. The sizes of the wild-type restriction fragments are given in numerals above the corresponding pieces. The sizes of the fragments of the hypovirulent strains are indicated in parentheses. InC9 was cloned as the 5.8 kb *Hind*III-*Eco*RI segment from KFC9-E6.

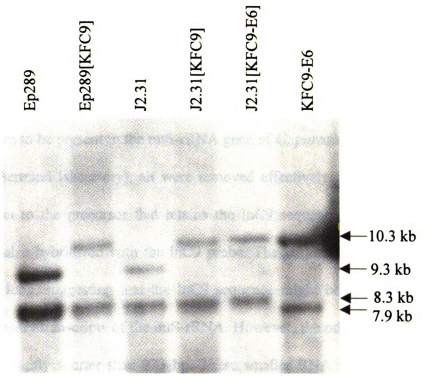

Figure 3.1B. Restriction mapping of the region bearing InC9 in the KFC9-E6 strain. Southern blot of EcoRI digested genomic DNAs was hybridized with a probe generated from the wild-type 10.5-kb *Hind*III fragment (see Figure 3.1A). Whereas the 10.3-kb *Eco*RI piece that contains InC9 is duly transmitted with the senescence phenotype from KFC9 to the recipients, the adjacent 7.9-kb *Eco*RI fragment is not transferred.

electrophoresis, it appeared that KFC9 and Ep155 had comparable amounts of small- and large-subunit rRNA (Figure 3.2A). It was not possible discriminate whether or not the concentration of these RNAs was higher, lower or equal to their concentration in the mitochondria of the wild-type. However, when hybridized against a probe which was derived from the InC9 sequence, several species of RNA appeared in KFC9 that were absent in Ep155 (Figure 3.2B). One of these RNAs is approximately 1.0 kb larger than the mature mtS-rRNA. Since this species is relatively abundant, it seems that the InC9 sequence is not readily spliced from the precursor transcript. In contrast, the four large introns that are known to be present in the mtS-rRNA gene of *C. parasitica* (unpublished observation in the Bertrand laboratory), all were removed effectively from the primary transcript. In addition to the precursor that retains the InC9 sequence, three relatively small RNA species also hybridized with the InC9 probe. The largest of these RNAs is approximately 1-kb long, suggesting that the InC9 sequence might be spliced from at least some of the primary transcripts of the mtS-rRNA. However, the other two RNAs in this group are significantly shorter than 973 bp. These smaller RNA species could be produced either through premature termination of transcription within the InC9 insert in the mtDNA, or by RNA processing events that remove only part of the InC9 sequence from the primary transcript. The latter of these two possibilities appears less likely than the former because rRNA molecules that retain part of the insert are not apparent on the northern blot in the region between the precursor that retains the entire InC9 sequence and the mature mtS-rRNA. Therefore, it is also possible that the 1-kb RNA species is a product of transcription termination events that occur near the downstream end of the

InC9 DNA, or of an inaccurate splicing activity near the 3' end of the insert in the primary mtS-rRNA transcript.

To determine whether or not the InC9 sequence might be removed slowly from the mitochondria in strains that have the KFC9 cytoplasm, a series of RT-PCR experiments were performed. Using equal amounts of mitochondrial RNA from Ep155 and KFC9 as templates, the first experiment was performed with a pair of primers (P1 and P2) that were homologous to the exon sequences flanking the InC9 element. These primers were expected to produce a 215-bp product if the InC9 sequence is spliced from the mtS-rRNA transcript and an 1188-bp product if InC9 is retained in the transcript. As shown in Figure 3.3, 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 produced high amounts of the 1188-bp product together with some of the 215-bp product. This result indicates that the InC9 sequence is spliced either very inefficiently or not at all from the predominant class of the mtS-rRNA transcripts. Since senescing cultures of the KFC9 strain tend to be heteroplasmic for the mutant and wild-type forms of mtDNA (Baidyaroy *et al.*, 2000), it is likely that most or all of the 215-bp product originated from the normal mtS-rRNA molecules that also are present in the mitochondria of this strain.

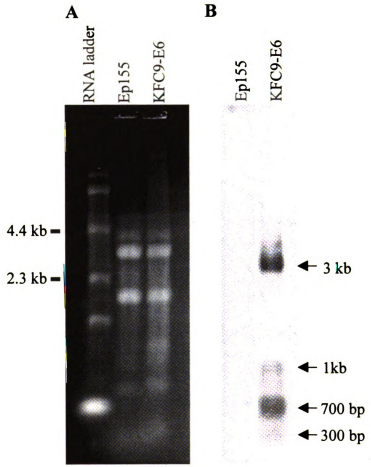

Figure 3.2. Expression of InC9. A Mitochondrial RNA extracted from wild type strain Ep155 and the mutant KFC9-E6 was separated on formaldehyde-containing agarose gel and stained with ethidium bromide. The molecular weight markers are shown towards the left margin. The 1.7-kb abundant RNA molecule is the small subunit ribosomal RNA. **B** Northern blot of KFC9-E6 mitochondrial RNA probed with InC9 sequence showing novel RNA molecules.

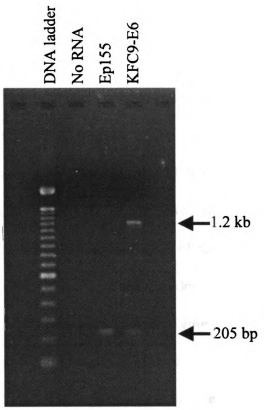

Figure 3.3. RT-PCR analysis of KFC9-E6 and Ep155 mitochondrial RNAs using a primer pair that are located on the exon sequence but flank the element InC9. The 205-bp molecule is generated from the processed transcript whereas the 1.2-kb product is derived from unprocessed mtS-rRNA transcripts.

KFC9 cannot splice InC9 but can splice introns. To determine whether the KFC9 strain has a defect that affects mitochondrial RNA splicing in general or merely cannot remove the InC9 RNA segment from the primary transcript of the mtS-rRNA, RT-PCR was performed with two pairs of primers, P1 and P2 flanking InC9 and P3 and P4 flanking the downstream *bona fide* intron 1. The design of the primers was based on the complete nucleotide sequence of the mtS-rRNA gene of *C. parasitica* (GenBank accession number AF029891). On the basis of this sequence, the primers flanking intron1 were expected to produce a 140-bp RT-PCR product from mature mtS-rRNA transcripts and a 2340-bp product from unspliced precursor mtS-rRNA molecules. Agarose gel electrophoresis of the RT-PCR products revealed that, whereas there were significant amounts of the product generated from unprocessed RNA of KFC9 with primers P1 and P2, the primers P3 and P4, that flank intron 1 produced detectable products only from processed RNAs for both the KFC9 and Ep155 strains (Figure 3.4A). Southern blots of similar electrophoretically-separated products generated from similar amounts of mitochondrial RNA from Ep155 and KFC9 also were hybridized with a mixture of two probes, one derived entirely from the InC9 insert and the other overlapping the exon1/intron1 junction (Figure 3.4B). As expected from the results presented in the previous section, the RT-PCR reaction with the primers flanking InC9 produced a high amount of the 1188-bp product from KFC9 RNA, and none from Ep155 RNA. In contrast, the reactions using primers that flank intron 1 generated equal amounts of the 140-bp and 2340-bp products from the KFC9 RNA as from and Ep155 RNA. In the case of KFC9, the amount of the 1188-bp product derived from the InC9 insert was

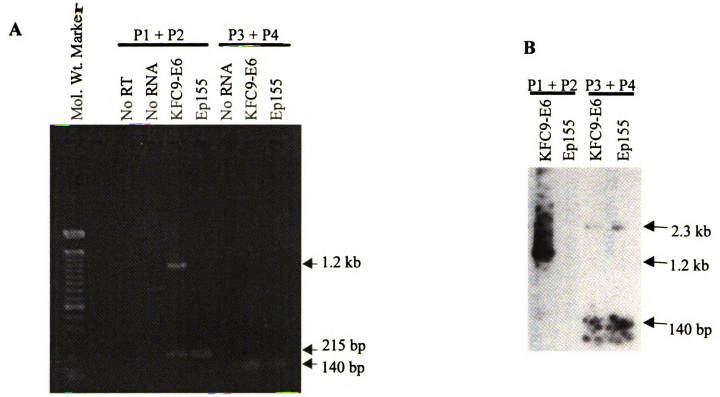

Figure 3.4. Comparison of splicing activity of InC9 and the downstream intron1. A RT-PCR products from Ep155 and KFC9-E6 mitochondrial RNAs using primers P1 and P2 that flank InC9, and P3 and P4 that flank intron1.**B** RT-PCR products were run on an agarose gel and blotted on to a nylon membrane. The Southern blot was hybridized using a mixture of two probes, one derived entirely from InC9 sequence and the other derived from the region spanning the 5' exon1-intron1 junction. The 2.3-kb and the 1.2-kb fragments are the ones derived from unprocessed mtS-rRNA transcripts.

noticeably higher than the amount of the 2340-bp product generated from precursor RNA molecules that retained the intron 1 sequence. Collectively, these results indicate that the InC9 RNA segment is spliced very slowly, if at all, from the mtS-rRNA precursor, whereas the *bona fide* intron 1 RNA segment is spliced as rapidly and efficiency in the KFC9 mutant as in the Ep155 wild type.

Rare splicing of the InC9 sequence from mtS-rRNA precursor is incomplete. As observed in the Northern hybridizations and confirmed by the RT-PCR studies, there is some mature mtS-rRNA present in the mitochondria of the KFC9 mutant. This could be the manifestation either of heteroplasmy for mutant and some normal mtDNA or of the occasional removal of the InC9 sequence from a portion of the mtS-rRNA primary transcripts. To distinguish between these two alternatives, primer-extension experiments were performed on DNaseI-treated mitochondrial RNA templates using an antisense primer, P5, which anneals to a sequence that begins 64 nucleotides downstream of the site where the 5' end of the InC9 sequence is joined to the mtS-rRNA. For the determination of the size of the products, they were separated on a sequencing gel simultaneously with the products of a standard di-deoxy sequencing reaction primed by P5 on the corresponding cloned segment of KFC9 mtDNA. As shown in Figure 3.5A, the primer-extension product was two nucleotides shorter than the product which was expected if the InC9 sequence would have been spliced accurately. This result not only demonstrated that the first two nucleotides are lost from the 5' end of the InC9 sequence whenever it is removed from the primary transcript, but also indicated that the same two nucleotides

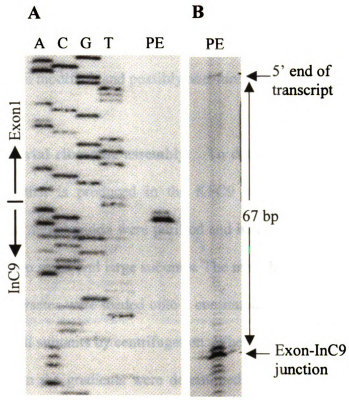

Figure 3.5 Primer extension analysis for spliced InC9 molecules. A The primer extension reaction was run next to a sequencing reaction performed using the same primer (P5; see Figure 3.1A) for accurate determination of the primer extension product size. **B** A primer extension reaction using the antisense primer P5 that anneals to the InC9 sequence shows two distinct products; the smaller product being the 5' end of the spliced InC9 molecules while the larger molecule is the run-off product obtained at the 5' end of the mtS-rRNA transcript.

might be left behind in the completely processed mtS-rRNA molecules that appeared in the KFC9 strain. An additional larger product, which is actually the run-off product at the 5' end of the RNA molecule, was also obtained (Figure 3.5B). In agreement with previous results, this observation indicates that a significant amount of mtS-rRNA molecules do not have InC9 spliced out. Thus, on the rare occasions when the Inc9 sequence is removed from the precursor transcript, it appears to be spliced inaccurately and by a process that yields a modified, and possibly non-functional, ribosomal RNA.

KFC9 lacks in mitochondrial ribosome assembly. To determine whether or not the mitochondrial mtS-rRNA that is produced in the KFC9 strain is incorporated into ribonucleoprotein particles, mitochondria were purified and incubated with puromycin to dissociate the ribosomes into small and large subunits. The mitochondria were then lysed with Triton X100 and the lysates were loaded onto a continuous sucrose gradient for the separation of large and small subunits by centrifugation. When the amounts and positions of nucleoprotein particles in the gradients were determined by UV-absorbance (Figure 3.6), 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 mitochondria of the mutant. The deficiency in small subunits was anticipated from the results indicating that most of the mtS-rRNA is abnormal in the mutant, but the absence of large subunits was not expected and can not be explained at present.

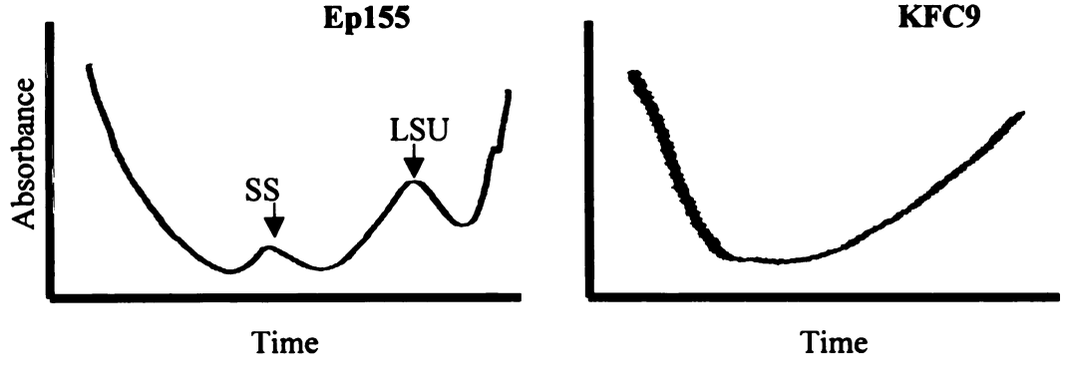

Figure 3.6. Ribosome profiles of the wild-type Ep155 strain and the mutant KFC9-E6. The peaks for the large subunit of ribosomes and the small subunit of ribosomes are represented by LSU and SSU respectively.

Transmission of InC9. The InC9 sequence does not have features that identify it as a mobile element, yet, it 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 *Eco*RI-*Hind*III fragment that contains InC9 (Figure 3.1A). The *Eco*RI 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 (Figure 3.1B). To explore if InC9 moves on its own or in conjunction with flanking segments of the mitochondrial chromosome, the regions of mtDNA that are located upstream and downstream of the insertion point of this element were cloned and sequenced as *Eco*RI-*Hin*dIII mtDNA fragments from several strains to discover sequence polymorphisms that could be used to resolve this question. Relative to the KFC9 mtDNA, a single base deletion was found only 80 bp upstream of the insertion point in the virulent J2.31 strain (Figure 3.7). However, no sequence variants could be identified downstream from the insertion point. In the converted hypovirulent form of J2.31, the upstream single base-pair deletion was absent and the sequence resembled that of KFC9. In contrast, the RFLPs that are characteristic of the mtDNA of the virulent form of J2.31 were retained by the converted strain. Thus, it appears that at least some of the mtDNA that is located upstream of the point of insertion is transmitted into the recipient strains along with InC9. The lack of an appropriate genetic marker has hampered the determination of whether or

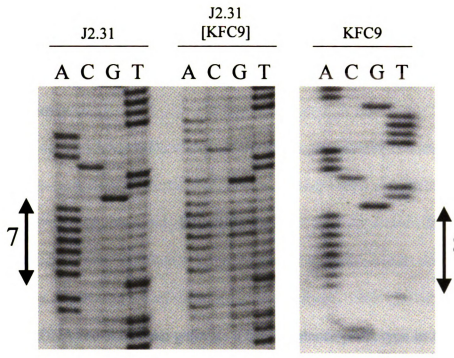

Figure 3.7. Transmission of upstream mtDNA sequences with InC9 along with the transmission of hypovirulence. Dideoxy chain-termination sequencing products, using the same primer, were separated on a polyacrylamide gel. Whereas the wild-type J2.31 strain has a stretch of 7 adenosines, the KFC9-E6 strain, as well as the hypovirulent J2.31[KFC9] strain has 8 adenosines in that same region, which is only 80 bp upstream of the InC9 sequence.

not sequences that are located downstream also are transferred with the InC9 element. However, the results suggest that the conversion of virulent strains to the hypovirulent state after contacts with KFC9 is caused by the suppressive accumulation of mtDNA molecules that retain the innocuous RFLP markers of the recipient type, but have acquired InC9 together with an undetermined length of the flanking sequences by recombination with mtDNA molecules that were introduced from the donor through hyphal anastomoses.

DISCUSSION

That mtDNA mutations can generate a hypovirulence phenotype in *C. parasitica* was established conclusively through the analysis of artificially-induced mutations that produced cytoplasmically-transmissible, maternally-inherited respiratory defects and cytochrome-deficiencies in the Ep155 virulent laboratory strain (Monteiro-Vitorello *et al.*, 1995). Not only did these mutants have cytoplasmically-transmissible and maternally-inherited hypovirulence phenotypes, but they proved to be even less aggressive than strains that were attenuated by infections with dsRNA hypoviruses. On the basis of these observations, it was predicted that the infectious hypovirulence phenotype of the virus-free KFC9-E6 strain of *C. parasitica*, which was isolated from a healing canker on a tree in the Kellogg Forest in Michigan and shows a senescence phenotype that is associated with respiratory deficiencies, might be caused by a "suppressive" mtDNA mutation

(Baidyaroy *et al.*, 2000). In this study, we have located this mutation and have shown that it is a 973-bp insertion named InC9, in the mtS-rRNA gene that most likely produces senescence and hypovirulence by blocking mitochondrial protein synthesis through interference in the assembly of mitochondrial ribosomes. This is the first definitive identification and molecular characterization of a spontaneous mtDNA mutation that gives rise to the mitochondrial hypovirulence trait in a phytopathogenic fungus in a natural ecological setting.

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 diseased isolates to virulent strains. Such transfers result in the conversion of virulent recipients to the hypovirulent state which is accompanied by the appearance of the associated senescence traits. These observations confirm that InC9 is a "suppressive" mtDNA mutation. Suppressiveness was initially defined by Ephrussi *et al.* (1955) as the capacity of mutant alleles of cytoplasmic genes to eliminate the corresponding wild-type alleles in zygotic cells of the yeast *Saccharomyces cerevisiae*. In the filamentous fungi, suppressiveness is attributed to mtDNA mutations that cause the gradual disappearance of functional organelles and extinction of the wild-type phenotype. While the mechanism that causes the gradual displacement of wild-type mtDNA by deleterious mutant forms in growing mycelia, i.e. "suppressiveness", still is the subject of some controversy (Jamet-Vierny *et al.*, 1999), at least in *Neurospora* it has been shown that the phenomenon is elicited by mtDNA point-mutations, deletions and insertions that disrupt cytochrome-dependent electron-transport activity (Bertrand 1995). 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 they are functionally crippled by InC9. Thus, the "renegade" mutant mitochondria of the diseased strains can "infect" virulent strains and convert them to the hypovirulent state through aggressive invasion of the coenocytic hyphae.

Senescence phenotypes in filamentous fungi are caused by a variety of mtDNA mutations. In *Podospora anserina*, senescence is commonly associated with the excision, circularization and subsequent amplification of small segments of mtDNA (Cummings *et al.*, 1979; Jamet-Vierney *et al.*, 1980). Certain *Neurospora* strains senesce because of integration of linear (Bertrand *et al.*, 1986) and circular (Akins *et al.*, 1986) plasmids into the mtDNA, whereas large deletions in mtDNA have been detected in *stopper* mutants of *N. crassa* (Bertrand *et al.*, 1980; de Vries *et al.*, 1981), the *ragged* mutants of *Aspergillus amstelodami* (Lazarus *et al.*, 1980; Lazarus and Küntzel, 1981) and in *Podospora curvicolla* (Böckelmann and Esser, 1986). In the context of all the different types of mutations that so far have been implicated in fungal senescence, the mutation in KFC9 is unique in that it is a disruption of the mtS-rRNA gene by insertion of a relatively long nucleotide sequence of unknown origin or function. The intriguing aspect is that an infectious type of hypovirulence, which remotely resembles that which is caused by hypoviruses, is an integral part of the phenotype that is produced by this mutation. Repeated sampling of the site has produced data indicating not only that the mutation has been retained during the past ten years in the population of *C. parasitica* at the Kellogg Forest site, but also that the proportion of isolates that have InC9 in their mtDNAs and have the mitochondrial hypovirulence phenotype has grown steadily and now has surpassed the 90% mark (Baidyaroy *et al.* 2000). So far, the observations gathered from

the Kellogg Forest population strongly support the conclusion that a spontaneous mtDNA mutation is effecting a natural control over chestnut blight because it is maintained as a stable infectious element by the population of *C. parasitica* at that site. It is possible that invasions of populations of filamentous fungi by deleterious mitochondrial genetic elements are fairly common, but are rarely recognized because the diagnosis requires manipulation of the organism in the laboratory.

As expected for a mutation or a gene that causes a particular effect, InC9 appeared in all the derivatives of virulent strains that had acquired hypovirulence and senescence traits 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 conidial 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 these single-conidial isolates became essentially homoplasmic for mtDNA molecules of the recipient type into which the InC9 sequence was inserted. This effect could be interpreted to 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 the converted strains suggests that recombination, rather than transposition, may account for the transfer of the InC9 sequence into the mitochondrial chromosomes of the recipients. It is likely that multiple rounds of reciprocal recombination in the heteroplasmons that were generated in the conversion experiments resulted in the transfer of InC9 from a small pool of donor mtDNA molecules into some units of the relatively large pool of recipient mtDNA

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molecules. Since mtDNA recombination has been observed in artificially-generated *C. parasitica* heteroplasmons (Polashock *et al.*, 1997), it is plausible that this process caused the separation of InC9 from the innocuous RFLPs that are present in the donor mtDNA and resulted in the dissipation of these markers into the pool of recipient mtDNA molecules. Since the suppressive nature of the disrupted mtS-rRNA gene causes strong selection for mtDNA molecules that contain InC9, it is not surprising that the mtDNA molecules that accumulate during the conversion process retain the RFLP markers of the recipient, but have acquired the insert together with an undetermined amount of flanking donor mtDNA through homologous recombination. Moreover, the results indicate that the acquisition of a very small amount of a 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.

The characterization of the nucleotide sequence of InC9 suggests that the element may be a piece of DNA that has no particular function. This notion is further supported by at least two additional observations. Firstly, the corresponding 973-bp RNA sequence remains firmly lodged in the mtS-rRNA that are produced from the mitochondrial gene in which the element is located, even though all the *bona fide* introns are effectively spliced from the corresponding primary transcript. Secondly, InC9 appears to be replicated and transmitted as an integral component of the mtS-rRNA gene rather than an autonomous or semi-autonomous genetic element, such as a plasmid or an active transposon. Thus, the integration of the InC9 sequence into the mtS-rRNA gene may have occurred as a one-time event that created the suppressive lethal mutation that now causes senescence by

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selection for the mutant mitochondrial gene in heteroplasmons. However, as a lethal allele, the ribosomal DNA that contains InC9 probably is maintained in the population of *C. parasitica* in the Kellogg Forest because there is a flow of mitochondria through hyphal anastomoses 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. Contributing to the establishment of this equilibrium might be factors such as the production of asexual spores, which is decidedly more abundant on healthy mycelia than senescent mycelia of the Kellogg Forest strains.

InC9 behaves genetically and functionally like a lethal mutation that disrupts the mtS-rRNA gene and abrogates the assembly of the small subunit of mitochondrial ribosomes, and thus prevents the synthesis of essential mitochondrial proteins. Included in this set of proteins is S5, a small-subunit protein that is encoded by an intron in the L-rRNA gene (Hausner *et al.*, 1999). Thus, the deficiency in the small subunits of the ribosomes by itself is sufficient cause for the appearance of the hypovirulence and senescence traits in the KF strains. However, it does not provide a rationale for the absence of the large subunit of ribosomes in the mutant. One possible explanation would be that the mtDNA of *C. parasitica* contains a gene that specifies a large-subunit ribosomal protein. However, no such gene has been found so far in the mtDNA of an ascomycetous fungus (Taylor and Smolich, 1985; Silliker and Cummings, 1990; Gillham, 1994; Bell *et al.*, 1996). Another possibility is that the stability of the large subunit somehow is linked to the assembly or presence of small subunits. This concept has not been explored experimentally at this time.

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Sexual transmission of the InC9 mitochondrial mutation so far has not been observed in the laboratory, mostly because senescent mycelia do not function as female parents in crosses. No attempts have been made yet to cross pre-senescent cultures that are heteroplasmic for the mutant and wild-type factors. However, lines of circumstantial evidence indicate that the mutant mtDNA may be transmitted sexually within the population of *C. parasitica* at the Kellogg Forest site. The first observation is that the isolates from that site are very heterogeneous with respect to their nuclear genotypes, but the mutant mtDNA appears in association with most, possibly all, the variants that are represented in the population (Baidyaroy *et al.*, 2000). The second observation is that the population of *C. parasitica* at the Kellogg Forest site presents the most diverse vegetative compatibility profile of all the populations that are present in blighted chestnut stands in Michigan (Likins, 1990). Even though the mutant mtDNA can move through hyphal anastomoses across many of the vegetative incompatibility barriers that restrict the movement of nuclei (Baidyaroy *et al.*, 2000), it simply appears unlikely, though not impossible, that it has invaded through this route alone all the incompatibility groups that are represented at the Kellogg Forest location. The third observation is that induced suppressive mtDNA mutations are maternally inherited in *C. parasitica* (Monteiro-Vitorello *et al.*, 1995). 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). Collectively, the observations on the asexual and sexual transmission of mitochondrial hypovirulence

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indicate that this trait potentially can be used as an alternative or a supplement to mycoviruses to control this and other phytopathogenic fungi.

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CHAPTER 4

A mitochondrial plasmid-like element and its role in induction of hypovirulence in *Cryphonectria parasitica*

ABSTRACT

In isolates of the chestnut-blight fungus *Cryphonectria parasitica* from the Kellogg Forest, Michigan, a cytoplasmically transmissible hypovirulence phenotype has been shown to be elicited by a debilitating mitochondrial DNA (mtDNA) mutation. This mutation consists of an insertion of a 973-bp nucleotide sequence, named InC9, into the mitochondrial small subunit ribosomal RNA (mtS-rRNA) gene. An additional mitochondrial element, pleC9, has now been detected in the strains rendered hypovirulent by InC9. This is a circular DNA element, which is present as a 1.4-kb long monomer as well as multimeric forms consisting of head-to-tail tandem repeats of the same element. Only a small part of pleC9 (130 bp) is homologous to *C. parasitica* mtDNA, and the origin of the rest of this DNA remains unknown. The accumulation of this DNA in the mitochondria appears to adversely affect growth of the fungus on synthetic medium. However, the appearance of the pleC9 element did not always correlate with the hypovirulence phenotype. Thus, it appears that the pleC9 element is not the primary

cause of hypovirulence. Size variants of the pleC9 element were observed in different isolates of C. parasitica from the Kellogg Forest location. These variants could have been generated by intra- and inter-molecular recombination of pleC9 because it contains several duplicated segments of DNA.

INTRODUCTION

The chestnut blight disease caused by the fungus *Cryphonectria parasitica* has resulted in virtual elimination of the entire population of the North American chestnut, *Castanea dentata*. Some degree of biological control of this fungus can be achieved by using double stranded RNA (dsRNA) mycoviruses that cause a cytoplasmic hypovirulence syndrome (Nuss, 1992). However, several occurrences of hypovirulent *C. parasitica* strains devoid of any mycoviruses have been reported (Fulbright, 1990; Mahanti *et al.*, 1993; Baidyaroy *et al.*, 2000a). The hypovirulence phenotypes of these strains were found to be cytoplasmically transmissible (Baidyaroy *et al.*, 2000a). Furthermore, all of these virus-free, attenuated strains were found to have increased levels of alternative oxidase activity (cyanide-resistant respiration) suggesting that this type of hypovirulence is associated with genetic alterations in the mitochondria that cause deficiencies in cytochrome-mediated respiration. The phenotypic characteristics of this type of hypovirulence (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000a) suggest that it is comparable to debilitating phenotypes caused by mtDNA

mutations in *Neurospora*, *Podospora* and *Aspergillus* (reviewed in Griffiths, 1992; Bertrand, 2000).

After the mtDNA of the standard laboratory wild-type strain Ep155 was mutagenized, two hypovirulent mutants were isolated which had high levels of alternative oxidase activity (Monteiro-Vitorello *et al.*, 1995). The hypovirulence phenotype was found to be cytoplasmically transmissible and hence, these laboratory-generated mutants mimicked the dsRNA-free hypovirulent strains from nature. The phenotype of hypovirulence and high alternative oxidase activity were maternally inherited in sexual crosses, thereby confirming that the genetic determinants were indeed located in the mitochondria. Upon analysis of the mtDNA, a highly amplified, plasmid-like element was detected in these mutants, which further established the role of dysfunctional mitochondria in these hypovirulent strains (Monteiro-Vitorello *et al.*, 1995). These results also indicated that it was possible that the genetic determinant of the cytoplasmically transmissible, virus-free hypovirulence phenotype found in nature are debilitating mtDNA mutations. Based on these observations, this type of virus-free hypovirulence has been referred to as "mitochondrial hypovirulence" (Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000a).

Hypovirulent, dsRNA-free strains have been isolated from a grove of American chestnut trees at Kellogg Forest in Augusta, Michigan (Baidyaroy *et al.*, 2000a). This particular grove has been found to be recovering from blight with the appearance of a high number of swollen (healing) cankers. The hypovirulence phenotype of these isolates is cytoplasmically transmissible and associated with elevated levels of cyanide-resistant respiration (Baidyaroy *et al.*, 2000a). Thus, the strains exhibited all the features of

mitochondrial hypovirulence. A specific region of the mtDNA was found to contain an insertion, referred to as InC9, which was identified as the mutation causing the disease syndrome in these strains (Chapter 3 of this thesis). The InC9 element is a 973-bp long nucleotide sequence of unknown function and origin. It is located in the mtS-rRNA gene, and disrupts the assembly of mitochondrial ribosomes (Chapter 3 of this thesis). However, apart from InC9, some of the hypovirulent strains were found to contain amplified, plasmid-like DNAs derived entirely or partially from the mtDNA. In this study, this novel element has been analyzed to gain a better understanding of its role, if any, in mediating hypovirulence in the strains from Kellogg Forest. Moreover, a population of *C. parasitica* strains from this location were analyzed to establish the prevalence and dispersal of this element in nature.

MATERIALS AND METHODS

Fungal strains, culturing conditions, respiration assays and virulence tests. The *C. parasitica* strains of used in this study are listed in Table 4.1. Cultures were maintained on Endothia complete medium (ECM) (Puhalla and Anagnostakis, 1971). Methionine was added to the medium at a concentration of 0.1 mg/ml to grow the methionine-deficient strain Ep289 *met*. Tests for alternative oxidase activity were performed as described by Monteiro-Vitorello *et al.* (1995). Virulence tests were performed

Table 4.1. Important features of strains used in this study.

[a] Virulence status of the strains are designated by 'V' for virulent and 'HV' for hypovirulent.

[b] Presence and absence of the element is indicated by '+' and a '–', respectively.

[c] For the strains containing pleC9 or its derivatives, the sizes of different elements homologous to pleC9 are given in kilo-base pairs. When a strain contains more than one element of a particular size, and one of those predominates the others, the predominant element is marked with a '*' sign. When a certain element is present in the mitochondria in very low amounts, it is designated by a '†' sign. Elements that do not contain a *Xba*I site in their sequence are mentioned as 'no *Xba*I'. Absence of pleC9 is represented by the '–' symbol.

Table 4.1. Important features of strains used in this study.

Strain	Source	Virulence Status[a]	InC9[b]	pleC9[c]
Ep155	ATCC 38755	V	–	–
KFC9-E6	Kellogg Forest	HV	+	1.4
Ep289	Baidyaroy *et al.*, (2000a)	V	–	–
Ep289[KFC9]	Baidyaroy *et al.*, (2000a)	HV	+	1.4
J2.31	Baidyaroy *et al.*, (2000a)	V	–	–
J2.31[KFC9]	Baidyaroy *et al.*, (2000a)	HV	+	1.4, 1.3
J2.31[KFC9-E6]	Baidyaroy *et al.*, (2000a)	HV	+	1.4, 1.3
F2.36	Baidyaroy *et al.*, (2000a)	V	–	–
F2.36[KFC9-E6]	Baidyaroy *et al.*, (2000a)	HV	+	1.4*, 1.3, 0.75
KFD9	Kellogg Forest	HV	+	1.3†
KFD10	Kellogg Forest	HV	+	–
KFD18	Kellogg Forest	HV	+	1.3
KFD19.1	Kellogg Forest	HV	+	No *Xba*I
KFD19.2	Kellogg Forest	V	–	1.3
KFD27.1	Kellogg Forest	HV	+	No *Xba*I
KFD27.2	Kellogg Forest	HV	+	0.8, 1.3†, 1.1†
KFD27.4	Kellogg Forest	HV	+	1.3, 1.7†
KFD27.10	Kellogg Forest	HV	+	0.8, 1.3†, 1.2†
KFC27.2	Kellogg Forest	HV	+	1.0†
KFC27.4	Kellogg Forest	HV	+	1.3, 1.7†
3V1	Kellogg Forest	HV	+	No *Xba*I
4V1	Kellogg Forest	HV	+	–
8HV1	Kellogg Forest	HV	+	No *Xba*I
9HV3	Kellogg Forest	HV	+	1.7*, 2.1, 1.3†
16V2	Kellogg Forest	HV	+	1.65, 2.05†, 1.4†, 1.25†
16V3	Kellogg Forest	HV	+	–
16HV1	Kellogg Forest	HV	+	1.3
16HV3	Kellogg Forest	HV	+	1.65,1.2, 2.05†
16HV4	Kellogg Forest	HV	+	–
18V1	Kellogg Forest	HV	+	1.4
18HV1	Kellogg Forest	HV	+	1.6, 1.3, 2.1†
30V2	Kellogg Forest	HV	+	No *Xba*I
WISC 23.2-Euro7	DWF collection	HV	–	Not tested

on apples (Fulbright, 1984), on live chestnut tissue (Lee *et al.*, 1992) and on live trees (Elliston, 1978).

Preparation of mitochondrial DNA and RNA and genomic DNA. Mitochondria were purified by sucrose flotation-gradient procedure (Lambowitz, 1979), and mtDNA was prepared as described by Bell *et al.* (1996) with an added purification step using cetyltrimethylammoniumbromide (Ausubel *et al.*, 1987). RNA was isolated from purified mitochondria by the SDS-diethylpyrocarbonate procedure (Solymosy *et al.*, 1968). Total genomic DNA from fungal cultures was isolated as described previously (Baidyaroy *et al.*, 2000a).

Molecular cloning and standard DNA and RNA manipulations. Restriction enzymes were obtained from Gibco BRL (Gaithersburg, Maryland). Enzymatic digestions of DNAs, molecular cloning, agarose gel electrophoresis and Southern blotting were done as described by Sambrook *et al.* (1989). Southern blot hybridizations were performed with chemiluminescent probes as directed by the manufacturer (Boehringer Mannheim, Indianapolis, Indiana). For nuclear DNA fingerprinting, Southern blots were hybridized with a plasmid (pMS5.1) containing a cloned moderately repetitive nuclear DNA fragment (Milgroom *et al.*, 1992).

DNA Sequencing. Sequencing was performed manually by the di-deoxy chain-termination method (Sanger *et al.*, 1977) with αP^{33}-labelled adenosine triphosphate (dATP) followed by resolution in polyacrylamide gels and visualization through

autoradiography. Automated sequencing of DNA was performed using the ABI Catalyst 800 kit for Taq cycle sequencing and the ABI 373 Sequencer. Initial sequences of inserts in the BluescriptKS+ vector were obtained using vector-specific forward and reverse primers. These initial sequences were subsequently extended with sequence-specific primers synthesized by the Michigan State University Macromolecular Synthesis Facility. Both strands were completely sequenced in all cases. Sequences were aligned and databases were searched using the MicroGenie™ MG-IM-5.0 (Queen and Korn, 1984) and BLAST (Altschul *et al.*, 1997) computer software, respectively.

Polymerase chain reaction (PCR). PCR was done as suggested by the manufacturer (Promega Inc., Madison, Wisconsin). Template DNAs were boiled for 5 min before being added to the reaction tubes. The DNA was denatured at 93°C for 3 min and subjected to PCR for 30 cycles, each cycle consisting of the following successive steps: 93°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The last cycle consisted of an extension reaction at 72°C for 10 min. The sequence of the primers used to detect the presence of InC9 in fungal isolates were designed on the basis of the nucleotide sequence of the mtS-rRNA gene of *Cryphonectria parasitica* (GenBank accession no. AF029891) and are as follows: 5'- GGTTGGTGATTCTTTCATGG-3' (forward primer) and 5'-TACACTCACCTGTACAC-3' (reverse primer). The sequence of the primers used to detect the presence of pleC9 in cultures are as follows: 5'-GTCTTAGCCCTGCATCC-3' (P1; forward primer) and 5'-CCTAAGAGGGGACCCTT-3' (P2; reverse primer). The positions of these two primers on pleC9 are shown in Figure 4.3A.

Two-dimensional gel electrophoresis. The two-dimensional gel electrophoresis experiments were performed as described by Brewer and Fangman (1988; 1991). The first-dimension of DNA was resolved on 0.5 % agarose gels at 1 V/cm for approximately 24 hr. The second-dimension was electrophoresced on a 1.2% agarose gel at 5V/cm for 14 hours at 4°C in the presence of ethidium bromide (0.4 μg/ml). The DNA was blotted on to positively-charged nylon membranes (Amersham) and hybridized with a probe derived from the cloned pleC9 DNA.

RESULTS

Identification of the plasmid-like element in hypovirulent strains. When genomic DNA from the mitochondrially-hypovirulent strain KFC9-E6 was digested with the enzyme *Xba*I and the pieces were separated on an agarose gel, a highly amplified 1.4-kb molecule was observed when stained with ethidium bromide (Figure 4.1A). However, this DNA species was not observed as a single dense band when the same DNA preparation was digested with a different restriction enzyme (Figure 4.1A). This phenomenon is a characteristic feature of highly amplified, circular DNA molecules, which exist as a population of multimeric forms and sizes, and hence are dispersed by electrophoresis throughout the gel as shown for this particular element in Figure 4.1A. These observations indicate that the hypovirulent strain KFC9-E6 contained an element that is highly amplified, probably circular in conformation, and exists as a series of

multimers *in vivo*. That this DNA was mitochondrial in location was ascertained when it was detected as a 1.4-kb fragment in mtDNA preparations of the same strain (Figure 4.1B). Since no other amplified fragments were visible upon digestion with *Xba*I, it was assumed that the 1.4-kb linear molecules represent a full-length repeat unit of the element, which has been named pleC9 (plasmid-like element from strain KFC9). The *Xba*I fragment was cloned from KFC9-E6 mtDNA and used as a probe in further experiments. When Southern blots of electrophoretically-separated fragments of *Xba*I digested genomic DNAs from several wild-type strains and their corresponding hypovirulent forms (Baidyaroy *et al.*, 2000a) were probed with this cloned element, only the hypovirulent strains were found to contain the pleC9 DNA (Figure 4.1C). The 2.8-kb molecule that hybridizes is the dimeric form of the element. Analysis of other fragments that can be seen in Figure 4.1C that hybridize with the same probe will be presented later in this study. Thus, pleC9 appears to be present and amplified only in strains rendered mitochondrially hypovirulent by contact with KFC9.

***In vivo* conformation of pleC9.** From the results described in the previous section, it seemed that pleC9 was a circular DNA element existing not only as a unit-length molecule but also in various multimeric forms of the unit-length element. This feature of pleC9 was further ascertained when a Southern blot of uncut mtDNAs from mitochondrially hypovirulent strains (Baidyaroy *et al.*, 2000a) were hybridized with the pleC9 probe (Figure 4.2A). The probe hybridized, in each lane, with numerous bands instead of one unique fragment. In addition, the intensity of hybridization was dissimilar for all of the different bands that hybridized. These observations suggest that

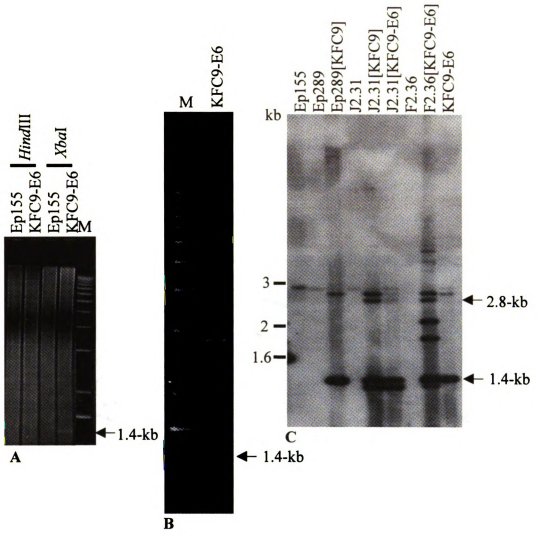

Figure 4.1. Identification of pleC9 DNA in hypovirulent strains. Lane M in both the panels A and B represents molecular weight markers. **A** The pleC9 element is visible as a 1.4-kb amplified fragment in KFC9-E6 when the DNA is digested with *Xba*I. If digested with other enzymes like *Hind*III, the element is not visible. It is absent in the wild-type strain Ep155. **B** pleC9 DNA is observed also in the mtDNA fraction of KFC9-E6 as amplified DNA when digested with *Xba*I. **C** Southern blot hybridization of XbaI digested genomic DNAs from virulent strains and their hypovirulent counter-parts with a pleC9 probe. The pleC9 element is visible as a 1.4-kb monomer or a 2.8-kb dimer only in the hypovirulent strains. The molecular weight marker is given at the margin for the precise determination of sizes.

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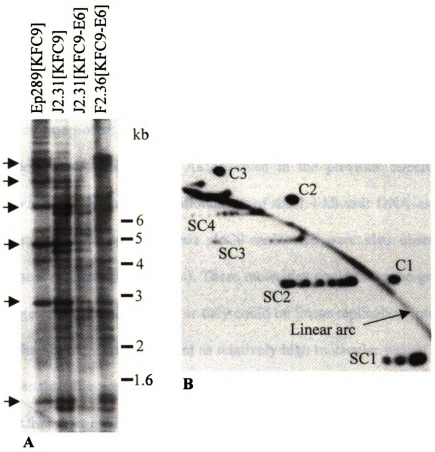

Figure 4.2. ***In vivo*** **conformation of pleC9 DNA. A** Southern blot hybridization of uncut mtDNAs from hypovirulent strain with a pleC9 probe. The unit length molecule and other multimers are indicated by arrows. The molecular weight marker is given at the margin for the precise determination of sizes. **B** Two-dimensional gel profile of pleC9 DNA was obtained by hybridizing a Southern blot of KFC9-E6 mtDNA separated in two dimensions with a pleC9 probe. The relaxed circular monomer, dimer and trimer are indicated as C1, C2 and C3 respectively. Similarly the corresponding supercoiled circular forms are indicated as SC1, SC2, etc.

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pleC9 is present as an element that exists in more than one particular size. The conformation of the element was established by electrophoresis where mtDNA from the hypovirulent strain KFC9-E6 was separated on two dimensions, blotted on to a membrane and hybridized with the pleC9 probe (Figure 4.2B). From the pattern of hybridization, it appears that a majority of the pleC9 element exists either as supercoiled, circular molecules or in relaxed circular forms. The different series of molecules observed in each group of supercoiled circular DNAs of a particular size correspond to the different degrees of supercoiling. As observed in the previous experiments, the element was found to exist as circular monomers of the 1.4-kb unit DNA as well as in multimeric forms. Some randomly sized pleC9 molecules were also observed to be migrating as linear molecules (linear arc). These molecules could either be generated by random breakage of the circular forms, or they could be linear replication intermediates. Since most of the linear forms are present as relatively high molecular weight molecules, which are larger than the predominant circular forms, and in a continuous gradient in terms of size rather than having some predominant forms, it might be concluded that a part of these linear forms probably are replication intermediates where pleC9 replicates predominantly by a rolling circle mechanism. This notion is further supported by the observation of multimeric circular forms of pleC9 that can be generated by rolling circle replication. However, the generation of a part of the linear forms by breakage of circular molecules during preparation and handling of the DNA cannot be ruled out.

DNA sequence and characteristics of pleC9. Both strands of the cloned 1.4-kb *Xba*I fragment representing the unit-length pleC9 was sequenced completely (GenBank

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accession no. AF218210; Appendix B). This ascertained the true length of the element at 1364 bp. The pleC9 sequence was unusually rich in its G-C content (44.1%) relative to known sequences of the mtDNA of *C. parasitica* (21%; Hausner *et al.*, 1999; Monteiro-Vitorello *et al.*, 2000), did not have any sizable open reading frames (ORF), and was not similar to any sequence known so far. There were many direct repeats of various sizes present on pleC9 as shown in Figure 4.3A. To determine whether or not all or part of pleC9 is generated from the mtDNA of *C. parasitica*, the pleC9 DNA was hybridized to restriction-enzyme digested mtDNA of the wild-type strain Ep155, which does not contain the pleC9 element. A *Pst*I restriction fragment of the Ep155 mtDNA was found to hybridize with pleC9 (Figure 4.3B). This fragment was cloned, and sequenced to find the corresponding region of homology to the pleC9 sequence. Only a short region of mtDNA (127 bp) was found to be similar to the pleC9 sequence. However, in pleC9, this homologous region was split by a 72-bp long intervening sequence. The homologous region of mtDNA was found to be a part of an ORF that encodes a 779 amino acid long maturase-like protein (GenBank accession no. AF218567; Appendix C).


Segments of homology
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Region of deletion in
J2.31[KFC9-E6] element

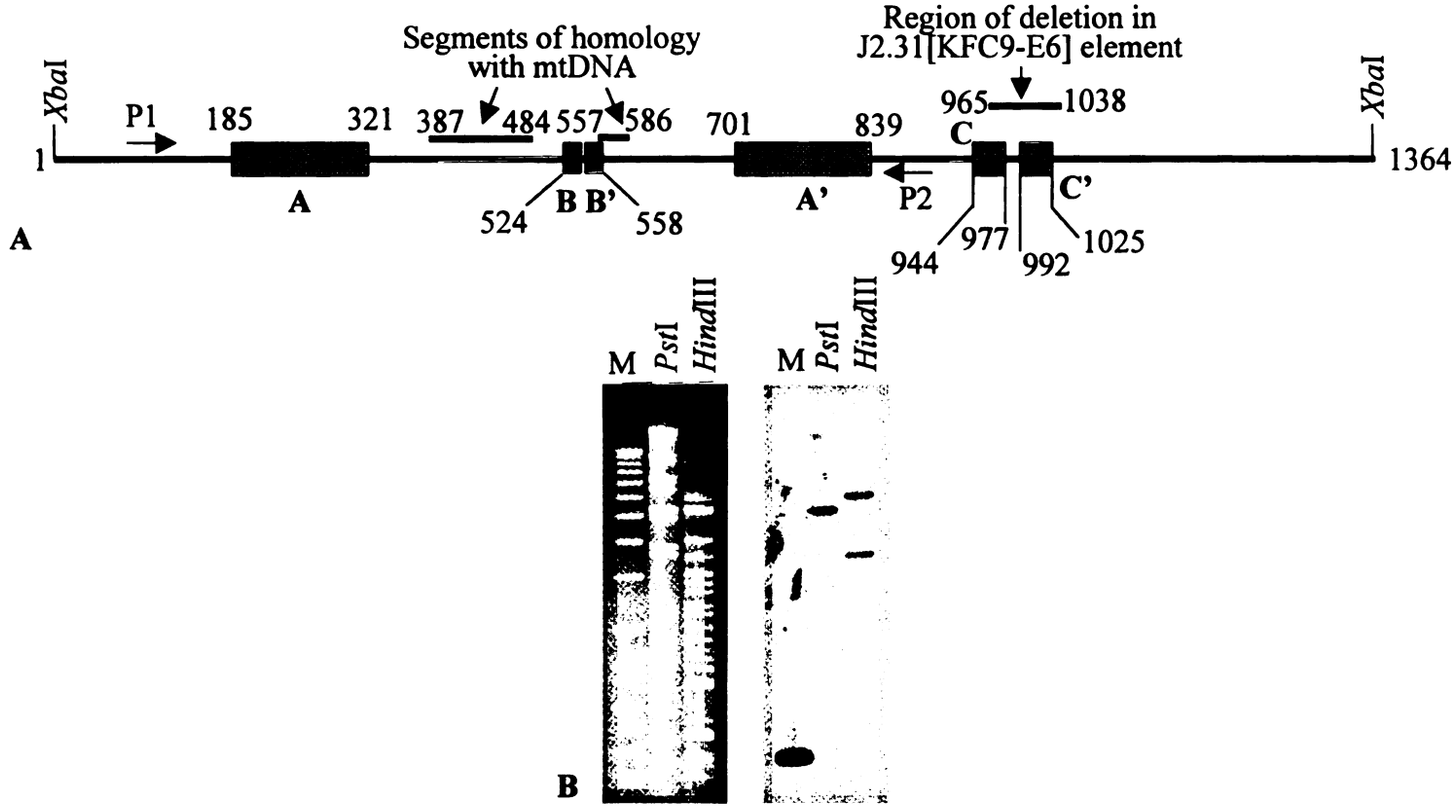

Figure 4.3. Sequence characteristics of the pleC9 element. A Schematic representation of pleC9 DNA. The element is shown as a 1363-bp long, linear XbaI clone. The repeats are given as the A and A', B and B', and C and C' elements. The position of the repeats is given in numericals. The region of homology to the *C. parasitica* mtDNA also is shown. The position of the two primers P1 and P2, used in this study, are given. **B** Homology of the pleC9 element with *C. parasitica* mtDNA. MtDNA from the wild-type strain Ep155, which is devoid of pleC9 DNA was digested with the enzymes *Pst*I and *Hind*III. Lane M represents molecular weight markers. The corresponding Southern blot was probed with pleC9 DNA.

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pleC9 and its role in induction of hypovirulence. Since the converted mitochondrially-hypovirulent strains (Baidyaroy *et al.*, 2000a) contained the element pleC9 as well as InC9, which has been described previously as the cause of hypovirulence in these strains (Chapter 3 of this thesis), it was difficult to evaluate the role of the pleC9 element in the induction of hypovirulence. However, different growth phenotypes of the strain KFC9-E6 helped in understanding the effect of pleC9 on the *C. parasitica* isolates. KFC9-E6 is a very unstable isolate in terms of growth characteristics (Figure 4.4A). When isolated from nature and cultured on ECM, it was a relatively fast-growing strain and hence, designated KFC9-F. Upon sub-culturing, KFC9-F acquired a relatively stable slow-growth phenotype (KFC9-FS). The KFC9-FS strain again produced a fast-growing sector (KFC9-FSF) which subsequently degenerated rapidly into slow-growing mycelium (KFC9-FSFS). That all of these derivatives of KFC9-E6 had essentially the same nuclear background was confirmed by hybridizing their genomic DNAs with a probe for repetitive DNA (Figure 4.4B). The nuclear DNAs of two randomly picked single-conidial isolates of KFC9-FS gave the same fingerprint patterns as the KFC9-E6 strains. All of these derivatives were hypovirulent on apples, chestnut bark and trees, and had elevated levels of alternative oxidase activity (Table 4.2). All the derivatives also were found to retain in their mtDNAs the hypovirulence-inducing InC9 element (Figure 4.4C).

Only the derivatives of KFC9-E6 that have a slow-growth phenotype on ECM were found to contain pleC9 (Figure 4.4D). The single-conidial isolates of KFC9-FS had a fast-growth phenotype on ECM (reverted to the KFC9-F morphology or proceeded to the KFC9-FSF growth phenotype) and were found to be free of the pleC9 element. When

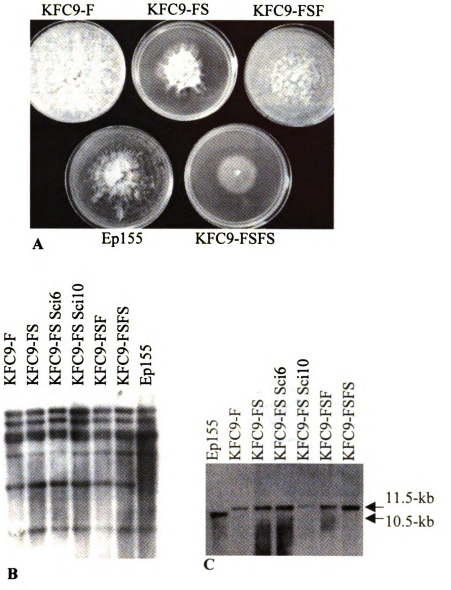

Figure 4.4. Effect of pleC9 DNA on *C. parasitica* strains. A Growth morphologies of different morphs of the mitochondrially hypovirulent strain KFC9-E6. **B** Nuclear fingerprint analysis by Southern blot hybridization of *Eco*RI digested genomic DNAs with a probe derived from a moderately repetitive nuclear DNA fragment (Milgroom *et al.,* 1992). **C** Presence of InC9 DNA in the KFC9-E6 derivatives is ascertained by hybridization of a larger 11.5-kb *Hind*III fragment as opposed to the 10.5-kb fragment in the wild-type strain Ep155, which does not have the additional 973-bp element. *Eco*RI digested genomic DNAs were separated on an agarose gel, Southern blotted, and probed with a probe derived from the cloned region of mitochondrial small subunit ribosomal gene of *C. parasitica* (Chapter 3 of this thesis).

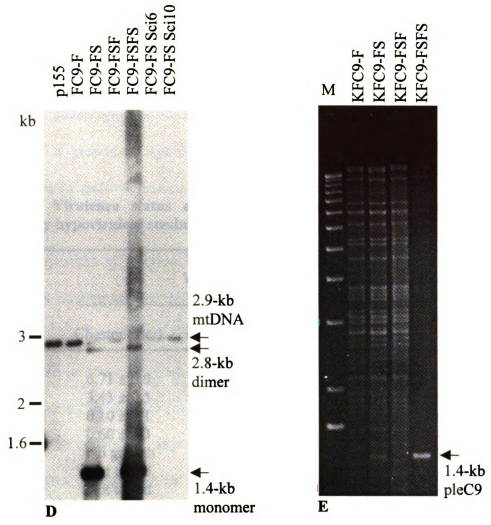

Figure 4.4. Effect of pleC9 DNA on *C. parasitica* strains. D Southern blot hybridization of *Xba*I digested genomic DNAs with a pleC9 probe indicating the presence of the 1.4-kb pleC9 DNA in only the slow-growing forms of KFC9-E6. The 2.8-kb large dimeric form of pleC9 is also observed. The 2.9-kb mtDNA fragment that hybridizes in all the strains contains the sequence that is homologous with the pleC9 element. The molecular weight marker is given at the margin for the precise determination of sizes. **E** MtDNAs were isolated from the KFC9-E6 derivatives and digested with XbaI and observed by ethidium bromide staining upon agarose gel electrophoresis. The pleC9 DNA is visible as a bright 1.4-kb fragment only in the slow-growing strains. In KFC9-FSFS, there is a considerable increase in the accumulation of the pleC9 element with concomitant loss of mtDNA, which is barely present now. MtDNA patterns of all the strains remained the same. Lane M contains the molecular-weight marker DNA.

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Table 4.2. Virulence status of the fast and slow-growing forms of the mitochondrially hypovirulent strain KFC9-E6.

Strain	Virulence[a] on			Alternative oxidase activity as % of total respiration
	Chestnut Bark	Apples	Tree	
Ep155	6.71 ± 0.9	24.19 ± 2.5	39.7 ± 7.1	7.5 ± 0.3
KFC9-F	3.43 ± 0.5	13.45 ± 3.1	6.98 ± 1.6	60.38 ± 15.9
KFC9-FS	0.10 ± 0.1	1.72 ± 0.7	No growth	59.65 ± 20.0
KFC9-FSF	0.60 ± 0.3	6.91 ± 2.3	No growth	54.01 ± 11.5
KFC9-FSF	No growth	No growth	No growth	91.73 ± 2.1
KFC9-FS Sci6	Not tested	6.35 ± 0.7	Not tested	Not tested
KFC9-FS Sci10	Not tested	6.36 ± 3.0	Not tested	Not tested
WISC 23.2-Euro7[b]	3.38 ± 0.7	Not tested	4.59 ± 2.5	Not tested

[a] Virulence is given as the area of lesion produced by a strain on either chestnut bark or apples.

[b] The WISC 23.2-Euro7 strain is a dsRNA virus-containing hypovirulent strain that has been used as a control.

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mtDNAs of the different morphological derivatives of KFC9-E6 were digested with *Xba*I, separated on an agarose gel, and stained with ethidium bromide, the absence of the amplified pleC9 DNA in the fast-growing strains was further confirmed (Figure 4.4E). However, in the slowest-growing form KFC9-FSFS, which has to be maintained in liquid medium, pleC9 is present in significantly higher amounts than in KFC9-FS, with a proportional loss in mtDNA content. The mtDNA profiles of the different KFC9-E6 derivatives were otherwise identical. Since, all the derivatives of KFC9-E6 were hypovirulent regardless of whether or not they contain pleC9, the primary cause of hypovirulence is attributed to the InC9 insert in the mtS-rRNA gene. However, with the appearance and subsequent amplification of pleC9 in the mitochondria, the strains were adversely affected in terms of growth (Figure 4.4A) on ECM and virulence (Table 4.2). In addition, the extent of reduction in growth potential due to pleC9 seems to be directly proportional to the amount of amplification and accumulation of the element in the mitochondria. This is indicated by KFC9-FSFS, the slowest-growing morph, which contains the highest amount of pleC9 (Figure 4.4E). Thus, it appears that pleC9 plays a secondary role in the generation of the hypovirulence phenotype, but is the primary cause of senescence.

From hybridization studies, it was apparent that the fast-growing derivatives of KFC9-E6 did not contain pleC9 in an amplified form. However, the slow-growing forms that were derived from these particular fast-growing strains were observed to contain pleC9. Thus, it must be that either the pleC9 DNA is generated *de novo* in the mitochondria of the slow-growing morphs, or it is present in the fast-growing strains in undetectably low amounts. To distinguish between these two alternatives, a segment of

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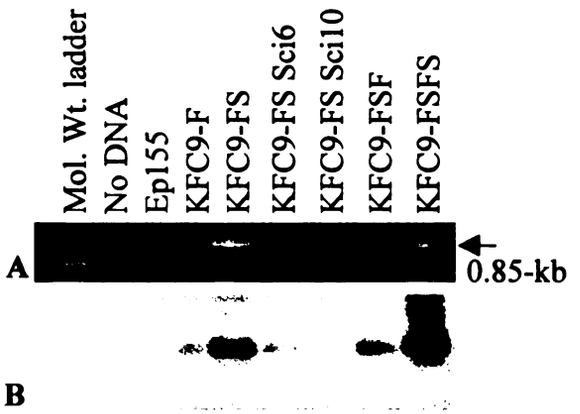

Figure 4.5. Detection of low amounts of the pleC9 element in the fast-growing derivatives of KFC9-E6. Panel **A** Part of the element was PCR amplified with the pleC9-specific primers P1 and P2 (Figure 4.3A) and the products were visualized by ethidium bromide staining upon agarose gel electrophoresis. Visible 850-bp large products were obtained only for the slow-growing forms. Lane M represents the molecular weight marker DNA. **B** The corresponding Southern blot, when hybridized to a pleC9 probe, revealed the presence of low amounts of products for the faster-growing derivatives also. No product was obtained with the wild-type Ep155.

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pleC9 was amplified by PCR from the DNA of the fast-growing derivatives of KFC9-E6 using primers that hybridize only to this element. The products were electrophoresced on an agarose gel and visualized by ethidium bromide staining (Figure 4.5A). As expected, the slow-growing derivatives produced the predicted product whereas no product was visible for the fast-growing derivatives or the wild-type EP155 strain. However, the corresponding Southern blot of this gel, when hybridized with a pleC9 probe, showed the presence of low amounts of the PCR product derived from pleC9 DNA in the fast-growing KFC9-E6 derivatives as well as in the fast-growing single conidial isolates of KFC9-FS (Figure 4.5B). The wild-type Ep155 strain was not observed to have any pleC9 DNA even on hybridization. Collectively, these observations suggest that the pleC9 plasmid-like element is maintained in very low amounts in the fast-growing derivatives of KFC9-E6 but are amplified in the slow-growing senescent forms.

Variations of pleC9 DNA and its dispersal in nature. A total of 23 strains, collected from Kellogg Forest, Michigan, in 1997 (Baidyaroy *et al.*, 2000a) and 1999 were tested for the presence of pleC9 DNA. Severely senescent isolates could not be included in this sample because sufficient mycelium for the extraction of DNA could not be generated. Genomic DNA from each isolate was digested with *Xba*I, separated on agarose gels, and the corresponding Southern blots were hybridized with the pleC9 probe (Figure 4.6). The 1.4-kb pleC9 DNA was found to be absent in 4 strains while in 2, it was maintained in very low amounts. There were several strains that had different variants of the element in terms of the observed size of the *Xba*I fragment. The different sizes of amplified DNAs

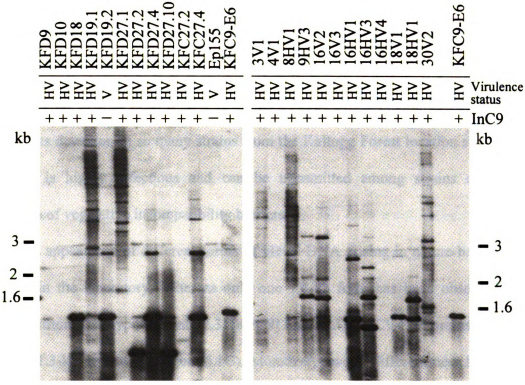

Figure 4.6. Identification of different variations of the pleC9 element and analysis of its dispersal in nature. Southern blot of *Xba*I digested genomic DNAs were hybridized with a pleC9 probe. The molecular weight markers are given at both margins for the precise determination of sizes of the variations of the pleC9 DNA. The presence or absence of InC9 in the strains are indicated by '+' or '-', respectively. The virulence status of each of the strains is also mentioned as either 'HV' for hypovirulent, or as 'V' for virulent.

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bearing homology to pleC9 DNA are listed in Table 4.1. The hybridization of a 2.9-kb DNA fragment was observed for all the strains because that fragment contains the mtDNA fragment that bears homology to a short segment of the pleC9 DNA. The presence or absence of pleC9 and its variants did not correlate with the occurrence of hypovirulence. For example, it was absent in the hypovirulent strain KFD10 (Baidyaroy *et al.*, 2000a) but present in the virulent strain KFD19.2, which has a normal respiratory phenotype and does not contain InC9 (Baidyaroy *et al.*, 2000a). Thus, it appears that pleC9 is not the genetic determinant of the KFC9-type of mitochondrial hypovirulence. However, its detection in so many strains from the Kellogg Forest location suggests that this DNA is highly infectious and can be transmitted among strains quite easily irrespective of vegetative incompatibility barriers.

The appearance of different forms of pleC9 DNA arising in nature has also been witnessed in the laboratory. Whereas only one 1.4-kb form has been observed in the KFC9-E6 strain, the hypovirulent J2.31[KFC9] and J2.31[KFC9-E6] strains contain an additional 1.3-kb derivative (Figure 4.1C). Moreover, several different new forms of the pleC9 DNA also were detected in the hypovirulent strain F2.36[KFC9-E6]. Upon cloning and sequencing of the new 1.3-kb derivative from J2.31[KFC9-E6], it was found that this new element was 1295-bp long and fundamentally identical to the pleC9 sequence (see Appendix B). However, it is shorter than pleC9 because it has lost part of the sequence that includes one copy of the B/B' repeat (Figure 4.3A) in its entirety and part of the other copy of the same repeat. There were also several differences between this new form and the pleC9 sequence that included three base deletions and two base substitutions. The 1.4-kb equivalent of pleC9 from J2.31[KFC9-E6] also was sequenced completely to

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compare it with the original pleC9 sequence. The five differences between the J2.31[KFC9-E6] element and pleC9 included one base deletion and four substitutions. When the 1.4-kb and 1.3-kb elements of J2.31[KFC9-E6] are compared, the differences lie not only in the deletion of the repeats B/B' in the 1.3-kb element, but two base deletions and six base substitutions also were observed. From all of the different variations of the pleC9 DNA observed among these strains, and the detection of a high number of differences among pleC9 and its derivatives in J2.31[KFC9-E6], it appears that this DNA is highly unstable, involved in a high number of errors in replication, and probably escapes mitochondrial DNA repair mechanisms.

DISCUSSION

The appearance of plasmid-like elements in the mitochondria of debilitated fungi has been reported before (Bertrand *et al.*, 1980; Gross *et al.*, 1989). In many fungi, emergence of amplified sequences is correlated with hyphal senescence (for reviews see Wolf and Del Guidice, 1988; Dujon and Belcour, 1989; Griffiths, 1992; Bertrand, 2000). Thus, the appearance of pleC9 in senescing mitochondrially-hypovirulent cultures of *C. parasitica* is not a surprise altogether. However, a major difference between amplified elements in the mitochondria of other fungi and pleC9 lies in the fact that unlike others, pleC9 is not entirely derived from the host mtDNA sequence. This feature can be explained by two possible mechanisms. The most likely explanation is that the mtDNA

haplotype from which pleC9 was originally derived has either degenerated in nature or unfortunately is absent from our collection. The other possible, but less likely, reason may be that this element has existed for a long time in nature and its nucleotide sequence has evolved to the extent that it now lacks resemblance to the DNA of its origin. Based on the appearance of new forms in the laboratory and occurrence of numerous different forms among the strains from Kellogg Forest, it may be surmised that this element is extremely unstable. The basis of this instability may be in part because of an abundance of direct repeats in its sequence that result in intra- and inter-molecular recombination generating new size variants of the element. Intra-molecular recombination in mitochondria producing sub-genomic mtDNA molecules has been observed in *Neurospora* (Gross *et al.*, 1984, 1989; Bertrand *et al.*, 1993) and Ophiostoma (Abu-Amero *et al.*, 1995).

Mitochondria of fungi have been shown to be capable of harboring both linear and circular epigenetic elements (Griffiths, 1995). Amplified mtDNA sequences arising in cytoplasmic respiratory mutants of *Neurospora* (Bertrand *et al.*, 1980: De Vries *et al.*, 1981; Gross *et al.*, 1984, 1989; Almasan and Mishra, 1991) and senescent *Podospora* (Dujon and Belcour, 1989; Griffiths, 1992) strains have been reported to be circular in conformation. In accordance with these observations, pleC9 was also observed to be predominantly circular. The abundance of linear molecules can be attributed to replication of the element by a rolling circle mechanism that also can account for the generation of multimeric forms of the element. The preponderance of this element in the mitochondria suggest that pleC9 may be replicated at a higher rate than *bona fide* mtDNA. This might lead to higher incidences of replication errors not being rectified if

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the mitochondrial repair machinery is not working in conjunction with the replication complex. However, this might also happen if the pleC9 element lacks sites that are required for binding of a complete replication-repair complex. This notion is supported by the detection of variable forms of this element among strains. Chances of partial or absolute lack of DNA repair for pleC9 can not however be ruled out.

The phenomenon of intermittent fast and slow growth phenotypes for the KFC9-E6 strain bears an uncanny resemblance to the "stop-start" growth phenotype of the 'stopper' mutants of *Neurospora crassa* (Bertrand and Pittenger, 1969; McDougall and Pittenger, 1969) and the 'ragged' mutants of *Aspergillus amstelodami* (Lazarus et al., 1980; Lazarus and Küntzel, 1981). The stoppers are named accordingly because they have an irregular growth phenotype in which mycelial elongation commences, then stops, and sometimes starts again. This pattern essentially is observed for the KFC9-E6 strain too. The initial strain KFC9-F is a relatively fast-growing strain that slows down to yield the KFC9-FS. The KFC9-FS in turn produced the fast-growing sector KFC9-FSF that eventually degenerates to the slow-growing KFC9-FSFS. In addition, defective mtDNA molecules vary in concentration in the stopper mutants depending on whether the mutant is fast-growing or not (Dujon and Belcour, 1989). During the stopped phase of growth, smaller circular DNA molecules predominate over wild-type mtDNA molecules. This feature is also manifested in KFC9-E6 in the accumulation of pleC9 DNA in the mitochondria only when the strain is growing slowly. However, unlike the stoppers, the slow-growing morphs of KFC9-E6 are relatively stable and can be perpetuated for prolonged periods of time. The cause for the slow-growth phenotypes of the stopper mutants have been identified as accumulation of mtDNA molecules that have large

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deletions (Almasan and Mishra, 1988, 1990; Bertrand *et al.*, 1980; de Vries et al., 1981; Gross *et al.*, 1984) rather than the accumulation of small plasmid-like elements. This seems to be the case also for KFC9-E6 where the primary mutation that causes hypovirulence appears to be the disruption of the mtS-rRNA gene by InC9 (Baidyaroy *et al.*, 2000a, Chapter 3 of this thesis) instead of the amplification of pleC9 DNA.

MtDNA isolated from senescent cultures of the fungus *Podospora anserina* was always found to contain numerous small circular molecules, named senDNAs (Dujon and Belcour, 1989; Griffiths, 1992; Bertrand, 2000). The senDNAs from different senescent cultures were heterogeneous in composition and derived entirely from various parts of the wild-type mtDNA molecules. Similarly, amplification of one or two specific regions of mtDNA and its maintenance as circular head-to-tail concatamers has been observed in the cytoplasmic ragged mutants of *Aspergillus amstelodami* (Lazarus *et al.*, 1980; Lazarus and Küntzel, 1981). The pleC9 element of *C. parasitica* is very similar in its pattern of appearance to these amplified sub-genomic circular DNAs of *Podospora* and *Aspergillus*. The pleC9 DNA is only amplified in strains that are very slow-growing with rapidly degenerating mycelia. This phenotype of the fungus is morphologically similar to the senescence phenotypes of *Podospora* and *Aspergillus*. Furthermore, another common feature shared by pleC9 with the senDNAs is that the mtDNA region that is homologous to pleC9 contains a part of an intron. The only property common to all known senDNAs is the presence of at least a partial intron (Dujon and Belcour, 1989). In fact, the maturase enzyme encoded by the mtDNA ORF that is homologous to the pleC9 sequence is similar to the one encoded by the α-senDNAs of *Podospora* (Michel and Lang, 1985; see Appendix C).

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The pleC9 element certainly has an effect on the growth morphology of the fungus on synthetic medium. This is evident because accumulation of this element in high amounts correlates with slow-growth whereas the suppression of the element produces faster growth. In this respect, pleC9 is again similar to the senDNAs of *Podospora* where senescent cultures have been rejuvenated by the removal of senDNAs through ethidium bromide treatment (Koll *et al.,* 1984). However, amplification of pleC9 appears to be only of secondary consequence as far as hypovirulence is concerned. The strains where this element appears already have dysfunctional mitochondria that are destabilized by the InC9 element (Chapter 3 of this thesis). Thus, the generation of pleC9 seems to be a stress-induced phenomenon. Similarly, in *Ophiostoma*, where a cytoplasmically transmissible disease is caused by mitochondrial mycoviruses, transmission of the disease results in the generation of amplified circular, plasmid-like molecules derived from the mtDNA (Charter *et al.,* 1993). That the senDNAs also are an effect of stress rather than the immediate cause of the stress is evident from the fact that these DNAs can appear in non-senescent cultures of *Podospora* (Silliker and Cummings, 1990) and that some nuclear mutations can abolish the accumulation of senDNAs without preventing senescence (Silar *et al.,* 1997).

Hypovirulence and slow-growth phenotypes appear to be controlled by mutually exclusive mechanisms, at least for the chestnut blight fungus *Cryphonectria parasitica.* Strains growing fast on synthetic medium do not necessarily behave as virulent isolates. Thus, the fast-growing forms of KFC9-E6 are still hypovirulent on apples as well as on chestnut bark. The element pleC9, though it effects a dramatic reduction in the growth rate of the fungus, does not produce similar effects on the virulence potential. For

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example, the strain KFD19.2, which does not carry the InC9 element (Baidyaroy *et al.*, 2000a), still contains pleC9 DNA but remains virulent. In addition, several strains from the Kellogg Forest site are hypovirulent despite the absence of pleC9 in their mitochondria. However, that pleC9 does not contribute to the severity of the hypovirulence phenotype can not be ruled out. The derivatives of KFC9-E6 that have high amounts of pleC9 have very low virulence (Table 4.2) when compared to the derivatives where pleC9 accumulation is suppressed. A possible mechanism by which pleC9 accomplishes this reduction in general growth and virulence is probably through the recruitment of the limited DNA replication machinery for its own replication, thereby reducing the normal rate of replication for the wild-type mtDNA. Indeed, an increase in the amount of pleC9 is always accompanied by a proportional decrease of indigenous mtDNA molecules (Figure 4.4E). However, the mechanism that controls the accumulation or suppression of pleC9 is not apparent.

Like the true mitochondrial plasmids of filamentous fungi (Griffiths, 1995), pleC9 is also infectious. This is explained by the detection of this element in almost all of the strains from the Kellogg Forest site. Since pleC9 is usually not inherited through conidia and because similar elements are often not inherited in sexual spores (G. Hausner and H. Bertrand, unpublished), it appears that pleC9 can only spread in a population through cytoplasmic transmission involving hyphal fusion. Movement of pleC9 in the laboratory can be inferred from the presence of this element in all of the converted hypovirulent strains that were infected with KFC9 (Baidyaroy *et al.*, 2000a). The strains from Kellogg Forest have already been shown to be very heterogeneous in terms of nuclear composition (Likins, 1990; Baidyaroy *et al.*, 2000a). Thus, it can be assumed that these

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strains would not be vegetatively compatible with one another. Collectively, these observations imply that pleC9 can get transmitted vegetatively across incompatibility barriers with sufficient ease so as to be present in almost all of the strains at the Kellogg Forest site. In this respect, pleC9 is similar to the *bona fide* mitochondrial plasmid pCRY1 of *C. parasitica* (Baidyaroy *et al.*, 2000b). The infectious characteristic of such molecules and their efficient maintenance in nature makes them a highly desired tool for induction of biological control of pathogenic fungi provided they elicit a debilitating phenotype.

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CHAPTER 5

Dynamics of asexual transmission of a mitochondrial plasmid in *Cryphonectria parasitica* and its effect on virulence of the fungus

ABSTRACT

In the chestnut blight fungus *Cryphonectria parasitica*, as in most fungi, little is known about the efficiency of the asexual transmission of optional mitochondrial plasmids, vertically through conidia, and horizontally through hyphal anastomoses. In this paper, we show that pCRY1, a circular mitochondrial plasmid, is transmitted vertically with 100 % efficiency through conidia. Moreover, the plasmid is transmitted horizontally through hyphal contact from donor strains to vegetatively compatible and most incompatible strains. An allelic difference between the donor and recipient strain at only one of the five nuclear incompatibility genes that were tested strongly inhibited, but did not absolutely prevent, the transfer of pCRY1 through hyphal fusions. In contrast, allelic differences in any one or several of the other four heterokaryon-compatibility loci suppressed the

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Note. The content of this chapter has been published as Baidyaroy, D., Glynn, J.M., and Bertrand, H. 2000. Dynamics of asexual transmission of a mitochondrial plasmid in *Cryphonectria parasitica*. Curr. Genet. (In press).

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transmission of the plasmid only partially or not at all. The plasmid was also transmitted among incompatible strains by protoplast fusion without the concomitant transfer of mitochondrial DNA (mtDNA). A comparison of plasmid-bearing with plasmid-free isogenic strains revealed that pCRY1 significantly diminishes the pathogenic potency of some strains of the fungus, but does not affect the virulence of others. Collectively, the observations indicate that the introduction of deleterious mitochondrial genetic elements into natural populations may be a means for managing fungal pathogens.

INTRODUCTION

Somatic incompatibilities that result in adverse reactions between cells of different genotypes, have been described in organisms ranging from protists to animals, plants and fungi (Esser and Blaich, 1973; Buss, 1982). In the filamentous fungi that have coenocytic mycelia, vegetative incompatibility causes antagonistic reactions at points of hyphal fusion between genotypically different strains and prevents the formation of stable heterokaryons. Formation of stable or transient heterokaryons in fungi has been shown to facilitate the transmission of extranuclear genetic elements, namely mtDNA (Saville *et al.,* 1998; Toth *et al.,* 1998), plasmids (Griffiths, 1995) and viruses (Huber, 1996; Liu and Milgroom ,1996). In this respect, it has been suggested that vegetative incompatibility is a mechanism that controls the movement of cytoplasmic elements through cell death when incompatible strains fuse vegetatively (Caten, 1972).

In most ascomycetes, incompatibility reactions result from allelic differences at either one or multiple vegetative incompatibility genes, generally referred to as *vic* or *het* (for heterokaryon incompatibility). Two strains are termed vegetatively compatible when they have similar alleles at all of their *vic* loci (Perkins, 1988). For fungi like *Neurospora*, *Podospora* and *Aspergillus*, up to 17 *het* loci have been identified so far (Begueret *et al.*, 1994; Glass and Kuldau, 1992; Leslie, 1993) and the existence of more of these genes in other fungi can be surmised based on the large number of vegetative compatibility types that have been observed (Huber, 1996; Guerber *et al.*, 1997). In ascomycetous fungi, most *vic* genes have only two alleles, but instances of loci having more than two alleles also have been reported (Dales *et al.*, 1993; Howlett *et al.*, 1993). For *Cryphonectria parasitica*, up to 7 *vic* loci have been identified so far (Cortesi and Milgroom, 1998; Huber, 1996) and only two alleles are known for each locus.

Despite the barriers presented by vegetative incompatibility, somatic transmission of genetic elements among incompatible strains has been observed. Not only cytoplasmic elements can be transferred among incompatible strains (Collins and Saville, 1990; Griffiths *et al.*, 1990; Debets *et al.*, 1994; Liu and Milgroom, 1996; van der Gaag *et al.*, 1998) but also nuclear factors have been shown to move from one incompatible strain to another (Masel *et al.*, 1996; Poplawski *et al.*, 1997). Even the transmission of a mitochondrial plasmid among unrelated fungi has been recorded (Kempken, 1995). In addition, viruses have been shown to be transmitted among incompatible strains through hyphal contact (Huber, 1996; Liu and Milgroom ,1996) as well as by protoplast fusion (van Diepeningen *et al.*, 1998). These observations suggest that infectious elements like viruses and plasmids frequently evade incompatibility reactions.

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Since infectious hypovirulence phenotypes that are caused either by double-stranded RNA viruses (Nuss 1992) or by deleterious mitochondrial elements (Monteiro-Vitorello *et al.,* 1995; Baidyaroy *et al.,* 2000) occur in *Cryphonectria parasitica*, an understanding of the effects of *vic* genes on the horizontal transmission of cytoplasmic genetic elements becomes relevant. Whereas some information regarding the role of individual *vic* genes in influencing transmission of free cytoplasmic mycoviruses in *C. parasitica* already exists (Huber, 1996; Liu and Milgroom, 1996), similar studies involving mitochondrial elements have not been reported. Hence, we have analyzed the movement of the mitochondrial plasmid pCRY1 (Monteiro-Vitorello *et al.,* 1995, 2000; Bell *et al.,* 1996) across incompatibility barriers in *C. parasitica*, as well as the effect of this plasmid on the virulence of the fungus. We also have explored the potential of effecting plasmid transmission by protoplast fusion between incompatible strains when the transfer of the plasmid could not be attained through hyphal contacts. Collectively, the observations impact the understanding of potential natural dissemination of deleterious mitochondrial plasmids which may be introduced for the biological control of harmful fungi, in this case, the chestnut blight agent *C. parasitica.*

MATERIALS AND METHODS

Fungal strains and growth conditions. The *C. parasitica* strains that were used in this study are listed in Table 5.1. The genotypes and allele designations of the strains are

Table 5

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Table 5.1. *Cryphonectria parasitica* strains used in this study.

Strains	Pigmentation	Vegetative incompatibility (*vic*) genotype[a]	Source	Presence of pCRY1 in nature[b]
		vic 1 2 3 4 5		
Ep155	Orange	2 2 1 1 -[c]	ATCC 38755	-
Ep289	Orange	1 2 1 1 1	Conn. Agri. Exp.	+
Ep339	Orange	Unknown	Conn. Agri. Exp.	+
Ep388	Orange	2 1 1 1 1	ATCC 38979	+
389.7	Cream	1 1 1 1 1	MSU collection	-
22508	Orange	2 2 1 1 1	MSU collection	-
A1.13	Cream	1 2 1 1 1	MSU collection	-
F2.2	Brown	2 1 1 2 2	MSU collection	-
F2.36	Brown	1 1 1 1 1	MSU collection	-
F3.15	Brown	2 1 1 1 2	MSU collection	-
F3.39	Brown	1 1 1 2 2	MSU collection	-
F4.9	Brown	1 1 1 1 2	MSU collection	-
J1.27	Brown	2 2 1 1 1	MSU collection	-
J2.31	Brown	1 2 1 2 1	MSU collection	-
J2.43	Brown	1 2 1 1 1	MSU collection	-
M1.5	Brown	1 2 2 1 1	MSU collection	-
M1.6	Cream	1 2 2 1 1	MSU collection	-
KFC9-E6	Orange	Unknown	MSU collection	-

Strains listed as obtained from MSU collection are described by Huber (1996) and were kindly made available for us by him from Dr. Dennis Fulbright's laboratory, Michigan State University.

[a] For brevity, vegetative incompatibility (*vic*) genotypes are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as 1 and 2, are known for each *vic* locus.

[b] Presence of pCRY1 plasmid in a strain in nature is denoted with a '+' sign and its absence is marked with a '-' sign.

[c] Allele for *vic* 5 is unknown.

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presented in a format consistent with nomenclature used for *Neurospora* as described by Perkins (1999). The specific genes are designated by the name (*vic*) followed by a number representing the particular locus (e.g., *vic*-1, *vic*-2, etc.) according to the nomenclature used by Huber (1996). Alleles are represented as superscripted numbers. For example, the two alleles for *vic*-1 are represented as *vic*-1^1 and *vic*-1^2. The strains were maintained in Petri plates on Endothia complete medium (ECM) as described by Puhalla and Anagnostakis (1971). Methionine was added to the medium at a final concentration of 0.1 mg/ml, when required.

Transmission of mitochondrial plasmid through hyphal anastomoses. The plasmid-containing donor strains were co-grown with the appropriate compatible or incompatible recipient strains on ECM in Petri-plates. The inoculum plugs, typically ranging between 3 and 4 mm^2 in size, were placed near the walls of the Petri-plates (Figure 5.1A) and the cultures were allowed to grow until their hyphal tips reached the opposite side of the plates. For each transmission experiment, the donor and recipient strains differed in mycelial pigmentation (orange, brown or cream) which provided the traits required for the subsequent recovery of the homokaryotic conidial isolates from heterokaryons. At least three different plates were set up for each pair of strains and conidia from all of the plates were analyzed for the transmission of the plasmid. For the analysis of the invasiveness of the plasmid, which is defined as the ability of the plasmid to spread into the mycelium of the recipient strain beyond the zone of contact with the donor, the plugs of donor and recipient mycelium were placed either side by side (Figure 5.1A) or diametrically opposite to each other, near the walls of Petri-plates (Figure 5.1B).

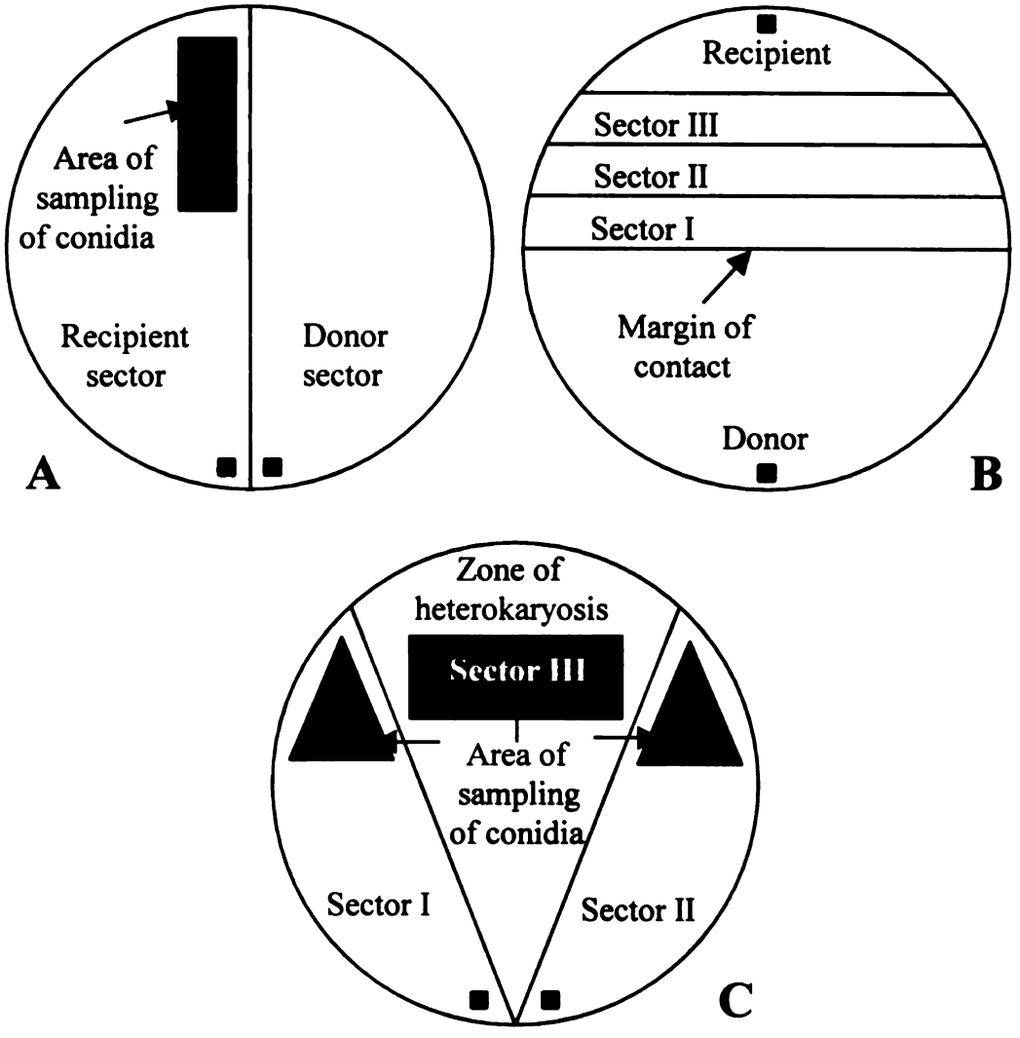

Figure 5.1. Position of inoculum plugs on plates used to study vegetative transmission of pCRY1. Panel **A** shows the orientation of the inoculum plugs for the asexual transmission of the plasmid and the area from which conidia were sampled. Panel **B** indicates the position of inoculum plugs and zones of sampling on plates used for the analysis of invasiveness of the plasmid. Panel **C** shows the position of inoculum plugs for the analysis of the dynamics of plasmid transmission and the area from which conidia were sampled.

Recovery of single conidial isolates of the recipient strains. Conidia were scraped with a sterile loop from the area near the growth front of the recipient sector adjacent to the zone of interaction of the donor and recipient mycelia (Figure 5.1A). For the study of the dynamics of transmission of plasmid pCRY1, conidia were not only taken from the recipient sector, but also from the donor sector and the zone of heterokaryosis (Figure 5.1C).

The conidia were suspended in 5 ml of sterile water, mixed thoroughly, diluted serially in five-fold steps, and spread on ECM-agar in Petri-plates. The plates were incubated at room temperature for 4 to 5 days or until the conidia germinated and formed small visible colonies. Colonies formed from hyphal contaminants in the suspension were easily detected because they were visible in 1 to 2 days, whereas colonies formed from isolated conidia appeared only after 4 days of incubation. Germinated conidia were transferred onto fresh medium and grown until the mycelia showed the pigmentation characteristic of the donor and recipient strains.

In the experiment assessing the invasiveness of pCRY1, mycelial plugs were taken from three 0.5 cm wide zones within the recipient sector on the experimental plates (Figure 5.1B), labeled I, II and III, progressively away from the line of contact between the two strains. The mycelial plugs were subcultured separately and single conidial isolates were generated from the resulting cultures. In this case, only one recipient single conidial isolate per subculture was tested for the presence of pCRY1.

Extraction of DNA from conidial isolates. Mycelial plugs from conidial isolates were grown in 0.5 ml of liquid ECM in 1.5 ml Eppendorf tubes for 2-3 days. The medium was

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carefully removed from the tubes and the mycelia were placed on ice and rinsed with cold, sterile water. The water was subsequently discarded and each sample was then incubated with 0.5 ml of 1 M sorbitol containing 0.5 g of Novozym 234 (Novo Biolabs) for 2 hr at 37°C with gentle shaking at approximately 100 rpm. The tubes were afterwards placed on ice and the liquid was very gently removed with a pipette. At this stage, 0.25 ml of proteinase K, at a concentration of 2 mg/ml, was added to each sample in a buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 M NaCl, 0.5% SDS, pH 8.0, and the tubes were incubated at 37°C for 16-18 hr. The DNA was then purified by a phenol:chloroform extraction, precipitated with sodium acetate and alcohol, dried under vacuum, re-dissolved at 37°C for 1 hr in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with RNaseA (10 µg/ml) and stored at –20°C.

Detection of pCRY1 DNA in conidial isolates. The presence of pCRY1 DNA in the DNA isolated from the single conidial isolates was detected by two different techniques: (1) the polymerase chain reaction (PCR) using plasmid-specific primers and/or (2) hybridization of a probe derived from cloned pCRY1 DNA with dot blots of total DNA extracted from mycelia of single conidial isolates.

For the detection of pCRY1 through PCR, two primers, 5' - CATACTCAGCGAATGCC - 3' and 5' - GGTGTGGATAATAGTAGG - 3', were designed on the basis of the published nucleotide sequence (Monteiro-Vitorello *et al.*, 2000), and used to amplify a 1.5-kb region of the plasmid. Template DNAs were boiled for 5 min before being added to the reaction tubes. The PCR reaction consisted of 30 cycles of denaturation at 93°C for 1 min, annealing at 54°C for 1 min, and amplification

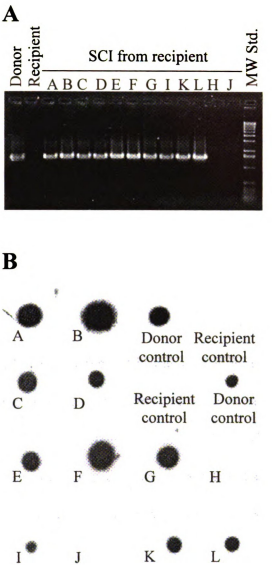

Figure 5.2. Detection of the presence of pCRY1 in conidial isolates. Panel **A** shows a sample of data produced by detection of the plasmid by PCR. Presence of the plasmid in the donor strain and some of the recipient single conidial isolates (sci) is indicated by the 1.5-kb DNA fragment. The recipients shown in lanes H and J did not acquire the plasmid. Panel **B** shows the same donor, recipient and single conidial isolates as in panel A, but pCRY1 was detected by dot blot hybridization.

at 72°C for 1 min. An extension period at 72°C for 10 min was included after the 30 cycles of PCR. The products were separated on a 0.8% agarose gel and visualized by ethidium bromide (EtBr) staining. An example of the PCR products obtained and detected by this protocol is depicted in Figure 5.2A. There was no product found if the plasmid was absent. For detection of pCRY1 by hybridization, a chemiluminescent probe labeled with dig-oxigenin-dUTP was generated by random primed labeling of the cloned complete unit of the plasmid DNA (Monteiro-Vitorello *et al.*, 2000) using the Genius kit and by following the procedure suggested by the manufacturer (Boehringer-Mannheim). For the preparation of dot blots, each sample of *C. parasitica* DNA was boiled for 6-8 min and cooled rapidly on ice. For each sample, 15 µl of the boiled DNA was spotted onto a nylon membrane (Amersham Inc.), allowed to dry, and then cross-linked under UV-light. Hybridization and washes were performed at 65°C as described by Sambrook *et al.* (1989). Binding of the probe was detected with anti-digoxigenin Fab-alkaline phosphatase conjugate using CDP-star as directed by the manufacturer (Boehringer-Mannheim). A sample of the signals obtained using this procedure is shown in Figure 5.2B.

Generation of a hygromycin-resistant Cryphonectria strain and protoplast fusion. For the generation of a hygromycin-resistant strain of *C. parasitica*, spheroplasts were prepared as described by Churchill *et al.* (1990) and transformed with 5 µg of DNA of the plasmid pCB1003 (Carroll *et al.*, 1994) using methods described by Selitrennikoff and Sachs (1991). The transformants were selected on hygromycin-containing (150 µg/ml)

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ECM-agar and the hygromycin-resistant Ep339-HGR1 strain was chosen to be used for the protoplast fusion experiments.

For the protoplast fusion experiments, spheroplasts were also prepared by the method of Churchill *et al.* (1990) from the donor Ep339-HGR1 and the recipient Ep155-BEN3 (Monteiro-Vitorello *et al.*, 1995) strains. Donor and recipient spheroplasts were mixed in a ratio of 10 : 1 and then collected by centrifugation at 4,500 × g at 4°C for 10 min. After discarding the supernatant, 500 µl of fusion buffer (30 % PEG 6000, 0.75 mM $CaCl_2$, 0.05 mM glycine, pH 7.5) were added to the protoplasts and the mixture was incubated at 28°C for 15 min. The mixture was then plated in molten ECM-agar containing hygromycin (150 µg/ml) and benomyl (0.5 µg/ml), and the plates were incubated at room temperature until individual colonies were visible. Since the donor and the recipient were incompatible, their nuclei segregated rapidly and the fusion products died when left on the medium containing hygromycin and benomyl for periods longer than two days after the first appearance of visible colonies. Therefore, germinated spheroplasts were transferred onto ECM plates containing only benomyl as soon as they appeared. The absence of the donor nuclei was confirmed by sensitivity of the isolates to hygromycin.

Isolation and manipulation of mitochondrial DNA. Mitochondrial DNA was isolated as described by Bell *et al.* (1996). The DNA was digested with restriction enzymes, fractionated by electrophoresis on 0.8% agarose gels and was visualized under UV-light after staining with EtBr. Enzymatic digestions, Southern blotting and DNA hybridizations were carried out according to procedures described by Sambrook *et al.* (1989). Specific

DNA fragments were excised from agarose gels and purified using the Elu-Quik DNA purification kit of Schleicher and Schuell.

Respiration tests, growth rate measurements and virulence assays. Respiration tests were conducted as described by Monetiro-Vitorello *et al,* (1995). Growth rates of *C. parasitica* strains were measured according to the method of Ryan *et al.* (1943) over a period of 42 days. Strains were tested for virulence on Golden Delicious apples (Fulbright, 1984), on chestnut bark (Lee *et al.,* 1992), or on live chestnut trees (Elliston, 1978). The strains were grown for 15 days on apples and 60 days on trees before the areas of the lesions were determined as measures of virulence.

RESULTS

Vertical transmission of the pCRY1 plasmid through conidia. For the determination of the frequency of transmission of pCRY1 through conidia, single conidial isolates were prepared from two strains: Ep289, which contains the plasmids in nature, and F4.9, into which pCRY1 was introduced in the laboratory by hyphal transmission. The experiment was designed to study the transmission of pCRY1through conidia and also to determine if there was any difference in the patterns of transmission between strains that contained the plasmid naturally and strains into which it had been introduced artificially.

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The strains were grown on ECM for 5-7 days and then conidia from the cultures were plated for the recovery of single conidial isolates. The resulting colonies were then checked for the presence of pCRY1. The plasmid was found to be present in all of the 20 single conidial isolates checked from each of the strains. Hence, pCRY1 appears to be transmitted very efficiently through conidia and without regards to its acquisition by the fungus under natural ecological conditions or by artificial manipulation in the laboratory.

Horizontal transmission of pCRY1 among vegetatively compatible strains by hyphal contacts. To determine the rate of transmission of pCRY1 from a donor strain to a vegetatively-compatible recipient by hyphal contact, single conidial isolates prepared from the heterokaryotic zone (Figure 5.1C) formed between the mycelia of the brown strain, F2.36 *br*, and the compatible cream-colored strain, 389.7 *cre*, were checked for the presence of the plasmid (Table 5.2). When strain F2.36 *br* [pCRY1] was used as the donor, the plasmid was present in all 15 cream-colored single conidial isolates representing the recipient 389.7 strain. For the reciprocal transmission-experiment, where the 389.7 *cre* [pCRY1] was used as the donor and F2.36 *br* served as the recipient, the plasmid appeared in all the brown single conidial isolates that were recovered from the heterokaryotic zone. Homokaryosis in the single conidial isolates was ensured because the conidia of *Cryphonectria* are uninucleate (Puhalla and Anagnostakis, 1971). These observations indicate that hyphal fusions among compatible strains occur frequently enough to permit invasion of the recipient by pCRY1. This data also implies that mitochondrial plasmids like pCRY1 behave like infectious elements and rapidly colonize

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the plasmid-free mitochondria of the recipient strain once they have been transferred through hyphal anastomoses.

Effect of individual vegetative incompatibility (*vic*) genes on horizontal transmission of pCRY1. To test the extent of transmission of pCRY1 among vegetatively incompatible strains, plasmid-containing donors were inoculated side by side with plasmid-free recipients. Single conidial isolates, recovered from the zone of recipient mycelia as depicted in Figure 5.1A, were screened for the plasmid. For each transmission experiment, the donor and the recipient strains had identical *vic* genotypes, except at the locus being tested for its effect on transmission. The impact of the different *vic* genes on horizontal transmission of pCRY1 was variable (Table 5.2) and can be represented in the following three categories. First, some *vic* genes do not appear to inhibit the transmission of pCRY1. For example, an allelic difference between the donor and the recipient at *vic*-5 did not inhibit plasmid transmission in either direction, i.e. either from vic-5^1 to vic-5^2 or *vice versa*. However, that *vic*-5 is a *bona fide* incompatibility gene is evident from the fact that strains differing in alleles at this locus failed to form heterokaryons (Huber, 1996; this study). Secondly, *vic* genes can have a strong inhibitory effect on transmission of pCRY1, as observed for *vic*-2. Virtually no transmission was observed either from *vic*-2^1 to *vic*-2^2 or *vic*-2^2 to *vic*-2^1 (Table 5.2). Finally, the remaining three loci that were tested, namely *vic*-1, *vic*-3 and *vic*-4 inhibited pCRY1 transmission in one direction only (Table 5.2). For *vic*-1, transmission occurred from the *vic*-1^2 donors to *vic*-1^1 recipients, but not from *vic*-1^1 donors to *vic*-1^2 recipients. For *vic*-3 and *vic*-4, a high amount of transmission of pCRY1 was observed only from allele 1 to allele 2. Although the amount of

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transmission of the plasmid from *vic*-4^2 to *vic*-4^1 appeared to be low, it was still high enough (16 %) to indicate that *vic*-4 is not as strong an inhibitor as *vic*-3. Transmission of pCRY1 was completely inhibited only in two combinations: from *vic*-1^1 donors to *vic*-1^2 recipients and from *vic*-3^2 donors to *vic*-3^1 recipients.

Transmission of pCRY1 among strains differing at multiple *vic* genes. The cumulative effects of vegetative incompatibility, as it may occur most frequently under natural ecological conditions, was assessed in part by measuring the extent of transmission of pCRY1 among strains that differ in more than one *vic* gene. In this scenario, transmission of pCRY1 generally followed the patterns observed in transfers of the plasmid among strains with allelic differences at only one *vic* gene. Thus, transfer between strains that differ at *vic*-2, which has been found to be the strongest inhibitor of cytoplasmic transfer, is always low irrespective of the number of differences or similarities at other loci (Table 5.2). Similarly, transmission between strains with allelic differences at two *vic* genes can vary widely from a high level of transmission to no transfer at all, depending on the specific allele combinations of the donor and the recipient. Pairs of strains, which differ at two loci involving the 'strong' incompatibility genes, namely *vic*-1, *vic*-2 and *vic*-3, have low rates of transmission of the plasmid among themselves. If two strains differ at the two relatively weak loci, *vic*-4 and *vic*-5, a high amount of transfer of pCRY1 occurs. Nonetheless, when the donor and recipient strains differed at *vic*-4 and *vic*-3, with a *vic*-3 allele combination that allowed transmission and an allele combination in *vic*-4 that inhibits transmission, pCRY1 was detected in approximately 27 % of the recipient single conidial isolates. This observation further

supports the notion that *vic*-4 also can be categorized as a weak inhibitor of cytoplasmic transmission. In experiments involving two strains that differed at three *vic* genes (*vic*-1, *vic*-4 and *vic*-5), pCRY1 was recovered in 50 % of the conidia of the recipient, probably because the combination of the alleles in the donor and the recipient favored transmission. Thus, it appears that transmission between strains differing at multiple *vic* loci is dependent on the sum total of the effects of individual *vic* genes and allele combinations rather than the number of different loci.

Table 5.2. Effect of vegetative incompatibility (*vic*) loci on asexual transmission of pCRY1 among strains.

vic locus tested	Donor		Recipient		Transmission of pCRY1[b]
	Strain	*vic*[a] 1 2 3 4 5	Strain	*vic* 1 2 3 4 5	
-	F2.36	1 1 1 1 1	389.7	1 1 1 1 1	15/15
	389.7	1 1 1 1 1	F2.36	1 1 1 1 1	15/15
1	Ep289	1 2 1 1 1	J1.27	2 2 1 1 1	0/24
	Ep388	2 1 1 1 1	389.7	1 1 1 1 1	13/18
2	389.7	1 1 1 1 1	J2.43	1 2 1 1 1	1/18
	Ep289	1 2 1 1 1	F2.36	1 1 1 1 1	1/24
3	Ep289	1 2 1 1 1	M1.5	1 2 2 1 1	9/29
	M1.5	1 2 2 1 1	A1.13	1 2 1 1 1	0/26
4	Ep289	1 2 1 1 1	J2.31	1 2 1 2 1	22/29
	J2.31	1 2 1 2 1	A1.13	1 2 1 1 1	3/18
5	389.7	1 1 1 1 1	F4.9	1 1 1 1 2	14/15
	F4.9	1 1 1 1 2	389.7	1 1 1 1 1	8/8
1,2	F2.36	1 1 1 1 1	22508	2 2 1 1 1	0/15
2,3	389.7	1 1 1 1 1	M1.5	1 2 2 1 1	1/19
3,4	J2.31	1 2 1 2 1	M1.6	1 2 2 1 1	4/15
4,5	Ep388	2 1 1 1 1	F2.2	2 1 1 2 2	11/18
1,2,5	Ep289	1 2 1 1 1	F3.15	2 1 1 1 2	1/22
1,4,5	Ep388	2 1 1 1 1	F3.39	1 1 1 2 2	9/18
1,2,4,5	Ep289	1 2 1 1 1	F2.2	2 1 1 2 2	0/22

[a] For brevity, *vic* loci are designated as in Table 5.1.

[b] Transmission of pCRY1 is given as the proportion of single conidial isolates in which the plasmid was detected relative to the total number of single conidial isolates tested.

Dynamics and invasiveness of transmission of pCRY1 among compatible strains. To determine if the mode of growth of *C. parasitica* has an effect on the transmission of pCRY1, the vegetatively compatible donor and recipient strains were grown either side-by-side (Figure 5.1C) or confrontationally (Figure 5.1B). When the vegetatively compatible, brown-pigmented F2.36 [pCRY1] donor and the cream-colored 389.7 recipient strains were grown side-by-side, with both the strains essentially growing in the same direction, orange-pigmented zones representing stable heterokaryons were formed between the mycelia along the line of contact (Figure 5.1C). In this case, all of the conidia sampled from the donor (sector I), recipient (sector II) and the heterokaryotic sectors (Sector III) (Figure 5.1C) contained the plasmid (Table 5.3). That the plasmid was detected not only in the region of contact but also in the recipient sector indicates that it is actively transported through the hyphae of the recipient strain.

However, when the same strains were grown towards each other so that the direction of hyphal growth of the donor is opposite to that of the recipient (Figure 5.1B), the distance of migration of the plasmid into the recipient mycelium, which we define as invasiveness, is drastically reduced (Table 5.3). The plasmid was detected in only the zone of the recipient mycelium which was in direct contact with the donor (Sector I). For a compatible pair of strains, all the samples from this zone contained the plasmid whereas for a pair of strains that differed at one *vic* locus, *vic*-3, only 40 % of the single conidial isolates of the recipient contained pCRY1. Even for the compatible pair, the plasmid was detected in the recipient within the contact zone only 5 days after hyphal contact was established. In contrast, when the strains differed at one *vic* locus, the appearance of

Table 5.3. Invasiveness of the pCRY1 plasmid in recipient mycelia of *C. parasitica*.

Donor		Recipient		Days after hyphal contact	Transmission of pCRY1[b]		
Strain	*vic*[a] 1 2 3 4 5	Strain	*vic* 1 2 3 4 5		Sector I	Sector II	Sector III
Strains grown side-by-side (Sector I is the zone of contact as shown in Figure 5.1C)							
F2.36	1 1 1 1 1	389.7	1 1 1 1 1	NA[c]	15/15	15/15	15/15
Strains grown confrontationally (Sectors as shown in Figure 5.1B)							
F2.36	1 1 1 1 1	389.7	1 1 1 1 1	0[d]	0/10	NT[e]	NT
				5	10/10	0/10	NT
				10	10/10	0/10	1/10
Ep289	1 2 1 1 1	M1.5	1 2 2 1 1	0	0/10	NT	NT
				5	0/10	0/10	NT
				10	4/10	0/10	0/10

[a] For brevity, *vic* loci are designated as in Table 5.1.
[b] Transmission of pCRY1 is described as under Table 5.2.
[c] Not applicable.
[d] The day the growing hyphal tips of the two strains came in complete contact with each other throughout the growth front was taken as day zero.
[e] Not tested.

pCRY1 in the recipient was delayed to 10 days after hyphal contact. Thus, vegetative incompatibility might influence not only the frequency of successful transfers of cytoplasmic elements but also the amount of cytoplasm that is transferred through hyphal anastomoses. However, a delay in the colonization of the hyphae by the plasmid after its transfer into the recipient also can account for this result. The absence of the plasmid in the sectors that are located further away from the region of contact indicates that pCRY1 is not transported effectively into the recipient mycelium against the direction of growth. This observation also implies that, for *Cryphonectria* and probably other filamentous fungi as well, growth of mycelia in opposite directions does not allow effective intermingling of their hyphae regardless of their vegetative compatibility characteristics.

Transmission of pCRY1 and mitochondrial DNA among incompatible strains through protoplast fusion. To determine if pCRY1 can be transferred among extremely incompatible strains by artificial means, and afterwards is stably maintained in the recipient, protoplasts were prepared from strains Ep339 [pCRY1] and Ep155 and fused with each other. These strains were chosen specifically because a strong incompatibility reaction was seen when they were grown on plates. In fact, when the two strains were cultured side by side or confrontationally, the growing margins of their mycelia never appeared to touch each other. Since both strains were orange-pigmented, the plasmid-containing Ep339 was transformed with plasmid pCB1003 (Carroll *et al.*, 1994) to generate a hygromycin-resistant derivative, Ep339-HGR1 [pCRY1]. A benomyl-resistant mutant derived from the standard laboratory wild-type strain Ep155, Ep155-

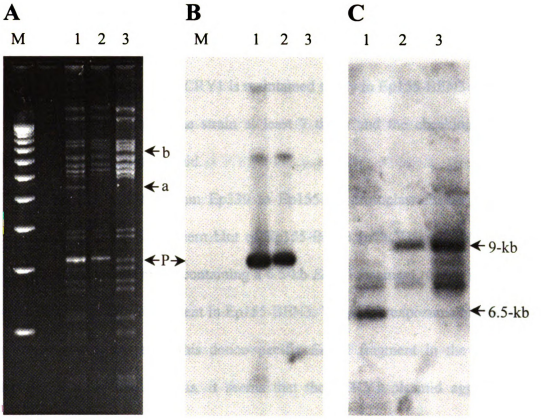

Figure 5.3. ***Eco*RI digestion patterns of mitochondrial DNAs from strains involved in the transfer of pCRY1 through protoplast fusion.** *Lane 1* donor Ep339-HGR1 [pCRY1]; *lane 2* Ep155-BEN3 [pCRY1]; *lane 3* recipient Ep155-BEN3; *lane M* 1-kb DNA ladder. **A** Ethidium bromide stained agarose gel. The position of pCRY1 is denoted by 'P' and the positions of the polymorphic mtDNA fragments are denoted as 'a' (6.5-kb) and 'b' (9-kb). **B** Hybridization of the Southern blot of the gel in panel A with a pCRY1 probe. **C** Southern blot hybridization of *Eco*RI digested mtDNAs with the 6.5-kb donor-specific *Eco*RI fragment. The probe hybridizes to the 6.5-kb fragment in Ep339-HGR1 and to the 9-kb fragments in the recipient. No signal is found for the 6.5-kb fragment in Ep155-BEN3 [pCRY1].

BEN3 (Monteiro-Vitorello *et al.*, 1995), was used as the recipient. Seven benomyl-resistant isolates were checked for the presence of pCRY1 by PCR and one was found to contain the plasmid (Ep155-BEN3 [pCRY1]). The presence of the plasmid was further confirmed by agarose-gel electrophoresis of mtDNA prepared from Ep155-BEN3 [pCRY1] (Figure 5.3A) and hybridization of the Southern blot of the gel against a pCRY1 probe (Figure 5.3B). That pCRY1 is maintained stably in Ep155-BEN3 [pCRY1] was ascertained by subculturing the strain at least 7 times and the checking of each culture for the presence of the plasmid.

The transfer of mtDNA from Ep339 to Ep155 by protoplast fusion also was assessed. For this purpose, a Southern blot of Ep155-BEN3 [pCRY1] mtDNA (Figure 5.3C) was hybridized with a probe containing a 6.5-kb *Eco*RI fragment, which is present in Ep339 and Ep339-HGR1 but absent in Ep155-BEN3. Very high exposures of this blot failed to reveal the presence of this donor-specific *Eco*RI fragment in the recipient Ep155-BEN3 [pCRY1] strain. Thus, it seems that the pCRY1 plasmid aggressively colonized the mitochondria of the recipient without the concomitant transfer or accumulation of mtDNA from the donor.

Virulence of isolates containing pCRY1. In *C. parasitica*, cytoplasmic as well as mitochondrial double-stranded RNA elements have been shown to effectively reduce virulence (Nuss, 1992; Polashock *et al.*, 1997). In addition, in *Neurospora*, mitochondrial plasmids are known to cause instabilities in the mitochondrial genome resulting in senescence (Griffiths, 1992; 1995). In an effort to understand whether pCRY1 plays any role in the pathogenicity of the fungus, isogenic strains with and without the plasmid,

obtained during the vegetative transmission experiments, were assayed for their virulence on apples, chestnut bark and live trees. The results obtained seemed to vary according to the medium used for the tests. Whereas the virulence of only the strains F2.36 and M1.5 seemed to be negatively affected because of pCRY1 in apple tests, tests on chestnut bark revealed that pCRY1 causes a reduction of virulence in Ep155 as well (Table 5.4). The hypovirulent status of these strains was maintained on chestnut trees (Table 5.4). However, there was no apparent reduction in the virulence of F3.39 due to pCRY1 in any of the tests. Thus, it seems that pCRY1 can negatively influence virulence in *Cryphonectria* but only in a strain-specific manner. Moreover, it is apparent that the apple test did not accurately predict the capacity of the different strains to cause disease on chestnut trees.

It has been shown previously that *Cryphonectria* strains that are hypovirulent because of mitochondrial malfunctioning often have elevated levels of alternative oxidase activity (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000). Since pCRY1 is mitochondrial in location, tests were conducted to see if the plasmid-mediated attenuation was phenomenologically similar to the previously-described mitochondrial hypovirulence syndromes. To explore if the plasmid attenuates the strains F2.36 and M1.5 by interfering with the cytochrome-mediated respiratory pathway, the amount of respiration through the inducible alternative oxidase was estimated in these strains as cyanide-insensitive oxygen consumption (Table 5.4). The plasmid-containing and plasmid-free F2.36 and M1.5 strains demonstrated similar low levels of cyanide-resistant respiration (Table 5.4). Hence, there is no evidence that pCRY1 causes hypovirulence by disrupting respiratory pathways. In addition, the strains containing

Table 5.4. Comparisons of alternative oxidase activities, virulence and growth rates of mitochondrially-hypovirulent and pCRY1-attenuated strains of *C. parasitica*.

Strains	Description	pCRY1 present/absent	Alternative oxidase activity as percentage of total respiration	Growth rate[a]	Virulence tests[b] on		
					apples	bark	trees
Ep155	wild-type	absent	7.34 ± 0.34	5.40 ± 0.18	28.8 ± 5.6	5.6 ± 0.6	39.7 ± 7.1
Ep155	attenuated	present	NT[c]	NT	27.0 ± 6.4	1.0 ± 0.4	NT
KFC9-E6[d]	hypovirulent	absent	36.03 ± 7.1	>1.5	>1.0	NG[e]	NG
M1.5	wild-type	absent	11.11 ± 4.3	NT	21.9 ± 1.7	6.2 ± 1.2	28.5 ±10.8
M1.5	attenuated	present	4.85 ± 4.4	NT	13.5 ± 1.4	1.9 ± 0.3	7.8 ± 3.9
F2.36	wild-type	absent	11.26 ± 7.8	5.85 ± 0.17	19.9 ± 6.9	8.7 ± 1.1	24.5 ± 5.2
F2.36	attenuated	present	9.46 ± 8.2	5.86 ± 0.02	8.3 ± 1.3	1.0 ± 0.3	6.8 ± 3.6
J2.31	wild-type	absent	NT	5.88 ± 0.05	NT	NT	NT
J2.31	wild-type	present	NT	5.82 ± 0.22	NT	NT	NT
F3.39	wild-type	absent	NT	NT	8.9 ± 0.4	3.3 ± 1.0	NT
F3.39	wild-type	present	NT	NT	7.1 ± 1.5	3.4 ± 0.7	NT

[a] Measured as growth of mycelial front in growth tubes in mm per day.
[b] Virulence measured as area of lesion in cm^2.
[c] Not tested.
[d] KFC9-E6 is a mitochondrially hypovirulent natural isolate (Baidyaroy *et al.*, 2000).
[e] No growth.

pCRY1 had similar rates of growth (Table 5.4) on ECM-agar when compared to their respective counterparts that did not contain the plasmid. Thus, it seems that the pCRY1-mediated attenuation of virulence is different from the mitochondrial-hypovirulence syndromes that are associated with slow growth-rates and high levels of cyanide-resistant respiration (Mahanti *et al.,* 1993; Monteiro-Vitorello *et al.,* 1995; Baidyaroy *et al.,* 2000).

DISCUSSION

Like other mitochondrial genetic elements, namely dsRNA in *C. parasitica* (Polashock *et al.,* 1997) and plasmids in *Neurospora* (Debets *et al.,* 1995), pCRY1 is extensively transmitted through asexual spores. In this respect, it differs from cytoplasmic elements that are non-mitochondrial in location. For example, cytoplasmic mycoviruses in *Cryphonectria* are transmitted regularly to only a fraction of the conidia (Russin and Shain, 1985; Enebak *et al.,* 1994). Thus, vertical transmission of cytoplasmic elements through conidia can vary depending on their sub-cellular localization. This phenomenon might reflect the lack of existence of an active mechanism for inclusion of such elements in the conidia. Whereas mitochondria are functionally indispensable and of necessity are present in viable spores, dsRNA viruses are non-essential and can be distributed randomly without affecting viability. The plasmid pCRY1 also has been shown to be maternally inherited in sexual crosses (Monteiro-Vitorello *et al.,* 1995; 2000). Collectively, these observations imply that mitochondrial elements like pCRY1, once

they take up residence in a strain, are stably maintained and transmitted to the future generations. In contrast, most cytoplasmic dsRNA viruses of *C. parasitica* are not inherited sexually (Anagnostakis, 1988; Nuss, 1992). Due to this advantage that mitochondrial elements have over other cytosolic elements regarding vertical transmission, the hypovirulence phenotypes in *C. parasitica* that are caused by mitochondrial elements, namely mtDNA mutations (Mahanti *et al.,* 1993; Monteiro-Vitorello *et al.,* 1995; Baidyaroy *et al.,* 2000), plasmids (this study) or dsRNA molecules (Polashock *et al.,* 1997), probably have a better chance of survival and dissemination in nature than virus-mediated types of hypovirulence.

Vegetative incompatibility (*vic*) genes play an important role in the biology in the filamentous fungi because they affect the exchange of genetic elements among individuals through hyphal fusion. Incompatibility genes act as barriers to this transmission possibly because they provide a degree of immunity to the transmission of foreign elements such as viruses and transposons. However, based on the observations in this and other studies (Collins and Saville, 1990; Griffiths *et al.,* 1990; Debets *et al.,* 1994), it is clear that the restriction in the trafficking of cytoplasmic genetic elements by *vic* genes is not absolute. Of the total of seven *vic* genes known for *Cryphonectria* (Huber, 1996; Cortesi and Milgroom, 1998), we have studied the individual effects of the five genes described by Huber (1996) with respect to their effects on transmission of the mitochondrial plasmid pCRY1. The remaining two genes were described only recently (Cortesi and Milgroom, 1998). As shown previously for *Neurospora* (Debets *et al.,* 1994), individual *vic* genes in *C. parasitica* differ in their strength of inhibition of cytoplasmic transmission, with *vic*-2 being the strongest inhibitor and *vic*-5 the weakest.

That inhibition of transfer by particular genes sometimes can only occur in one direction, i.e. from one particular allele to another and not *vice versa*, is demonstrated in the effect of *vic*-1, *vic*-3 and *vic*-4 (Table 5.2) on the transmission of pCRY1. The same phenomenon was observed in studies on the effects of *vic* genes on the transmission of the non-mitochondrial, cytoplasmic dsRNA hypoviruses of *C. parasitica* (Huber, 1996, Liu and Milgroom, 1996). The significant observation in these transfer experiments is that, in all combinations except two, the plasmid was detected in at least one of the recipient single conidial isolates even though the sample sizes were relatively small. This included combinations between strains differing at even the strongest inhibitor, *vic*-2. This data suggests that mitochondrial plasmids like pCRY1 can be transmitted among highly incompatible strains and potentially can colonize populations of genetically different strains in nature. Since the strains used in this study are not isogenic with respect to genes other than the *vic* loci, the role of other hitherto unknown factors in affecting transmission cannot be ruled out at present. However, the results obtained here resemble the effects that the same *vic* genes have on the transmission of another type of cytoplasmic element, namely hypoviruses (Huber, 1996). Since the hypoviruses are dsRNAs that are located outside of the mitochondria, it can be expected that their intracellular proliferation and maintenance depends on a set of cellular functions that is quite different from that which is necessary for the maintenance and proliferation of the pCRY1 mitochondrial DNA plasmid. Hence, it is very likely that our observations provide a reasonably accurate account of the qualitative effects that the different *vic* genes have on the asexual transmission of the plasmid, and possibly mitochondria.

The level of cytoplasmic exchange between strains having allelic differences at multiple *vic* loci seemed to be a function of epistasis of individual genes rather than a complex set of additive interactions between genes. For example, when two strains differed at two loci where one allows transmission and the other does not, transmission usually did not occur. Also, when two strains differed at two different loci, each of which individually allows transmission of the plasmid, then the movement of pCRY1 from the donor to the recipient still occurred and there was no indication of increased inhibition of transfer due to additive negative effects. The observations, therefore, suggest that transmission of cytoplasmic elements between strains differing at multiple loci are influenced more by the specific effects of a limited number of individual *vic* genes or alleles than actual number of the heteroallelic loci that differentiate the donor from the recipient strain. Although so far we have investigated only a very limited number of allelic and non-allelic interactions with respect to their influence on the transmission of pCRY1, the observations are consistent with some of the trends in the effects that different combinations of *vic* alleles and genes have on the transmission of cytoplasmic dsRNA viruses in *C. parasitica* (Huber, 1996, Liu and Milgroom, 1996).

The observation of pCRY1 acquisition by Ep155-BEN3 from the highly incompatible strain Ep339-HGR1 [pCRY1] by protoplast fusion, and the subsequent stable maintenance of the plasmid in the recipient strain, indicates that vegetative incompatibility has little or no influence on the establishment of infectious genetic elements that colonize the mitochondria of coenocytic fungi. Despite the potential diversity in vegetative compatibility types owing to the large number of *vic* genes in natural populations of fungi, the number of the compatibility groups that so far have been

identified in individual populations tends to be small (Horn and Green, 1995; Milgroom and Brasier, 1997; Cortesi and Milgroom, 1998). This lack of diversity may help mitochondrial plasmids to easily colonize a regional population and to be stably maintained therein. The lack of diversity in compatibility types also indicates that many populations might be clonal in nature because of a lack of movement of genotypes between populations. This may also explain why infectious elements like mitochondrial plasmids are not absolutely widespread in distribution (Griffiths, 1995; Monteiro-Vitorello *et al.*, 2000).

The transmission of different cytoplasmic factors may vary depending on their particular nature and intracellular location. For example, whereas the transfer of pCRY1 from *vic*-4^2 donor to a *vic*-4^1 recipient was relatively low, Huber (1996) found that the transmission of double-stranded RNA viruses from *vic*-4^2 donor to a *vic*-4^1 recipient was relatively frequent. The pattern of transmission of a mitochondrial plasmid can even be different from that of the mitochondrial DNA. Mitochondrial DNA appears to be transmitted with much lower efficiency than plasmids, since phenotypes or RFLPs associated with the mtDNA of donors were not observed to be transmitted to the recipients even when mitochondrial plasmids were transferred (Debets *et al.*, 1994; van der Gaag *et al.*, 1998; this paper). For *Fusarium*, isolates in the same vegetative compatibility group were always found to be associated with the same mitochondrial DNA haplotype (Gordon and Okamoto, 1991). An extension of this phenomenon was observed in the transfer of pCRY1 by protoplast fusion, where the pCRY1 plasmid, but not the mtDNA was acquired and stably maintained by the recipient strain. Collectively, these observations suggest that the mitochondrial hypovirulence phenotypes caused by

interactions of plasmids with mitochondrial DNA, as observed for senescence-causing plasmids in *Neurospora* (Akins *et al.*, 1986; Bertrand *et al.*, 1986; Court *et al.*, 1991), can probably be more effectively transmitted, horizontally or vertically, than other types of cytoplasmic hypovirulence factors. However, mtDNA mutations that are suppressive have been shown to be transmitted at least as effectively as plasmids and also behave as infectious elements (Bertrand, 1995; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000).

The attenuation of *C. parasitica* by pCRY1 was found to be strain-specific since not all the strains that contained the plasmid were hypovirulent. Thus, the adverse effect that pCRY1 can have on its host strain may depend on physiological conditions determined by nuclear genetic factors. Two aspects of the virulence-depressing activity of pCRY1 are puzzling. The first enigma is that it has any effect at all, for the related Fiji and LaBelle elements are the only two well-characterized mitochondrial plasmids of *Neurospora* that appear to have no effect at all on the growth and longevity of this fungus (reviewed by Griffiths, 1995). In contrast, the circular Mauriceville and Varkud and linear *kalilo* and *maranhar* plasmids all are known to induce a degenerative mitochondrial syndrome described as senescence (reviewed by Griffiths, 1992; 1995). Thus, it is possible that related plasmids interact in different ways with their respective fungal hosts. This view is supported not only by the observation that variants of the senescence-eliciting kalilo plasmid found in several species of *Neurospora* and closely related genera do not integrate with equal efficiency into the mtDNAs of all the host species (Griffiths, 1998), but also by the finding that pCRY1 interacts in different ways with different strains of *C. parasitica*. The second paradox is that the plasmid depresses the virulence of

the Ep155, F2.36 and M1.5 strains of *C. parasitica* without having a noticeable effect on the growth rate, reproductive potential, pigmentation, respiratory phenotypes, or any other obvious characteristics of the fungus. In this respect, the pCRY1-mediated form of hypovirulence is different from the other mitochondrial hypovirulence syndromes that have been described to date (Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995; Baidyaroy *et al.*, 2000). In these cases, the afflicted cultures manifest slow growth rates and abnormal respiratory phenotypes indicative of mtDNA mutations that inhibit cytochrome-mediated electron-transport activity. At this point, there is no indication that pCRY1 affects virulence by interfering directly with mitochondrial energy metabolism.

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CHAPTER 6

In vivo conformation and replication of circular mitochondrial plasmids and mitochondrial DNA of filamentous fungi

ABSTRACT

In this study, replication mechanisms that can be sustained in the mitochondria of filamentous fungi were ascertained. For this purpose, we have analyzed the replication intermediates of circular mitochondrial plasmids were analyzed by two-dimensional (2D) gel electrophoresis and electron microscopy (EM). Both the two different classes of circular plasmids, namely plasmids that encode reverse transcriptase genes (Mauriceville and Varkud of *Neurospora* species) and plasmids that encode DNA polymerase genes (Fiji and LaBelle of *N. intermedia* and pCRY1 of *Cryphonectria parasitica*) were included in this study. Contrary to previous studies, the circular plasmids were found to predominantly exist *in vivo* as circular molecules instead of in linear forms. Whereas all the plasmids were found to be replicating predominantly by rolling circle mechanisms, the Mauriceville plasmid of *N. crassa* was observed to be also able to replicate by reverse transcriptase-mediated mechanisms. Furthermore, unlike the DNA polymerase-containing plasmids, the Mauriceville plasmid seemed to have a fixed origin for rolling circle

replication. A plasmid-like element, named pleC9, of *C. parasitica*, which contains short sequences that had homology to the mtDNA, was found also to be replicating by a rolling circle mechanism. An analysis of *in vivo* conformation of the *N. crassa* mtDNA revealed that, concomitant to previous observations in other systems, a majority of mtDNA molecules exist in linear forms with heterogeneous ends. However, a few circular mtDNA molecules were also detected. On the basis of these results, a replication mechanism for *Neurospora* spp. mtDNA that is most consistent with a rolling circle model is suggested.

INTRODUCTION

Mitochondrial DNA (mtDNA) is the main form of extra-nuclear genetic material in filamentous fungi encoding several genes responsible for the functioning of the electron transport chain (Gillham, 1994). Since most fungi are obligate aerobes, these genes are essential, and hence perpetuation and maintenance of mtDNA is an absolute necessity in these organisms. In the filamentous fungi, this aspect is of enhanced significance because abnormalities of mtDNA maintenance resulting in debilitating mutations are infectious (Bertrand, 1995) on account of their suppressive nature and the coenocytic nature of the mycelia. Such mutations can rapidly spread in epidemic fashion through a population (Baidyaroy *et al.*, 2000a). These observations suggest that the faithful replication, and repair, of the mtDNAs are of utmost importance for the survival

of these organisms. Despite a significant amount of progress in the investigation of mtDNA replication in vertebrates (reviewed by Shadel and Clayton, 1997) and yeasts (Maleszka *et al.*, 1991, Han and Stachow, 1994), similar in-depth studies have not been conducted in filamentous fungi. In this study, an attempt has been made to understand the mechanisms of replication that can be sustained in the mitochondria of a model filamentous fungus, namely *Neurospora* spp. and the chestnut blight agent *Cryphonectria parasitica.*

Apart from the presence of *bona fide* mtDNA molecules, the mitochondria of filamentous fungi can also harbor true plasmids (Fecikova, 1992; Griffiths, 1995; Kempken, 1995). Whereas restriction-mapping data suggests a circular map for the fungal mtDNA (Taylor and Smolich, 1985; Bell *et al.*, 1996; Silliker and Cummings, 1990), both linear and circular mitochondrial plasmids (Griffiths, 1995) have been detected in these organisms. The linear plasmids are very different in structure from the mtDNA in terms of organization because most have terminal inverted repeats and terminal proteins, much like linear bacteriophages (Yoshikawa *et al.*, 1986; Savilhati and Bamford, 1986), adenoviruses (reviewed by Salas, 1991) and similar elements in yeasts (Worsham and Bolen, 1990; Kikuchi *et al.*, 1984), plants (Kemble and Thompson, 1982; Pring and Smith, 1985), and prokaryotes (Kinashi and Shimaji-Murayama, 1991; Keen *et al.*, 1988). They also seemingly contain the machinery for their own perpetuation in terms of encoding DNA polymerases, RNA polymerases and sometimes even reverse transcriptases (Walther and Kennell, 1999). Thus, it appears that the linear plasmids probably replicate in a fashion similar to the well-described protein-primed system of the adenovirus (Salas, 1991), and hence, are quite independent of the mtDNA replication

machinery. However, the circular plasmids found in filamentous fungi either encode either a DNA polymerase or a reverse transcriptase. Therefore, these elements obviously need the mitochondrial transcription apparatus for the expression of their genes. Thus, the circular plasmids probably share some common features of replication of *bona fide* mtDNA despite the fact that mtDNA replication is controlled at the nuclear level whereas plasmid replication is not.

In many filamentous fungi, plasmid-like molecules have been detected as amplified elements in the mitochondria. These elements can be derived either entirely (Silliker and Cummings, 1990) or partially (Chapter 4 of this study) from the mtDNA. The amplification of these sub-genomic circles have been seen in a variety of filamentous fungi, namely *Podospora anserina* (Silliker and Cummings, 1990), *Neurospora crassa* (Bertrand *et al.*, 1980), *Aspergillus amstelodami* (Lazarus *et al.*, 1980) *Cryphonectria parasitica* (Monteiro-Vitorello *et al.*, 1995) and *Ophiostoma ulmi* (Charter *et al.*, 1993), and are derived from different regions of mtDNA. Since these molecules appear to be circular, are essentially a part of the *bona fide* mitochondrial genome, and often do not code for any gene product, it is very likely that these elements are replicated in a fashion similar to the mtDNA. Thus, it seems logical that a thorough investigation into the mechanism of replication of such elements might help in the understanding of the replication mechanisms of the mtDNA.

Some circular mitochondrial plasmids of filamentous fungi can cause senescence without integration into the mtDNA (Stevenson *et al.*, 1999; Baidyaroy *et al.*, 2000b). This phenomenon has been observed for both reverse transcriptase and DNA polymerase encoding plasmids in two different fungi, namely *Neurospora crassa* and *Cryphonectria*

parasitica. For the pCRY1 plasmid-mediated attenuation of *C. parasitica*, the affected strains did not demonstrate elevated levels of cyanide-resistant respiration. This observation indicates that the mechanism of pCRY1-mediated attenuation is different than the one that results from suppressive mtDNA mutations that block oxidative phosphorylation pathways where the senescence syndrome is accompanied by high levels of cyanide-resistant respiration. One possible mechanism by which these plasmids can produce the senescence phenotype is through over-replication of its genome thereby competing with the host mtDNA for essential cellular factors. Thus, to gain a better understanding of the pathways by which these plasmids induce senescence, an investigation of the replication mechanisms of these elements and the host mtDNAs need to be initiated.

Since mtDNA mutations can act as infectious debilitating elements in filamentous fungi (Bertrand, 1995), the effect of similar mutations have been considered as potential bio-control agents of plant pathogenic fungi (Baidyaroy *et al.*, 2000a). It was found that suppressive mtDNA mutations can indeed result in a transmissible hypovirulence syndrome, thereby alleviating disease in nature (Monteiro-Vitorello *et al.*, 1995, Baidyaroy *et al.*, 2000a). Thus, it seems that delivery of suppressive mtDNA mutations into the mitochondria of pathogenic fungi can be an important tool in biological control of diseases. For the development of an effective gene delivery system it is necessary to examine replication mechanisms of fungal mitochondria and mitochondrial plasmids that can be utilized as shuttle vectors. For this purpose, we have analyzed replication intermediates of circular mitochondrial plasmids, as well as circular plasmid-like elements from the mitochondria in order to conceive the forms of replication that can be

supported within the fungal mitochondria. The replication intermediates were studied mainly by examining two-dimensional gel electrophoresis patterns and also by electron microscopic observations of plasmid DNAs. The *in vivo* conformation of mtDNA molecules was also analyzed for this purpose.

MATERIALS AND METHODS

Fungal strains and media. Strains that were used in this study were *Neurospora crassa* Mauriceville-1c (FGSC 2225), *Neurospora intermedia* strains Varkud-1c (FGSC 1823), LaBelle-1b (FGSC 1940) and Fiji N6-6 (FGSC 435), and a *Cryphonectria parasitica* strain Ep339 and a hypovirulent, natural isolate named KFC9-E6. The *Neurospora* strains were maintained on Vogel's medium (Vogel, 1956) while the *C. parasitica* strains were grown on Endothia complete medium as described by Puhalla and Anagnostakis (1971).

Preparation of mitochondrial DNA. The strains were grown in liquid medium while shaking at 200 rpm for 12-14 hr at room temperature. A conidial suspension was used as the inoculum for the *Neurospora* strains while homogenized mycelia were used for *C. parasitica*. Mycelia were harvested by filtration through a Schleicher and Schuell No. 470 filter, and washed first with cold deionized water and then with ice-cold isolation buffer (0.44 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 7.6). Purified mitochondria were obtained by the flotation-gradient procedure (Lambowitz, 1979). MtDNAs were purified

by following previously described techniques (Bell *et al.*, 1996). For the *Neurospora* strains, the mtDNAs were further purified with successive phenol, phenol-chloroform and chloroform extractions. MtDNAs from the *C. parasitica* strains were purified by a successive phenol and phenol-chloroform extraction followed by a treatment with CTAB to remove impurities that interfered with the activity of restriction enzymes (Ausubel *et al.*, 1987). This step was followed by two consecutive chloroform extractions to purify the DNA. The mtDNAs were precipitated with acetate-saturated ethanol (Ausubel *et al.*, 1987), washed with cold 70% ethanol, dried under vacuum, and dissolved in a small volume of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.6), supplemented with heat-treated RNaseA (10 μg/ml).

Preparation of mitochondrial DNA by alkaline lysis and standard procedures. *Neurospora* strains were grown for 4-5 days on solid Vogel's medium (Vogel 1956) in three 250 ml Erlenmeyer flasks with 50 ml of medium in each flask. The conidia were harvested by washing the cultures with approximately 150 ml sterile distilled water. The water was gently swirled around the inside of the flasks to collect as much conidia as possible. This conidial suspension was used to inoculate 2 liters of liquid Vogel's medium and the strain was grown for 12-14 hr while shaking at 200 rpm. The mycelia was harvested and processed as described above. Mitochondria obtained from the flotation gradients were re-suspended uniformly in cold isolation buffer, measured and divided equally. One half was used to prepare mtDNA as described in the previous section. The other half was centrifuged in the cold at 16,000 rpm for 40 min and the resultant supernatant was discarded. A standard alkaline lysis reaction, as used for the

extraction of plasmids from bacteria (Sambrook *et al.*, 1989), was performed with the isolated mitochondria with the following modifications. The mitochondria were re-suspended very gently with a teflon pestle in 2 ml of ice-cold solution I (re-suspension buffer; 50 mM Tris-HCl, pH 7.5, 10 mM EDTA). The mitochondria were lysed for 5 min with 4 ml solution II (lysis solution; 0.2 M NaOH, 1 % SDS) and neutralized with 3 ml solution III (neutralization buffer; 1.32 M potassium acetate) for 15 min. All of the above procedures were carried out on ice. The resultant DNA was precipitated at room temperature with iso-propanol for 16 hr. The DNA obtained by alkaline lysis, and by the usual procedures as described in the previous section, were dissolved in equal volumes of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.6), supplemented with heat-treated RNaseA (10 μg/ml).

Enzymatic digestions of mitochondrial DNAs. DNA was digested with restriction endonucleases as recommended by the manufacturer (Gibco, BRL). For DNaseI digestion, the mtDNA was treated with 3 units of the enzyme for 7 min at room temperature in the presence of a digestion buffer (0.05 M Tris-HCl, pH 8.0, 0.01 M $MgSO_4$, 2 mM EDTA). The enzyme was heat-killed at 65°C for 5 min after the reaction was completed. S1 endonuclease treatments were performed with 4 units of the enzyme and the reaction mixtures were incubated at 37°C for 30 min in the presence of a digestion buffer (0.2 M NaCl, 0.05 M Na-acetate, 1 mM $ZnSO_4$, 0.5% glycerol). The enzyme reaction was terminated at 65°C for 10 min. RNaseH digestions were carried out with 3 units of the enzyme and the reaction was performed at 37°C and the reaction was terminated by addition of the stop-dye mixture (Brewer and Fangman 1988). Exonuclease

III digestions were performed with 10 units of the enzyme per reaction and incubated 37°C for 16 hr. The enzyme was heat-killed at 65°C for 5 min after the reaction was completed. No particular buffer solution was used for RNaseH or exonuclease III treatments. For digestions that involved multiple enzymes that require different buffers, the DNA was precipitated after digestion with the first enzyme and re-suspended in water and appropriate buffer for subsequent treatments.

Neutral/neutral two-dimensional (2D) agarose gel electrophoresis. The 2D gel electrophoresis experiments were performed as described by Brewer and Fangman (1988; 1991). The first dimension was resolved on 0.4-0.5 % agarose gels, depending upon the size of the molecule of interest, at 1 V/cm. The second dimension was separated on 1-1.2 % agarose gels at 5 V/cm in the presence of ethidium bromide (0.4 μg/ml) in the cold. The concentration of agarose in the gels and the period of electrophoreses varied and were determined according to the molecular weight of the molecule of interest.

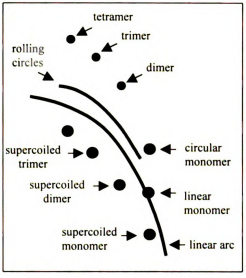
Southern blotting and hybridization. The DNA was blotted on to positively charged nylon membranes (Amersham) as described by Sambrook *et al.* (1989). Hybridizations and washes were performed at 70°C. The probes were labeled with dig-oxigenin-dUTP by random primed labeling using the Genius kit following the procedure suggested by the manufacturer (Boehringer-Mannheim). Binding of the probe was detected with anti-digoxigenin Fab-alkaline phosphatase conjugate using CDP-star as directed by the manufacturer (Boehringer-Mannheim).

Electron microscopy (EM). A modification of the micro-method for spontaneous adsorption was used to prepare DNA samples for examination under the transmission electron microscope (Coggins, 1987). Drops (200 µl) of solution containing 1ng/µl DNA, 14 ng/µl cytochrome c and 0.3M ammonium acetate were allowed to stand on parafilm for 15 min. Formvar-coated grids were then touched to the drops for 2 sec, immediately stained with a uranyl acetate solution (0.5 % uranyl acetate in 75% ethanol) for 20 sec, and washed in a 90% ethanol solution for 20 sec. Grids were then shadow-casted with 250 Hz of platinum evaporated in the electron beam gun. Platinum deposition was monitored with a Quartz Crystal Monitor, and controlled with a Control Unit EVM052. The angle of platinum deposition was 7 degrees. Grids were examined with a Philips CM10 transmission electron microscope operating at 100 kV and micrographs recorded.

RESULTS

***In vivo* conformation of plasmid DNAs in the mitochondria.** Previous studies (Maleszka, 1992) using pulse field gel electrophoresis suggested that plasmid DNA molecules exist predominantly in linear forms despite restriction mapping and sequence data indicating that they have a circular structure. When mtDNA preparations from a strain known to contain the circular mitochondrial plasmid Mauriceville (pMAU), which encodes a reverse transcriptase gene, was subjected to 2D gel electrophoresis, and the corresponding Southern blot hybridized to a plasmid probe, a novel pattern was observed.

The signals are explained schematically (Figure 6.1A) as described in previous studies (Brewer and Fangman, 1987; 1988; 1991). Relaxed circular forms were observed migrating at a slower rate than the linear forms that formed a continuous arc of low molecular weight molecules to high molecular weight molecules (Figure 6.1B). However, the bulk of the DNA was observed as elements migrating faster than the linear molecules, and in multiple sizes, which were distributed in an arc of their own. That these molecules were actually supercoiled circular plasmid molecules was ascertained by treating the DNA sample briefly with DNaseI. Digestion with DNaseI resulted in the nicking of the supercoiled DNAs and their conversion to the corresponding relaxed circular forms (Figure 6.1C). Moreover, supercoiled plasmids of a particular size were present as different species of molecules that migrate at the same rate in the second dimension but had separated differently in the first. A likely explanation for this phenomenon is that these are molecules of the same size but have different degrees of supercoils. Collectively, these observations imply that, unlike the findings of the previous study (Maleszka, 1992), the majority of the plasmid molecules were present *in vivo* either as circular supercoiled elements, or in relaxed circular forms (Figure 6.1B). The linear molecules could either be generated by random breakage of the circular forms, or they could be *bona fide* linear replication intermediates where the plasmid is replicating by a rolling circle mechanism. Since the presence of predominant linear molecules of sizes that correspond to the monomer and multimeric plasmid forms are observed, it can be argued that some of this linear DNA is a result of degradation of circular forms. A similar pattern was also observed for other circular mitochondrial plasmids as shown for the DNA polymerase-encoding Fiji plasmid (pFIJ) of *N. intermedia* (Figure 6.1D). An



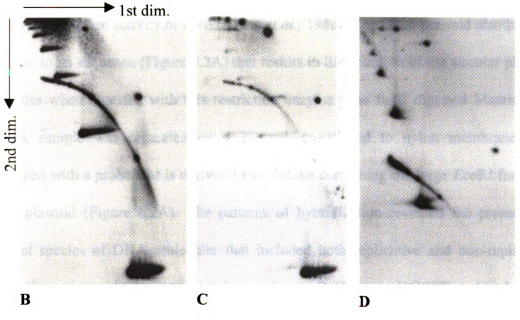


Figure 6.1. ***In vivo*** **conformation of circular mitochondrial plasmids analyzed by 2D gel electrophoresis.** The signals obtained were interpreted (**A**) according to Brewer and Fangman (1987, 1988, and 1991) and Backert *et al.*, (1997). Uncut mtDNA from the Mauriceville-1c strain, untreated (**B**) or briefly treated (**C**) with DNaseI, was separated in two dimensions, blotted and hybridized with the Mauriceville plasmid probe. Uncut mtDNA from the Fiji N6-6 strain revealed similar pattern of signal (**D**) when hybridized with a Fiji plasmid probe.

important observation to note is that none of the samples showed any arc joining any circular species of a specific length with its adjacent concatamers. This arc, referred to as the 'eyebrow' (Brewer and Fangman, 1987; Preiser *et al.*, 1996), is indicative of molecules replicating by a θ mechanism, i.e. bi-directionally from a single origin (Brewer and Fangman, 1988).

Replication of the Mauriceville plasmid of *N. crassa*. The Mauriceville plasmid of *Neurospora crassa* is known to contain an ORF that encodes a reverse-transcriptase gene (Griffiths, 1995) and mitochondrial extracts from this strain have been shown to have reverse transcriptase activity *in vitro* (Akins *et al.*, 1986). The DNA plasmid also has one *Bgl*II site in its sequence (Figure 6.2A) that results in linearization of the circular plasmid molecules when digested with this restriction enzyme. The *Bgl*II digested Mauriceville mtDNA sample was separated on a 2D gel, transferred to nylon membranes and hybridized with a probe that is derived from a clone containing the large *Eco*RI fragment of the plasmid (Figure 6.2A). The patterns of hybridization revealed the presence of different species of DNA molecules that included both replicative and non-replicative forms. These patterns were interpreted according to Backert *et al.* (1997) and is depicted in Figure 6.2B. A very strong signal was observed in the position of the linearized monomer (Figure 6.2C). A much weaker but clearly visible spot occurred at the position of linearized dimers. Other linear molecules were expected to migrate on a straight line between these two spots. All hybridization signals above the line of the linear molecules represent forms of the pMAU DNA other than linear molecules and depict replicative DNA. A continuous arc of growing Y-shaped replication intermediates expanding from

the linear monomer to the dimer was observed. In addition, an arc of X-shaped (or the so-called double-Y) molecules is observed to extend from the dimer. The presence of such structures could be attributed to recombination intermediates between two linear restriction fragments (Brewer and Fangman, 1988) where molecules along the spike differ in the position of their cross-over points, with the slowest-migrating form having their cross-over points near the centers of the fragments analyzed and the fast-migrating forms having their cross-over points near one of the two ends. Such an arc also can represent fragments with replication bubbles at both ends cut by the enzyme (Brewer and Fangman, 1987; 1988; 1991), and to circles with two tails, as detected by electron microscopy (Backert and Börner, 1996). Circles with more than one tail can be generated, for example, by a second initiation of replication at the origin before completion of the first round of replication or by simultaneous initiation of replication at more than one origin (Backert *et al.,* 1996). A faint 'E' arc is observed extending from the arc made by the X-shaped molecules. This arc represents large single stranded molecules or double stranded molecules containing large or numerous single stranded regions (Han and Stachow, 1994). These molecules can be derived from cut circles with tails exceeding the contour length of the circles which were not cut (e.g., because of stretches of ssDNA at the cutting site or entirely single stranded tails). The patterns observed for pMAU DNA are not compatible with those obtained from intermediates of bi-directional replication from an internal origin. Intermediates, which could result from digestion of molecules with replication bubbles, were never observed. Thus, the pattern of the simple Y-shaped molecules appear to be the predominant replication intermediates, which can be explained as σ-like molecules arising because of replication by a rolling circle mechanism from an

origin which is either located near the *Bgl*II site (because of generation of a complete Y-arc when cut with *Bgl*II) or from multiple dispersed origins.

When digested with *Hinc*II, which cuts twice on the pMAU DNA and thus generates two fragments, the Y-arc was found to be incomplete for the larger fragment (Figure 6.2D). An incomplete Y-arc extending from the monomer but not reaching the dimer suggests initiation of replication from an origin distant from the sites of the restriction enzyme (Backert *et al.,* 1997). The Y-arc on the smaller fragment is not visible because the probe used was derived from the large *Eco*RI fragment and only marginally overlapped with that region. Moreover, an extended E-arc and some X-shaped molecules extending from the Y-arc are also visible (Figure 6.2D). Collectively these observations suggest that the pMAU replicates predominantly by a rolling circle mechanism from a fixed origin on the large *Hinc*II fragment, which is located near the *Bgl*II site, but distant from the adjacent *Hinc*II site (Figure 6.2A).

Since reverse transcriptase activity has been detected in the mitochondrial extracts of strains containing circular plasmids that encode reverse transcriptases (Akins *et al.,* 1986), the hypothesis that pMAU is at least partially replicated through reverse transcription was tested. Replication mediated by reverse transcription would involve synthesis of DNA on an RNA template. Hence, possible replication intermediates that are generated can include molecules that are DNA-RNA hybrids. When the DNA sample was cut with *Hinc*II and subsequently digested with RNaseH, which preferentially degrades RNAs from RNA-DNA hybrids, new species of DNA molecules that formed continuous arcs which migrated faster than the linear double stranded DNA were observed (Figure 6.3A). These DNA molecules were not observed when the sample was not treated with

RNaseH (Figure 6.2D). The new faster-moving molecules were determined to be single stranded DNA as they were found to be degraded when the sample was treated with S1 endo-nuclease after treatment with *Hinc*II and RNaseH (Figure 6.3B). Thus, it appears that the DNA sample contained detectable amounts of DNA-RNA hybrid molecules that could represent intermediates synthesized when pMAU is replicated through reverse transcription. Similar results were obtained for the VS plasmid DNA that is maintained only in strains containing the Varkud plasmid (Akins *et al.*, 1988; Griffiths, 1995) that also encodes a reverse transcriptase (data not shown).

The conclusions derived from the 2D studies with respect to replication mechanisms of the pMAU plasmid was corroborated by the data obtained from electron microscopic analyses of the plasmid DNA. The plasmid molecules were found as closed circles (Figure 6.4A), and as closed circles with long, variable tails (Figure 6.4B). The presence of the latter type of molecule is suggestive of rolling circle replication. That the circular molecules varied in sizes was in agreement with the observation from 2D analyses which showed the plasmid to be present as concatamers as well as in monomeric lengths. In addition, some linear molecules were observed in all the DNA preparations that were used for electron microscopic analyses. These molecules were predominantly of the size of the monomeric plasmid and often, branched structures were observed on them (Figure 6.4A). A possible explanation for the presence of these molecules lies in the fact that pMAU can replicate also through reverse transcription and these linear molecules are the cDNA-RNA hybrid intermediates of this type of replication. Thus, the linear molecules can be the RNA-DNA hybrid molecules that were detected in the

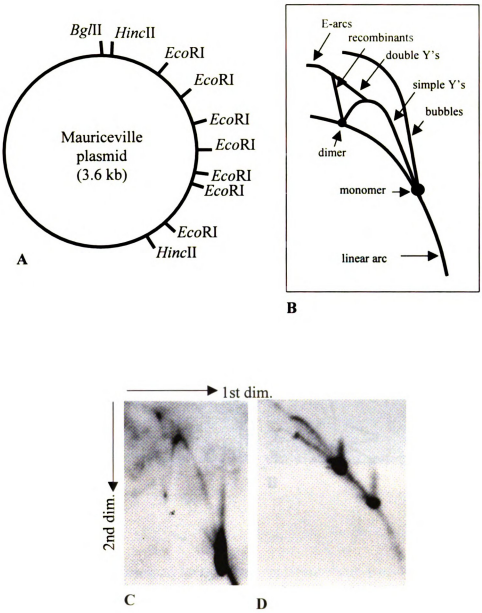


Figure 6.2. Rolling circle replication of the Mauriceville plasmid as detected by 2D gel electrophoresis. The DNA was digested with different restriction enzymes as required according to the restriction map of the plasmid (**A**). The patterns of hybridization obtained (**B**) are explained according to Brewer and Fangman (1987, 1988, and 1991) and Backert *et al.,* (1997). Southern blot hybridizations of *Bgl*II cut (**C**) or *Hinc*II cut (**D**) mtDNAs from the strain Mauriceville-1c were hybridized with a Mauriceville plasmid probe.

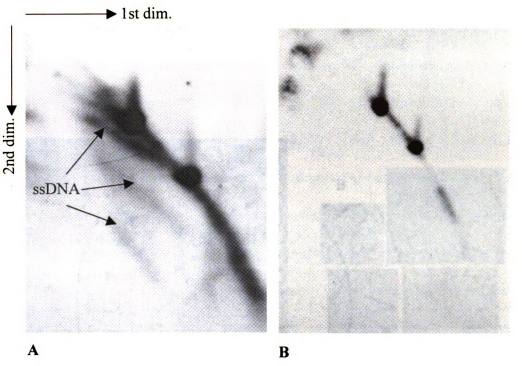


Figure 6.3. Reverse transcriptase-mediated replication of the Mauriceville plasmid. Single stranded DNA (ssDNA) arcs are observed when the *Hinc*II cut sample is treated with RNaseH (**A**). However the ssDNAs are degraded when the sample is subsequently treated with S1 endonuclease (**B**).

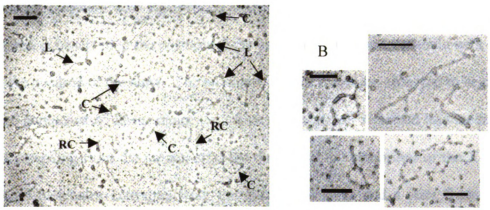


Figure 6.4. Electron micrographs showing replicative and non-replicative forms of the Mauriceville plasmid. In panel (A), circular DNAs (C), linear forms (L) and replicating circular forms (RC) are observed in the same field. The plasmid is observed to be replicating by rolling circle mechanism as indicated by characteristic tails (**B**) of variable lengths that are attached to circular plasmid molecules of various lengths. The bar in each panel represents a length of 200 nm.

2D patterns. However, generation of some of the linear molecules by random breakage of the circular plasmids cannot be ruled out.

Replication of the mitochondrial plasmids that encode a DNA polymerase gene. MtDNA from *C. parasitica* that contains the DNA polymerase encoding pCRY1 plasmid was linearized by restriction digestion with *Eco*RI, which cuts once on the pCRY1 sequence. The DNA sample was subjected to 2D gel electrophoresis, and the corresponding Southern blot was probed with pCRY1 DNA (Figure 6.5A). A strong hybridization is observed at the position of the monomeric plasmid molecule with a faint signal at the position of the dimer. The monomer and the dimer are observed to be present on the arc composed of linear molecules. A complete arc of Y-shaped intermediates is observed extending from the monomer to the dimer. An arc comprised of the X-shaped recombinant molecules extending from the dimer as a spike and a faint E-arc also is visible. These observations, in particular, are reminiscent of rolling circle replication (Backert *et al.*, 1997) commencing from an origin that is either near the *Eco*RI site or initiating from multiple dispersed origins throughout the plasmid DNA.

Both the DNA polymerase encoding circular plasmids of *Neurospora intermedia*, namely the Fiji and the LaBelle (pLAB) plasmids have one *Eco*RI site on their respective DNAs. When *Eco*RI digested mtDNA from a strain containing pFIJ was subjected to 2D gel electrophoresis and the corresponding Southern blot was hybridized to a probe derived from the pFIJ plasmid sequence, the pattern of signals obtained was suggestive of replication of the plasmid by a rolling circle mechanism (Figure 6.5B). Strong and weak hybridizations marked the positions of the monomer and the dimer molecules

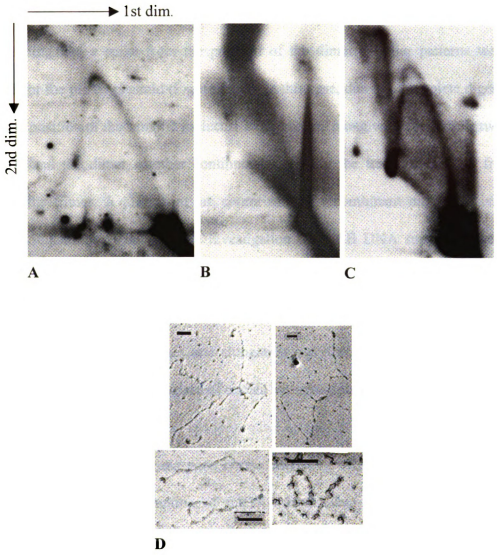


Figure 6.5. Replication mechanism of the DNA polymerase containing circular plasmids. Only simple Y-shaped molecules, recombinants and E-arcs are observed for the pCRY1 plasmid (**A**) of *C. parasitica*, the Fiji (**B**) and the LaBelle (**C**) plasmids of *Neurospora intermedia*. EM studies show circular LaBelle plasmid molecules of various lengths (**D**) with single or multiple tails of heterogeneous sizes, which is reminiscent of replication by a rolling circle model. The bar in each panel represents a length of 200 nm.

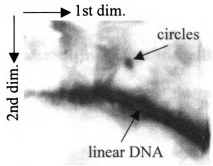
respectively. A complete Y-arc was suggestive of replication from an origin either near the *Eco*RI site or from multiple dispersed origins. Recombinant molecules formed a faint arc extending like a spike from the position of the dimer. Similar patterns were also obtained for the pLAB plasmid (Figure 6.5C). In this case, due to incomplete digestion of DNA, the position of the trimeric molecule is visible and along with an Y-arc between the monomer and the dimer, another continuous Y-arc can be traced extending from the dimer to the trimer. A prominent arc comprising of recombinant molecules is visible (Figure 6.5C). Electron microscopic investigation of pLAB DNA supported the notion that the plasmid replicates predominantly by a rolling circle mechanism. Circular plasmid molecules were observed to have tails of variable lengths (Figure 6.5D). That replication can be initiated at multiple times and elongation can occur simultaneously on a single molecule is indicated by the presence of circles with multiple tails.

***In vivo* conformation of *Neurospora crassa* mtDNA.** Restriction mapping data has suggested a circular conformation of mtDNA from *Neurospora crassa* (Taylor and Smolich, 1985) as well as from other filamentous fungi (Silliker and Cummings, 1990; Bell *et al.*, 1996). To ascertain the native conformation of mtDNA molecules *in vivo*, the uncut forms of the mtDNA from *N. crassa* were separated through 2D gel electrophoresis. The DNA was blotted and hybridized with a probe generated from *Taq*I digested *N. crassa* whole mtDNA. Hybridization patterns revealed that the bulk of the mtDNA molecules were linear in conformation and only a minute fraction of the total mtDNA migrated slowly in the second dimension like relaxed circles (Figure 6.6A). No other multimeric forms were observed. However, it is possible that the multimeric forms,

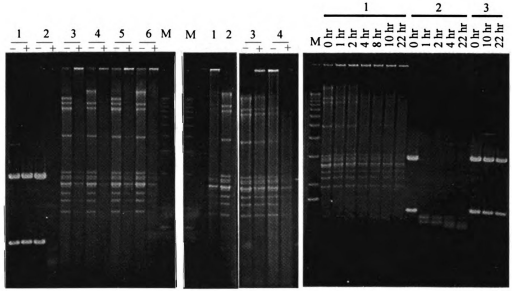
if any, might not be able to penetrate the gel under the conditions used. The linear fragments were not of any fixed length. Neither were there any particular sizes of the linear molecules that were predominant over the others. Instead, a continuous gradient, in terms of size, was observed. Some linear molecules were even larger in size than the circular form (Figure 6.6A). Similar results were always obtained whenever the experiment was repeated.

To confirm the findings obtained by 2D gel electrophoresis, mtDNA from *N. crassa* was treated with exonuclease III that can degrade only linear molecules. *N. crassa* mtDNA is known to contain a single *SalI* site (Taylor and Smolich, 1985) and hence digestion with *SalI* converts all mtDNA molecules to a linear form. Subsequent digestion with exonuclease III, hence, should degrade the entire DNA. However, when the mtDNA is not treated with *SalI* prior to exonuclease III digestion, the DNA that is circular will not be degraded. The exonuclease III treated mtDNA samples, when subsequently cut with *Eco*RI and separated on agarose gels would enable determination of DNA that is not degraded. There was no significant difference in the extent of degradation when the mtDNA was linearized prior to exonuclease III treatment and when it was not (Figure 6.6B). This result supports the observation obtained from 2D gel patterns that only a minute fraction of the mtDNA is present as covalently closed molecules. However, it was found that mtDNA can be isolated from purified mitochondria by alkaline lysis, a method employed for preferential enrichment for circular molecules. MtDNA was prepared using conventional methods and by alkaline lysis from equal amounts of mitochondria and the final DNA obtained from both the procedures was dissolved in equal volumes of buffer. Equal volumes of this DNA from both the samples were digested with *Eco*RI

Figure 6.6. ***In vivo*** **conformation of mtDNA of** ***Neurospora crassa***. 2D gel analysis (**A**) shows that only a very few molecules of mtDNA exist as circles whereas the bulk of the DNA migrates as linear molecules. This result was confirmed by exonucleaseIII digestion of the mtDNA samples (**B**). Sample 1 and 2 served as control where they are circular and linearized plasmid DNAs (linearized with *Xba*I before exonucleaseIII treatment) respectively. For each sample, (+) indicates the exonuclease treated set while (–) indicates that the sample was not treated with exonucleaseIII. Two different preparations of mtDNA (samples 3 & 4, and samples 5 & 6) gets equally degraded irrespective of prior linearization by *Sal*I (3 and 5) before exonucleaseIII treatment indicating that most of the DNA is present in the linear form. Some mtDNA can be isolated (**C**) by alkaline lysis of mitochondria (lane 1) though the amount obtained is much less when compared to amounts of mtDNA obtained by proteinase treatment (lane 2) of similar amounts of purified mitochondria. The mtDNA obtained by alkaline lysis is, however, insensitive to exonucleaseIII digestion (sample 3) whereas the mtDNA obtained by proteinase treatment of mitochondria (sample 4) is not. A time-course study (**D**) where total mtDNA (sample 1) was digested with exonucleaseIII over a period of time showed that the linear mtDNA molecules have heterogeneous ends as no particular fragment was observed to be degraded at a faster rate than the others. Sample 2 (linearized plasmid DNA cut with *Xba*I prior to exonuclease III digestion) and sample 3 (circular plasmid DNA, cut with *Xba*I after exonuclease III treatment) served as controls. All the mtDNA samples were digested with *Eco*RI after exonucleaseIII treatment. Lane M represented the molecular weight marker.


1st dim.
2nd dim.
circles
linear DNA

A


1 2 3 4 5 6 M
− + − + − + − + − + − +
M 1 2 3 4
− + − + − +
1
2
3
M 0 hr 1 hr 2 hr 4 hr 10 hr 22 hr 1 hr 2 hr 4 hr 22 hr 0 hr 10 hr 22 hr

B C D

and separated on a gel, and only a low amount of DNA was observed for mtDNA prepared by alkaline lysis when compared with the corresponding sample prepared using standard methods (Figure 6.6C). The mtDNA isolated by alkaline lysis was however insensitive to exonuclease III digestion while on the contrary the bulk of the DNA obtained by usual methods was degraded by this enzyme (Figure 6.6C). Collectively, these observations imply that a small fraction of the mtDNA of *N. crassa* is indeed present as covalently closed, circular molecules, but the majority of the molecules are present in linear forms.

To determine if the linear mtDNA molecules had telomere-like fixed ends, a time-course experiment was performed where mtDNA samples were treated with exonuclease III over a period of different time intervals, and subsequently digested with *Eco*RI and separated on agarose gels. The fast degradation of specific restriction fragments over other fragments would indicate that the linear molecules indeed have fixed ends with the fragments disappearing faster than the others being nearer to the termination points and the fragments disappearing slower than the rest being distant. However, this phenomenon was not observed when *N. crassa* mtDNA was subjected to this treatment: all the fragments were degraded in similar fashion (Figure 6.6D). The larger *Eco*RI pieces are observed to degrade more rapidly than the smaller ones since if there are no fixed ends, all the regions of the mtDNA will be equally susceptible to nuclease attack, and this phenomenon will be detected faster for the larger restriction fragments than the smaller ones.

***In vivo* conformation and replication of a plasmid-like element in the mitochondria.** A circular, plasmid-like element (pleC9) was detected in the mitochondria of the *Cryphonectria parasitica* strain KFC9-E6. This DNA had a unit length of only 1.4-kb (Chapter 4 of this thesis). Sequence analysis of pleC9 had revealed that only a small part of this element is homologous to the *bona fide* mtDNA of *C. parasitica* whereas the remaining DNA being of an unknown origin. When uncut mtDNA was separated in two dimensions and the corresponding Southern blot was hybridized with a probe derived from the cloned pleC9 element, a pattern similar to the mitochondrial plasmids was observed (Figure 6.7A). Oligomeric forms of the pleC9 DNA were observed with the concatamers being as large as hexamers. Larger molecules probably exist but were not detected in this particular experiment. In addition, similar to the mitochondrial plasmids, a majority of the pleC9 DNA was present as supercoiled circles rather than in relaxed forms. Moreover, a curve between the linear arc and the relaxed circular molecules, almost parallel to the linear arc, was detected. As described before in previous studies (Backert *et al.,* 1997), this signal represents plasmid molecules with growing tails of multiple contour lengths of the corresponding circle. Therefore, this arc could represent derivatives of σ-like pleC9 molecules. The signal on the linear DNA arc appears to be much stronger at the position of the linear monomer and just above that. This observation, together with the finding that the arc representing σ-like molecules extends from the monomer, indicates that the monomeric forms are probably the predominant templates for pleC9 replication. Similar observations have been also recorded for a mitochondrial rolling circle-plasmid from higher plants (Backert *et al.,* 1997). The multimeric forms of can be obtained from intra- and inter-molecular homologous

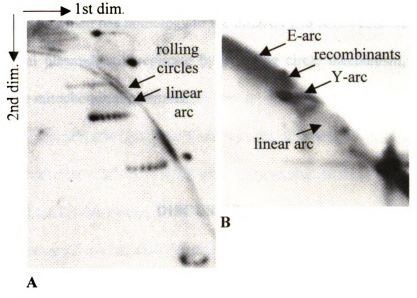


Figure 6.7. Analysis of replication intermediates and the *in vivo* conformation of the circular plasmid-like element pleC9 of *C. parasitica*. The pleC9 molecules exist *in vivo*, as mostly circular elements (**A**) and the observation of a complete Y-arc, a strong arc formed by recombinants and a long E-arc (**B**), when linearized by digestion with *Xba*I, are reminiscent of rolling circle replication.

recombination of the pleC9 molecules. Indeed a strong recombinant arc is detected when the pleC9 molecules are digested with *XbaI* that cuts once on the pleC9 sequence (Figure 6.7B). Apart from that, a very strong E-arc also corroborates the previous finding (Figure 6.7A) of a separate group of molecules with long tails. A complete Y-arc is observed extending from the linear monomer to the dimer. Collectively, these observations suggest that the pleC9 DNA, which has homology to the mtDNA and is not considered as a *bona fide* mitochondrial plasmid, still replicate by a rolling circle mechanism, in a fashion similar to the true mitochondrial plasmids.

DISCUSSION

A study of differential migration patterns of DNA molecules when separated in two dimensions and the analyses of these patterns according to previous studies (Brewer and Fangman 1987; 1988; 1991) have helped us in determining the native conformation of circular plasmid molecules in the mitochondria of filamentous fungi. Plasmids, whose circular structures were deduced from restriction mapping data and sequence information, were found to be present mostly in linear form when these molecules were analyzed using pulse field gel electrophoresis (Maleszka, 1992). In fact, only 8-9 % of the plasmid DNAs was found to migrate as circles (Maleszka, 1992). Contrary to this study, when we analyze these same molecules using 2D gel electrophoresis, the bulk of the plasmid molecules were found as circles with most of the molecules being oligomers of the unit

length plasmids. Even octamers of both the Mauriceville and the Fiji plasmids were detected with the possibility of larger forms being present as well. However, most of the plasmids were present in supercoiled forms with molecules of the same size varying in degrees of supercoils having different migration patterns in the first dimension. It appears that a circular molecule with the most number of supercoils would migrate faster than similar molecules with less number of supercoils when separated in the first dimension (Figures 6.1A, 6.1B, and 6.1C). However, all the supercoiled molecules of the same size migrate similarly in the second dimension. For all the different plasmids analyzed, a relatively low amount of molecules were found as relaxed circles. Thus, it seems that the relaxed circular forms are relatively a rarity with the predominant form of these molecules being supercoiled circles. However, generation of a fraction of the relaxed circular forms by accidental nicking of supercoiled plasmids during preparation and handling of the DNA samples cannot be ruled out. Linear forms of the plasmid DNAs were also present in comparatively low amounts. While it can be argued that some of this linear double-stranded DNA represents *bona fide* replication intermediates, the generation of a part of this DNA by random breakage of circular forms remains a possibility.

In this study, we have presented evidence supporting that different mechanisms of replication can be sustained by the fungal mitochondria. Instances of different kinds of replication, probably independent of each other, being supported and maintained in the mitochondria can be inferred from the presence of linear plasmids in these organelles (Griffiths, 1995). Linear plasmids represent elements that are more similar to adenoviruses (Salas, 1991) in structure than they resemble the native mtDNA. That these linear elements encode their own DNA and RNA polymerases, have terminal inverted

repeats, and have terminal proteins, indicate that these molecules are replicated in a fashion more similar to the protein-primed replication mechanism of adenoviruses than that of mtDNA molecules. However, in this study, we show that not only can different mechanisms of replication co-exist in the fungal mitochondria, but also different mechanisms of replication can account for maintenance of the same plasmid. For the pMAU plasmid, which encodes for a reverse transcriptase gene (Nargang *et al.*, 1984; Akins et al., 1988), we have detected both reverse transcriptase-mediated replication and replication by rolling circle mechanisms from a putative fixed origin. This data is congruent with the observation of the involvement of reverse transcriptase activity in mediation of integration events for pMAU into the mtDNA (Akins *et al.,* 1986). The observation of a strong Y-arc in the 2D gel patterns for the pMAU plasmid implies that a rolling circle mechanism also is involved in the replication of a significant amount of the plasmid DNA. Presence of circular molecules with variable tails, detected by electron microscopy, attests to this fact. Moreover, the observation of an unusually large number of linear molecules of unit length, which are often branched may represent linear Mauriceville plasmid molecules generated by reverse transcription and undergoing second strand synthesis.

Rolling circle replication of the pMAU DNA is surely mediated by the mitochondrial DNA polymerase enzyme because the plasmid lacks its own DNA-dependent DNA polymerase. Previous studies have shown that sub-genomic circular molecules can co-exist with mtDNA in the mitochondria despite not coding their own polymerases (Bertrand *et al.,* 1980; Lazarus *et al.,* 1980; Silliker and Cummings, 1990; Charter *et al.,* 1993; Monteiro-Vitorello *et al.,* 1995; Baidyaroy *et al.,* 2000a). However,

these elements have always been either completely or partially derived from mtDNA sequences and hence were thought to contain the mitochondrial origin for replication. Therefore, they were assumed to be under the control of mitochondrial DNA polymerase-mediated replication. However, pMAU seems to be replicated by the mitochondrial enzyme despite an apparent lack of any homology to the mtDNA. Therefore, it appears that the mitochondrial DNA polymerase, and the replication machinery in the mitochondria, is not template-specific and also can use foreign DNA elements as templates.

The mitochondrial plasmids that contain ORFs encoding DNA polymerases were found to be replicating predominantly by a rolling circle mechanism as evident from the strong Y-arcs in their 2D gel patterns. Lack of DNA arcs that represent molecules containing bubble-like structures imply that these plasmids do not replicate bi-directionally from an internal origin by a θ-type mechanism. For the pLAB plasmid, Y-arcs are seen to be extending from the monomer to the dimer and then from the dimer to the trimer. This observation suggests that probably all the concatamers of the plasmid are used equally as templates for replication. A significant observation here is that complete Y-arcs are always seen extending from the monomer to the dimer for all of the three DNA polymerase containing plasmids when digested with *Eco*RI. It is extremely unlikely that for all the plasmids, the origin would be at or very near to the *Eco*RI site because the three plasmids lack any homology at the nucleotide sequence level. Thus, it seems logical to assume that these plasmids replicate from multiple dispersed sites on their DNAs rather than having a fixed origin of replication. This notion is supported by the

observation in EM studies of a pLAB molecule that has multiple tails of variable lengths (Figure 6.5D).

A prominent arc extending from the dimer molecule as a spike that represents X-shaped molecules is always observed for all the circular plasmids as well as for the plasmid-like element pleC9. According to previous studies (Brewer and Fangman, 1987; 1988; 1991; Lockshon *et al.,* 1995), this arc consists of recombination intermediates. This observation indicates that the plasmid molecules are not only replicating but also recombining actively as well. The high levels of recombination can account for the circularization of the long, linear, newly-replicated DNA produced by a rolling circle mechanism. That the recombination process that results in circularization of the linear forms is rapid is implied by the presence of comparatively low amounts of plasmid molecules in linear forms. In addition, the presence of plasmid molecules as head-to-tail oligomers can be partially explained by inter-molecular homologous recombination between circular forms of the plasmids. However, circular oligomers can be produced also by rolling circle replication of multiple contour lengths of the template and subsequent circularization of the linear tail by homologous recombination. Replication mechanisms can be recombination-dependent as established in the most extensively studied bacteriophage T4 model (Mosig, 1983; 1987), some bacterial plasmids (Viret *et al.,* 1991; Asai *et al.,* 1994) and the malarial mtDNA (Preiser *et al.,* 1996). The presence of significant amounts of recombination intermediates and the observation of replication initiation from multiple sites on the plasmid DNA can, therefore, also suggest a recombination-driven replication mechanism for these elements.

The pleC9 DNA, which is not a mitochondrial plasmid in the true sense, is observed also to be replicating by a rolling circle mechanism with apparent lack of replication intermediates that would suggest otherwise. In addition, the circular, true mitochondrial plasmids were observed to be replicating by rolling circles. That the mitochondrial replication machinery and the mitochondrial DNA polymerase is capable of sustaining replication by the rolling circle mechanism is evident because pMAU replicates by a rolling circle mechanism despite lacking its own DNA polymerase enzyme.. The *in vivo* conformation of mtDNA, which was found to be mostly linear with heterogeneous ends with a few circular molecules, is in agreement with previous studies of mtDNAs in other organisms (Han and Stachow, 1994; Preiser *et al.,* 1996). The linear molecules could be generated by rolling circle replication using the few circular molecules as template. The presence of some linear forms which are larger than the circular molecules (Figure 6.6A) attest to the fact that these were generated by rolling circle replication where the linear, newly-replicated tail extended more than the contour length of the circular template. These large linear DNAs can be also produced by breakages of multimeric circular DNAs. That the linear molecules had heterogeneous ends suggest that either the population of the linear forms were produced by random breakage of larger linear or circular forms, or replication was initiated and/or terminated at randomly dispersed sites. Collectively, these observations suggest that replication in the mitochondria of filamentous fungi occurs predominantly by a rolling circle mechanism.

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SUMMARY

The results that are presented in this thesis definitively establish the validity of a hypothesis that has emerged from a series of recently-published observations (Baidyaroy *et al.*, 2000a; Bell *et al.*, 1996; Mahanti *et al.*, 1993; Monteiro-Vitorello *et al.*, 1995) and indicates that mtDNA mutations and mitochondrial plasmids can elicit cytoplasmically-transmissible hypovirulence syndromes in the chestnut blight pathogen *Cryphonectria parasitica*. This study also confirms that the physiological and genetic processes that are involved in the appearance and transmission of 'mitochondrial hypovirulence' are distinctly different from those that are involved in the manifestation and inheritance of the hypovirulence syndromes that are caused by cytosolic and mitochondrial dsRNA elements (Anagnostakis, 1987; Fulbright, 1999; Nuss, 1992; Polashock *et al.*, 1997). In most respects, the traits that are associated with the mtDNA mutations that elicit hypovirulence in *C. parasitica* are similar to those which are characteristic of the many different kinds of suppressive mitochondrial mutations that cause respiratory deficiencies, and sometimes senescence, in other fungi ((Bertrand, 2000; Bertrand, 1995; Bertrand *et al.*, 1976; Griffiths, 1992; Jamet-Vierny *et al.*, 1999). However, the hypovirulence phenotype that is caused by the pCRY1 mitochondrial plasmid is unique, for it is not associated with a detectable mitochondrial dysfunction, morphological character, or growth phenotype. Perhaps the most important contribution of this thesis is the discovery that mutations that cause mitochondrial hypovirulence syndromes appear spontaneously and can be sustained in populations of *C. parasitica* in nature, where they contribute to

the regeneration of chestnut trees by diminishing the aggressiveness of the pathogen (Baidyaroy *et al.*, 2000a).

Even though the research into 'mitochondrial hypovirulence' has barely seen the dawn of its existence, it is already clear that the genetic factors that elicit such syndromes are quite diverse. They range from the disruption of an essential mitochondrial gene by an element of unknown origin and function, InC9 (Chapter 3 of this thesis), through likely point mutations in mitochondrial genes that secondarily elicit the amplification of short segments of the mtDNA (Monteiro-Vitorello *et al.*, 1995), to the simple presence of a mitochondrial plasmid, pCRY1 (Chapter 5 of this thesis), which appears to be little more than an inconsequential passenger that happens to prefer mitochondria as its domicile. When these observations are combined with available information on the rich diversity of the mtDNA mutations (Griffiths, 1996) and mitochondrial plasmids (Griffiths, 1992; Griffiths, 1995) that affect the vigor and longevity of non-pathogenic filamentous fungi, it can be anticipated that virtually every independent occurrence of a mitochondrial hypovirulence that is associated with an elevated level of alternative oxidase activity will be caused by a unique mtDNA mutation. Since the mtDNAs of pathogenic fungi such as *C. parasitica* often are quite large (Bell *et al.*, 1996) and many of these mutations might not involve gross changes in the size and arrangement of the mtDNA, the detection of the exact alteration that causes the phenotype may sometimes be difficult. Thus, it cannot be expected that a significant fraction of the mtDNA mutations that cause debilitation will be recognized through the use of a simple set of molecular probes, such as, for example, a pair of PCR primers that detect the InC9 element that is present in the *C. parasitica* isolates from the Kellogg Forest (Chapter 3 of this thesis). In

contrast, the number and types of different plasmids that elicit mitochondrial hypovirulence syndromes that are not accompanied by elevated levels of alternative oxidase activity may be quite limited. Hence, it may be possible to identify the most common plasmids that have this effect and to devise a set of molecular probes that can detect whether or not the appearance of hypovirulence in a local population of *C. parasitica*, and for that matter any other fungus, is due to the presence of one or more of these elements, as illustrated for pCRY1 in Chapter 5.

Hypovirulence caused by mitochondrial genetic elements are usually accompanied by elevated levels of alternative oxidase activity in the debilitated strain. However, the mitochondrial plasmid pCRY1 does not appear to do so. Generally, mitochondrial plasmids are known to cause senescence by integration to the mtDNA, thereby generating debilitating mutations (Akins *et al.*, 1986; Bertrand *et al.*, 1986) that disrupts the normal respiratory pathway resulting in increased alternative oxidase activity. In contrast, the pCRY1 plasmid of *C. parasitica* elicits a mitochondrial hypovirulence phenotype without integration to the mtDNA, and hence, possibly without interfering with the mitochondrial respiratory functions of the host strain (Baidyaroy *et al.*, 2000b; Chapter 5 of this thesis). Moreover, there has been a similar report of detection of a senescence phenotype that is generated by over-replication of a mitochondrial plasmid rather than by its integration to the host mtDNA (Stevenson *et al.*, 1999). Thus, it appears that certain mitochondrial hypovirulence syndromes that are associated with mitochondrial plasmids might not be accompanied by demonstration of elevated levels of alternative oxidase activity. Similarly, hypovirulence phenotypes

generated by mitochondrial dsRNAS in *C. parasitica* and *Ophiostoma novo-ulmi* also do not exhibit increased respiration *via* the alternative oxidase pathway.

While it has been widely assumed that hypovirulence is caused primarily by dsRNA elements that are located in the cytoplasm (Buck, 1998; Fulbright, 1999; Nuss, 1992), the experiments conducted in this thesis show that this state can be caused in *C. parasitica*, and probably other filamentous fungal pathogens, by a broader than anticipated variety of cytoplasmic genetic elements. Since "hypovirulence" by definition is caused by a cytoplasmic genetic element (Fulbright, 1999), the general nature of the causative agent can be determined by addressing a simple set of questions in a step-wise manner. Whenever a hypovirulence syndrome is detected in nature, perhaps the two simplest and most informative steps in the path of the identification of its genetic determinant might be assays for alternative oxidase activity and the presence of dsRNA viruses. If a dsRNA is present, its location can be approximated by testing for its co-purification with mitochondria, the cytosolic fraction, or nuclei. If no dsRNA is present and the debilitated strain has a high level of alternative oxidase activity, then the genetic determinant of hypovirulence possibly most likely is due to a suppressive mtDNA mutation. Some of these mutations could be caused by integration into the mtDNA of a mitochondrial plasmid (Bertrand, 2000), a situation that undoubtedly will be detected during the subsequent characterization of the mitochondrial genome. Finally, if the mutant does not contain a dsRNA and does not manifest an abnormal respiratory phenotype, then the agent of hypovirulence might be a mitochondrial plasmid, e.g. pCRY1 in *C. parasitica* (Baidyaroy *et al.*, 2000; Monteiro-Vitorello *et al.*, 1995, Chapter 5 of this thesis). In the case that none of the above steps reveal the identity of the genetic

determinant of hypovirulence, the debilitating agent probably is a genetic element that has not been associated hitherto with this phenotype.

In summary, the study conducted in this thesis identifies the occurrence of two different types of natural, cytoplasmically-transmissible hypovirulence factors that were not characterized before. At least for *C. parasitica*, it is now clear that hypovirulence in nature can be mediated not only by dsRNA elements, but also by mtDNA mutations (Chapters 2 and 3) and mitochondrial plasmids (Chapters 4 and 5). Certain diagnostic features that can detect these aforementioned agents have been identified and were successfully employed to identify the different genetic elements that cause superficially similar hypovirulence syndromes. It has also been shown that the hypovirulence syndromes caused by mitochondrial mutations and plasmids are successfully transmitted (Chapters 2 and 5) and are sustainable in nature (Chapter 2) to an extent where the debilitation of *C. parasitica* can contribute to the regeneration of chestnut trees in nature.

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APPENDICES

Appendix A

Figure A.1. Nucleotide sequence of the InC9 element (GenBank accession no. AF218209). The region that is similar to other known sequences is indicated in bold.

ATGTGCGCGGTAACGAGGCTTTATGTTGTGAATATCAAGGTATAACTGGTACTTGATAT
ATGTATCCCAGAACTTAAATACATGGTAATATATCGGGGCTAGGTAATCCCAATAAGAG
CCTAGTCTTAGAGCAGATATGTAAAAGGGGAGGCCGTATAATTAACTATACGCAGAATA
AGCGGTATGAAGCGATAATTAATTGCTAATACTTCTCCTCCTCCTAGCCGAGTCGATAT
GGTGACGGTAAGAAACTTGTAGGGATGATCCTAGACGTGTAGCTGTCACGAGTGAGCCC
ATACCTCCCCAACGTTAAGATAGAGTCGGGGTATGTCCTGTGGAGCTAAGTATGATCAT
GATTAGCGAGTACTTAGTTGTTCTGAACACAGGAAGCAGATATAATGAATATCCGAAAA
CACGCAAGCACCTAAGTCGATAAAAACCAGAAAGAAAAACACATAACATAAGGTAACTA
CCGGAAGGACGCGGGACCCTTAGAAGGCCGCCCCCAGAAGTCCCATAGTAGAGATAAGA
TAATTAATAACTATTCGCTATATTATAGCTCTCGAAGGGGAATAGTGTCCCACAGATAA
GGATATTTCTCTTGGTCTCAACATACTCTGGACCCAGATCTTGTGAGAAGCCACAAAAG
GTATATAATAGTTTCGAAATCACACAAGTTATCTATGTTTTATGCAAGGGATAAGCAGC
CGATAGAGCGCACTTAACCTTGTAGTTTAAACTTGTGGAAAGATGCGAACTATATAGAA
CAAAGAGAGAGTACTAACAAAGAAGATCATGAACAGCTACCTGTTGTATGAAGAAGGAA
ATCTTATCATAGGCCAAATACATGTGTTATAAAGTAATACCCAACATTATCACATGATG
GGTCGAATTAGGGG**GGCATGGAGAGCCGTGTGCCGGGAAACTCGCATGCACGGTTCGGA**
GGACAGGAAGCGGAAGCCTACTGAGTCCT

Table A.1. List of genes that have nucleotide homology with the InC9 sequence. Sequences that include regions that are at least 80 % identical to the region in bold in the InC9 sequence (see Figure A.1) are listed.

Organism	Gene name or sequence	Accession Number
Marchantia polymorpha	complete mtDNA	M68929
Scendesmus obliquus	mitochondrial LSUrRNA	X17375
Podospora anserina	mitochondrial group II intron	X04336
Podospora anserina	α sen DNA	X63085
Podospora anserina	mitochondrial *cox*1	M36911
Podospora comata	mitochondrial *cox*1	Z69899
E.coli plasmid p0157	complete sequence	AB011549.2

Appendix B

Figure A.2. Nucleotide sequence of the pleC9 DNA (GenBank accession no. AF218210). Regions of homology to the mitochondrial DNA are in bold. The repeat elements A and A', B and B' and C and C' are also mentioned.

TCTAGACCCAAATTTTCATTTTTCAACCCAGGGGGCAAGCCTGAGCGCCACTTTTAAAT
GACCGCTGGTTGAGTCGGGAGGGGTCTTAGCCCTGCATCCGTAGTCTAACTTTCCAGAC
ATCCGTGTGCCTGTTATATTATCCATCTTCCAGCATGAAATGGAAACTGCGACCATCCC
|----------
CAGCCATTATATCCCTTATTCCTCTCGTACTTTCACTCCCTCTTGGTTACGCCTAAGGT
------- **Repeat element A** ----------
CTTATCCATTTCTTTTACCAAAATGAGACCCGTGTCGGGGTTCCAACTATTTCTAGCGG
----------|
ATTTCGTCTAGTTAAGGGTGACCTTTCAGACCTCAGATCCAGAACGATTCCTTATATCT
CCTCTTTTTATATACCTTCTCATTTACACCTCG**TATTTTGCTCTGGTCCATAAGTTTTC**
TTGCAATAGTGGTGACACCGACTCGCTTAGCCACCTTCGCAGGTTTGACCTCGTCTGCC
|-----**Repeat**
AGCTTTTCTTGGGGGGGGGTTTGATTTATCTCTTTCGTTCTCTTAGTCATCTAAGTACC
element B---||----**Repeat element B'**----|
GGACCCCTCTATGTACCGGACCCCC**TCACAATCTAAGACGTCTACTTCCAGAGTT**TTAC
TTTGTTGCGCATTTCCACCATTTTCATTTCTTCCGTTGTTATAATCTCATAGCCTCACA
|----------
TTGGGGTCGACTCAATTTCTTTTCACGTTCCTTTTTCATAGATTAGTAAGGGTACATCC
----------**Repeat**
CTTAATCAATTAGTACTTTCACTCCCTCTTGGTTACGCCTAAGGTCTTATCCATTTCTT
element A' ----------
TTACCAAAATGAGACCCGTGTCGGGGTTCCAACTATTTCTAGCGGATTTCGTCTAGTTA
----------|
AGGGTGCACCTTTATTTGGGCTATTTATAAGGGTCCCCTCTTAGGTTTCACTGTCTTGT
|-
GACCGTTTTTTCCTAGGAGGTGGATCGTTACTGGACCTTGGGTAGGACTCTCTTTTGTT
----------**Repeat element C** ----------| |----------
CGGCCGCTAGTGACTAGGGGATTGTTTTACTTACGAGTGCATCATACTCGGCCGCTAGT
--**Repeat element C'** ----------|
GACTAGGGGATTTTTTAACTTAATCGACACGATTAGTGGATCATACGCCCTAAATGTTG
CTAAAGCATAAAATCTCGTTGATATATGATTAGATGTAAGTAACTACGTTCCTAAAACT
CTATTTTCATGAATGCTGTATAGAGCTCTTTTCCCTTACGAGGACCCGACTTCTCATCT
TTGGGTGAGATATACTCATCCATTGGCTAAACCGTCGTTTCTTCATAGGGGGAAAGATA
AGGGGCCTGAAATTCAACTCTGTTCCTTCCGGGCCGTCTTTCTATCCACCATTCACACT
AGCCATGTCTCGGCTTATTTAGGGCCGGATTTGTCCTAGCTATTTTAGATGGGCTAGAC
GAGCTTA

Figure A.3. Alignment of nucleotide sequences of three plasmid-like elements cloned from two hypovirulent strains. The differences in sequence of other elements from the KFC9-E6 plasmid-like element (pleC9) are shown as shaded nucleotides. The nucleotide sequences of the pleC9 element from the strain KFC9-E6, a similar element from the strain J2.31[KFC9-E6], and a smaller derivative from J2.31[KFC9-E6] are denoted as 1.s, 3.s and 2.s respectively.

```
1      TCTAGACCCAAATTTTCATTTTTCAACCCA  1.s
1      TCTAGACCCAAATTTTCATTTTTCAACCCA  2.s
1      TCTAGACCCAAATTTTCATTTTTCAACCCA  3.s

31     GGGGGCAAGCCTGAGCGCCACTTTTAAATG  1.s
31     GGGGGCAAGCCTGAGCGCCACTTTTAAATG  2.s
31     GGGGGCAAGCCTGAGCGCCACTTTTAAATG  3.s

61     ACCGCTGGTTGAGTCGGGAGGGGTCTTAGC  1.s
61     ACCGCTGGTTGAGTCGGGAGGGGTCTTAGC  2.s
61     ACCGCTGGTTGAGTCGGGAGGGGTCTTAGC  3.s

91     CCTGCATCCGTAGTCTAACTTTCCAGACAT  1.s
91     CCTGCATCCGTAGTCTAACTTTCCAGACAT  2.s
91     CCTGCATCCGTAGTCTAACTTTCCAGACAT  3.s

121    CCGTGTGCCTGTTATATTATCCATCTTCCA  1.s
121    CC TGTGCCTGTTATATTATCCATCTTCCA  2.s
121    CC TGTGCCTGTTATATTATCCATCTTCCA  3.s

151    GCATGAAATGGAAACTGCGACCATCCCCAG  1.s
151    GCATGAAATGGAAACTGCGACCATCCCCAG  2.s
151    GCATGAAATGGAAACTGCGACCATCCCCAG  3.s

181    CCATTATATCCCTTATTCCTCTCGTACTTT  1.s
181    CCATTATATCCCTTATTCCTCTCGTACTTT  2.s
181    CCATTATATCCCTTATTCCTCTCGTACTTT  3.s

211    CACTCCCTCTTGGTTACGCCTAAGGTCTTA  1.s
211    CACTCCCTCTTGGTTACGCCTAAGGTCTTA  2.s
211    CACTCCCTCTTGGTTACGCCTAAGGTCTTA  3.s

241    TCCATTTCTTTTACCAAAATGAGACCCGTG  1.s
241    TCCATTTCTTTTACCAAAATGAGACCCGTG  2.s
241    TCCATTTCTTTTACCAAAATGAGACCCGTG  3.s

271    TCGGGGTTCCAACTATTTCTAGCGGATTTC  1.s
271    TCGGGGTTCCAACTATTTCTAGCGGATTTC  2.s
271    TCGGGGTTCCAACTATTTCTAGCGGATTTC  3.s
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301    GTCTAGTTAAGGGTGACCTTTCAGACCTCA  1.se
301    GTCTAGTTAAGGGTGACCTTTCAGACCTCA  2.se
301    GTCTAGTTAAGGGTGACCTTTCAGACCTCA  3.se

331    GATCCAGAACGATTCCTTATATCTCCTCTT  1.se
331    GATCCAGAACGATTCCTTATATCTCCTCTT  2.se
331    GATCCAGAACGATTCCTTATATCTCCTCTT  3.se

361    TTTATATACCTTCTCATTTACACCTCGTAT  1.se
361    TTTATATACCTTCTCATTTACACCTCGTAT  2.se
361    TTTATATACCTTCTCATTTACACCTCGTAT  3.se

391    TTTGCTCTGGTCCATAAGTTTTCTTGCAAT  1.se
391    TTTGCTCTGGTCCATAAGTTTTCTTGCAAT  2.se
391    TTTGCTCTGGTCCATAAGTTTTCTTGCAAT  3.se

421    AGTGGTGACACCGACTCGCTTAGCCACCTT  1.se
421    AGTGGTGACACCGACTCGCTTAGCCACCTT  2.se
421    AGTGGTGACACCGACTCGCTTAGCCACCTT  3.se

451    CGCAGGTTTGACCTCGTCTGCCAGCTTTTC  1.se
451    CGCAGGTTTGACCTCGTCTGCCAGCTTTTC  2.se
451    CGCAGGTTTGACCTCGTCTGCCAGCTTTTC  3.se

481    TTGGGGGGGGGTTTGATTTATCTCTTTCGT  1.se
481    TTGGGGGGGGGG[redacted]ATTTATCTCTTTCGT  2.se
481    TTGGGGGGGGGG[redacted]ATTTATCTCTTTCGT  3.se

511    TCTCTTAGTCATCTAAGTACCGGACCCCTC  1.se
510    TCTCTTAGTCATCTAAGTACCGGACCCCTC  2.se
510    TCTCTTAGTCATCTAAGTACCGGACCCCTC  3.se

541    TATGTACCGGACCCCCTCACAATCTAAGAC  1.se
540    TATGTACCGGACCCCCTCACAATCTAAGAC  2.se
540    TATGTACCGGACCCCCTCACAATCTAAGAC  3.se

571    GTCTACTTCCAGAGTTTTACTTTGTTGCGC  1.se
570    GTCTACTTCCAGAGTTTTACTTTGTTGCGC  2.se
570    GTCTACTTCCAGAGTTTTACTTTGTTGCGC  3.se

601    ATTTCCACCATTTTCATTTCTTCCGTTGTT  1.se
600    ATTTCCACCATTTTCATTTCTTCCGTTGTT  2.se
600    ATTTCCACCATTTTCATTTCTTCCGTTGTT  3.se

631    ATAATCTCATAGCCTCACATTGGGGTCGAC  1.se
630    ATAATCTCATAGCCTCACATTGGGGTCGAC  2.se
630    ATAATCTCATAGCCTCACATTGGGGTCGAC  3.se

661    TCAATTTCTTTTCACGTTCCTTTTTTCATAG  1.se
660    TCAATTTCTTTTCACGTTCCTTTTTTCATAG  2.se
660    TCAATTTCTTTTCACGTTCCTTTTTTCATAG  3.se
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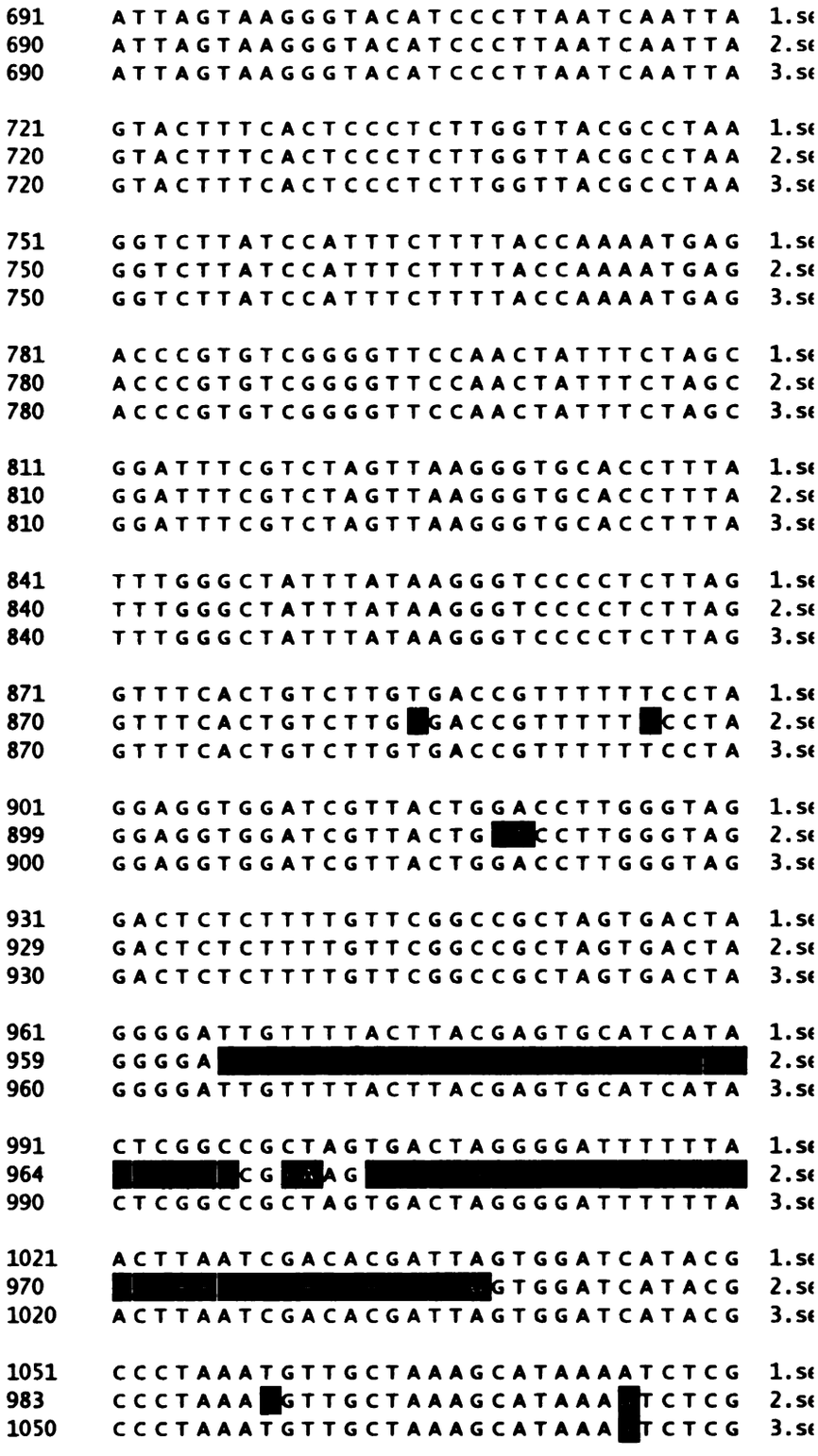

691 ATTAGTAAGGGTACATCCCTTAATCAATTA 1.se
690 ATTAGTAAGGGTACATCCCTTAATCAATTA 2.se
690 ATTAGTAAGGGTACATCCCTTAATCAATTA 3.se
721 GTACTTTCACTCCCTCTTGGTTACGCCTAA 1.se
720 GTACTTTCACTCCCTCTTGGTTACGCCTAA 2.se
720 GTACTTTCACTCCCTCTTGGTTACGCCTAA 3.se
751 GGTCTTATCCATTTCTTTTACCAAAATGAG 1.se
750 GGTCTTATCCATTTCTTTTACCAAAATGAG 2.se
750 GGTCTTATCCATTTCTTTTACCAAAATGAG 3.se
781 ACCCGTGTCGGGGTTCCAACTATTTCTAGC 1.se
780 ACCCGTGTCGGGGTTCCAACTATTTCTAGC 2.se
780 ACCCGTGTCGGGGTTCCAACTATTTCTAGC 3.se
811 GGATTTCGTCTAGTTAAGGGTGCACCTTTA 1.se
810 GGATTTCGTCTAGTTAAGGGTGCACCTTTA 2.se
810 GGATTTCGTCTAGTTAAGGGTGCACCTTTA 3.se
841 TTTGGGCTATTTATAAGGGTCCCCTCTTAG 1.se
840 TTTGGGCTATTTATAAGGGTCCCCTCTTAG 2.se
840 TTTGGGCTATTTATAAGGGTCCCCTCTTAG 3.se
871 GTTTCACTGTCTTGTGACCGTTTTTTTCCTA 1.se
870 GTTTCACTGTCTTG[redacted]GACCGTTTTTT[redacted]CCTA 2.se
870 GTTTCACTGTCTTGTGACCGTTTTTTTCCTA 3.se
901 GGAGGTGGATCGTTACTGGACCTTGGGTAG 1.se
899 GGAGGTGGATCGTTACTG[redacted]CCTTGGGTAG 2.se
900 GGAGGTGGATCGTTACTGGACCTTGGGTAG 3.se
931 GACTCTCTTTTGTTCGGCCGCTAGTGACTA 1.se
929 GACTCTCTTTTGTTCGGCCGCTAGTGACTA 2.se
930 GACTCTCTTTTGTTCGGCCGCTAGTGACTA 3.se
961 GGGGATTGTTTTACTTACGAGTGCATCATA 1.se
959 GGGGA[redacted] 2.se
960 GGGGATTGTTTTACTTACGAGTGCATCATA 3.se
991 CTCGGCCGCTAGTGACTAGGGGATTTTTTA 1.se
964 [redacted]CG[redacted]AG[redacted] 2.se
990 CTCGGCCGCTAGTGACTAGGGGATTTTTTA 3.se
1021 ACTTAATCGACACGATTAGTGGATCATACG 1.se
970 [redacted]GTGGATCATACG 2.se
1020 ACTTAATCGACACGATTAGTGGATCATACG 3.se
1051 CCCTAAATGTTGCTAAAGCATAAAATCTCG 1.se
983 CCCTAAA[redacted]GTTGCTAAAGCATAAA[redacted]TCTCG 2.se
1050 CCCTAAATGTTGCTAAAGCATAAA[redacted]TCTCG 3.se

```
1081    TTGATATATGATTAGATGTAAGTAACTACG  1.se
1013    TTGATATATGATTAGAT[ ]TA[ ]GTAACTACG  2.se
1080    TTGATATATGATTAGATGTAAGTAACTACG  3.se

1111    TTCCTAAAACTCTATTTTCATGAATGCTGT  1.se
1042    TTCCTAAAACTCTATTTTCATGAATGCTGT  2.se
1110    TTCCTAAAACTCTATTTTCATGAATGCTGT  3.se

1141    ATAGAGCTCTTTTCCCTTACGAGGACCCGA  1.se
1072    ATAGAGCTCTTTTCCCTTACGAGGACCCGA  2.se
1140    ATAGAGCTCTTTTCCCTTACGAGGACCCGA  3.se

1171    CTTCTCATCTTTGGGTGAGATATACTCATC  1.se
1102    CTTCTCATCTTTGGGTGAGATATACTCATC  2.se
1170    CTTCTCATCTTTGGGTGAGATATACTCATC  3.se

1201    CATTGGCTAAACCGTCGTTTCTTCATAGGG  1.se
1132    CATTGGCTAAACCGTCGTTTCTTCATAGGG  2.se
1200    CATTGGCTAAACCGTCGTTTCTTCATAGGG  3.se

1231    GGAAAGATAAGGGGCCTGAAATTCAACTCT  1.se
1162    GGAAAGATAAGGGGCCTGAAATTCAACTCT  2.se
1230    GGAAAGATAAGGGGCC[ ]GAAATTCAACTCT  3.se

1261    GTTCCTTCCGGGCCGTCTTTCTATCCACCA  1.se
1192    GTTCCTTCCGGGCCGTCTTTCTATCCACCA  2.se
1260    GTTCCTTCCGGGCCGTCTTTCTATCCACCA  3.se

1291    TTCACACTAGCCATGTCTCGGCTTATTTAG  1.se
1222    TTCACACTAGCCATGTCTCGGCTTATTTAG  2.se
1290    TTCACACTAGCCATGTCTCGGCTTATTTAG  3.se

1321    GGCCGGATTTGTCCTAGCTATTTTAGATGG  1.se
1252    GGCCGGATTTGTCCTAGCTATTTTAGATGG  2.se
1320    GGCCGGATTTGTCCTAGCTATTTTAGATGG  3.se

1351    GCTAGACGAGCTTA                  1.se
1282    GCTAGACGAGCTTA                  2.se
1350    GCTAGACGAGCTTA                  3.se
```

Appendix C

Figure A.4. Nucleotide sequence of the region of mitochondrial DNA that is homologous to the pleC9 DNA (GenBank accession no. AF218567). Sequences that are homologous to pleC9 are in bold. The initiation and termination codons of an ORF located in this region of the mtDNA are also indicated in bold.

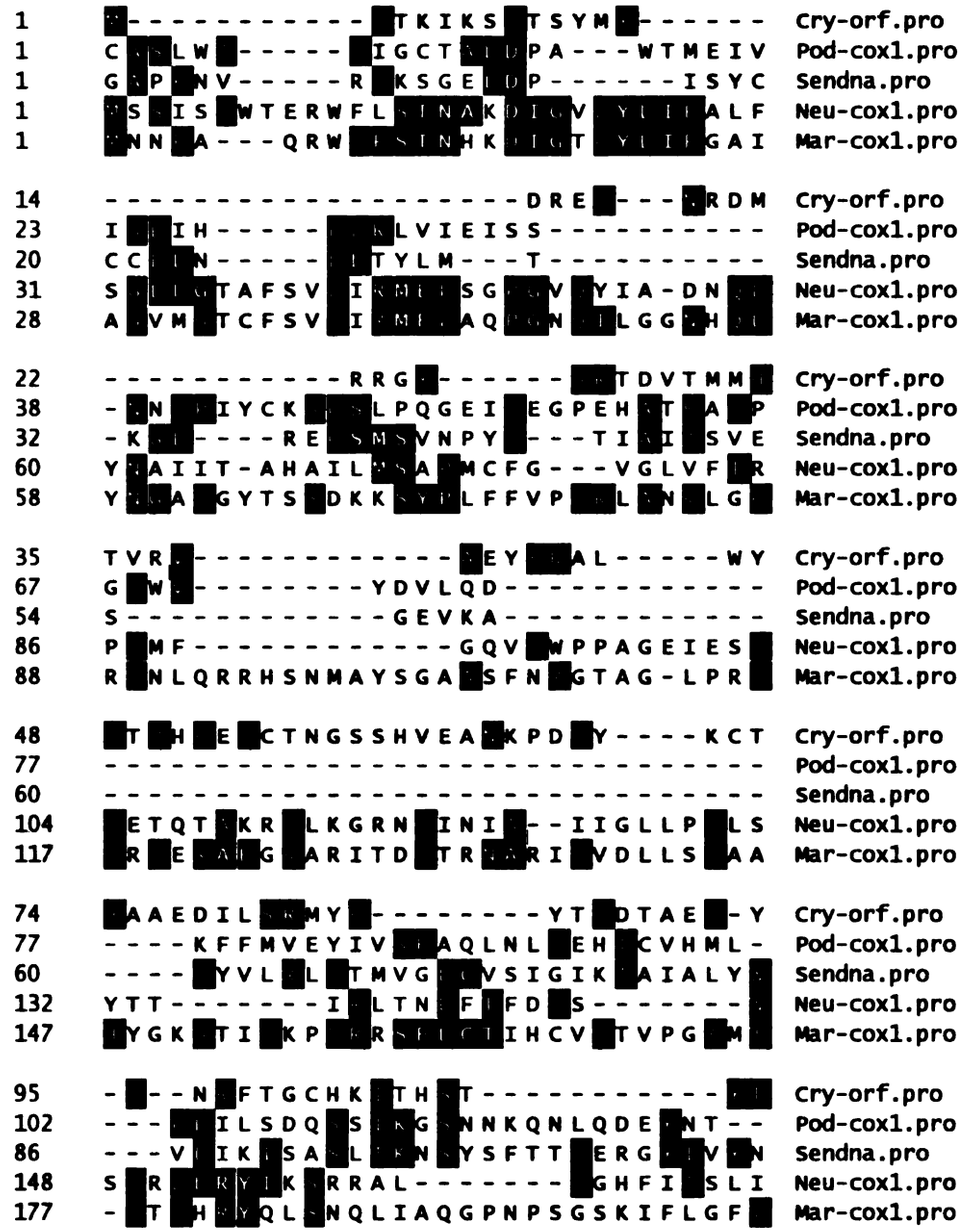
GTTGCCGTTAGCTTATTACGTGCGCCGGGCGAGGCCCTG**ATG**TTTCTTAAGATCAAGTC
CGATCTTTCGTATATATATGACCGTGAAATTAATCGCGATATACGCCGTGGGTATTCAC
GACTTGATGTGCTAATAATACATCTAGTACGGGTAGCTGAATACAAACGGGCATTATGA
TATGGACTGCCCCATAGAGAAGAATGCCTGAACGGGAGTAGTCATGTCGAGGCTAACAA
ACCTGATACTTATAAGTGCCTTCACGCTGCGGAAGATATCTTAAGCAGGATATATCGGT
ATCTTTGTGATCTGGCTGAGAAATATGGTAATAGATTTCTTGGTTGTCATAAAATTACA
CACTCTCTTTATACGAAGAGCCCACATGGGAATAAGCAGGCGGCTAAAACTACTTGGAC
TATAAACATAACATCAAGGCAACCCCGGGATCGAACCGCCTCTGTCAATTTATATATGA
GCATGCGGAGACGGAGTCTCCATAGTAGTGTCAACATGAAGGGAGATAGCAAATC**AACT**
CTGGAAGTAGACGTCTTAGATTGTGACCAAGAAAAGCTGGCAGACGAGGTCAAACCTGC
GAAGGTGGCTAAGCGAGTCGGTGTCACCACTATTGCAAGAAAACTTCTGGACCAGAGCA
AAATAGCTAACCAAAAATATTACAATATACTCAATGTACTAGCGGACCCCAACTTCTTA
ATAGCTTGCTATGACGAAATCAAGGGGAAACAGGGAAATATGACCAGGGGATATGACAA
AGCGACCTTGGATGGCTTGGATTATAACTGATTCGTGAAGACCGCAGGAGAGCTTAAGG
CTGGAAAATATAATTTCAAACCCAGTCGTCGAGTGGAAATACCCAAGGCCAACGGTAAA
ACACGACCGCTTGGAGTAGGGTCTCCCAGGGATAAAATTGTGCAAAAGGCGCTTCATGC
AATTCTAGAAGCAATATTTGAACCCCTGTTCTTGCCATCTTCGCATGGTTTCCGCCCTA
ATCGCTCCACACATTCCGCATTGCTTAAGGTATACCTTTCAGGGAATAAACATAACTGA
GTAATTCAAGGGGACATCACTAAATGCTTTGACTCAATACCCCACTCGATTATTTTAAA
GCGTATCGGTGCCCAGATTGGTGATAAGAAATATTTGAACTTGATAAGCAAATATCTTG
AAGCGGGACATATTGACCCCAAAACAGGAACGAAAGTTGTTCTGAACTATGGAACTCCG
CAGGGAGGTATATTGAGTCCTATTCTTAGTAATATCGTATTGCACGAGTTTGACAAGTA
TATGGCTAAGCTGTCGGAGAGTTTTCACAAGGGAAAGAAGAGAAGATGAAACCCGGCTT
ATAAAAGATTACTAGCCAGAAGGGGGAGAACCAAATCCCTCGAGGAGAAACAGACTCTT
CTTAAACAAATGAGAACTATGAGAAGTATAGATGCTTTTGATCCTAACTTCAGAAGGTT
GGATTACGTTCGGTATGCGGATGACTTCGTGGTCTTCATATCAGGTAGTTCAAAGGACG
CACTATTTATTAGAAATAACCTTAAAGATTATCTGAAAGTTAATTGCGGACTAGAGTTA
AACGTGGACAAAACAGCGATATCCAACTTAGCAACCGAAAAATGGAAATTCCTGGGGGC
CGAATTGTCTAAAATCAAACTGAACGCAAACTGACTTGTTAGCCACGGTAGAAAAAGAA
TTATTGGTACACCGATGTTACTAGTGAACGCACCTATTTCCGGTCTGATTAGTTCTCTT
AAGAAGGTTGGTATAGTTCGACAGAATCTTAAACAGAAGGTGTTCCCTCAAGGATTAAC
TTCCCTGGTCAACCTGTGTCACTATGATATTATAAGGTTCTATAACTCTAAAATTCATG
GAATCCTAAATTATTATAGTTTCGCAGCCAATCGGAACAGCTTACATTCTATAGTGTGA
TTGCTGAGAGCATCATGTGCTCTTACCCTTGCCCGTAAGTTCAAGTTAAGAACAATGAG
CAAGGCCTTTCAAAAGTTTGGTAGGGACTTAAAATGTCCGGGGACGGGTATAAGTATCT

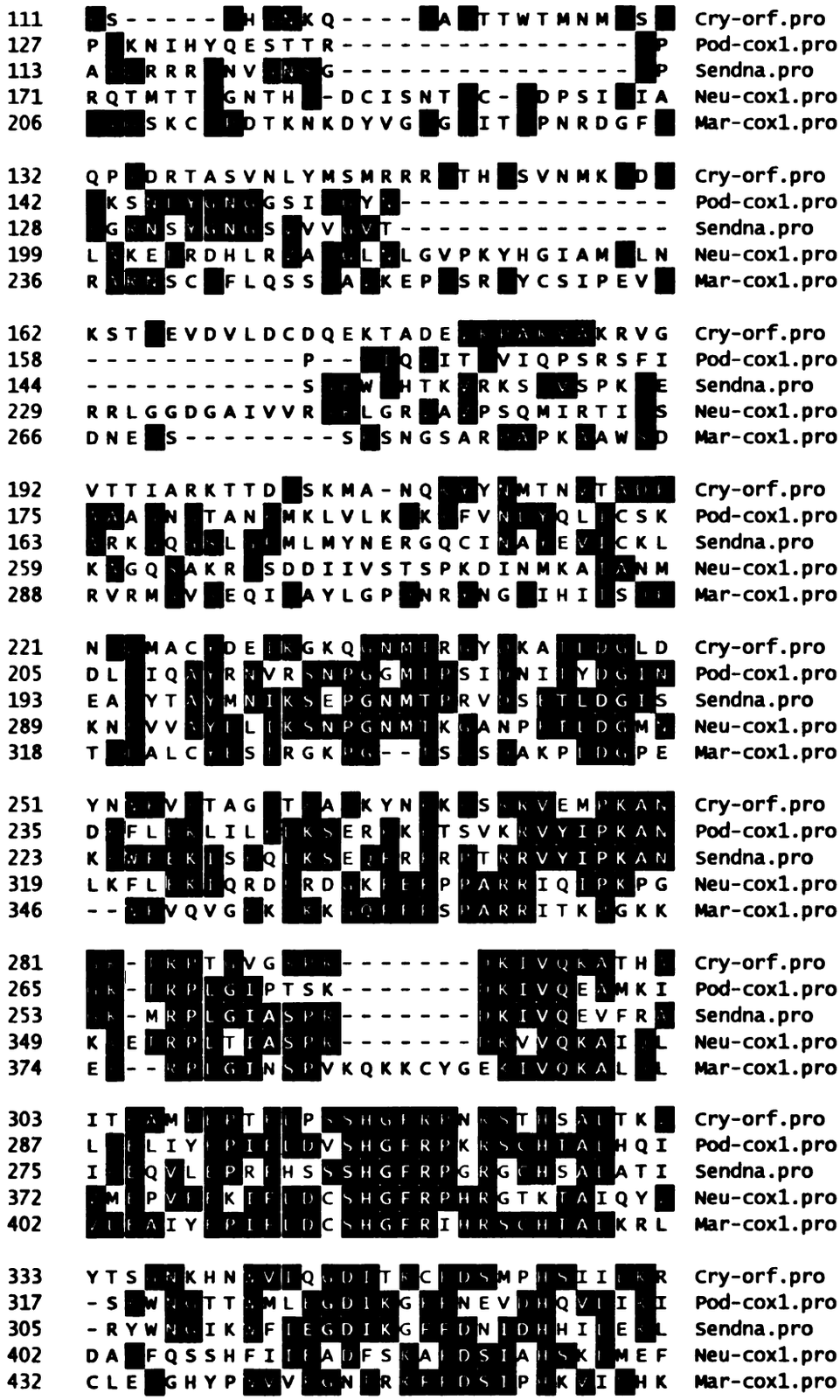
TCGACCCTGGATCCTTAAAGGCCATTCATGACTACAAGTCAGGTCCGGTCCCAGGGGTA
GAGGGTATAACCAACCAAGTCTGAAGCGGAAAACTTACAAAGTCCAGCTTTGGTCTGTC
GTGCGTAATTTGCGGTTCTACTACCAACCTTGAAATGCACCACTATAGATCGGTTAAGG
AAGTGAGAGCTAAGTTTCGGAAAGGTAACGAGATCTCTTTCGCAGAGTTCAGGGGAGCC
CTTCTACGTAAACAGATTCCACTATGTGAGTCCATCACAAATTATCCACAAAGGGGATC
TGCCGATACGAAC**TAG**AAAAATCGC

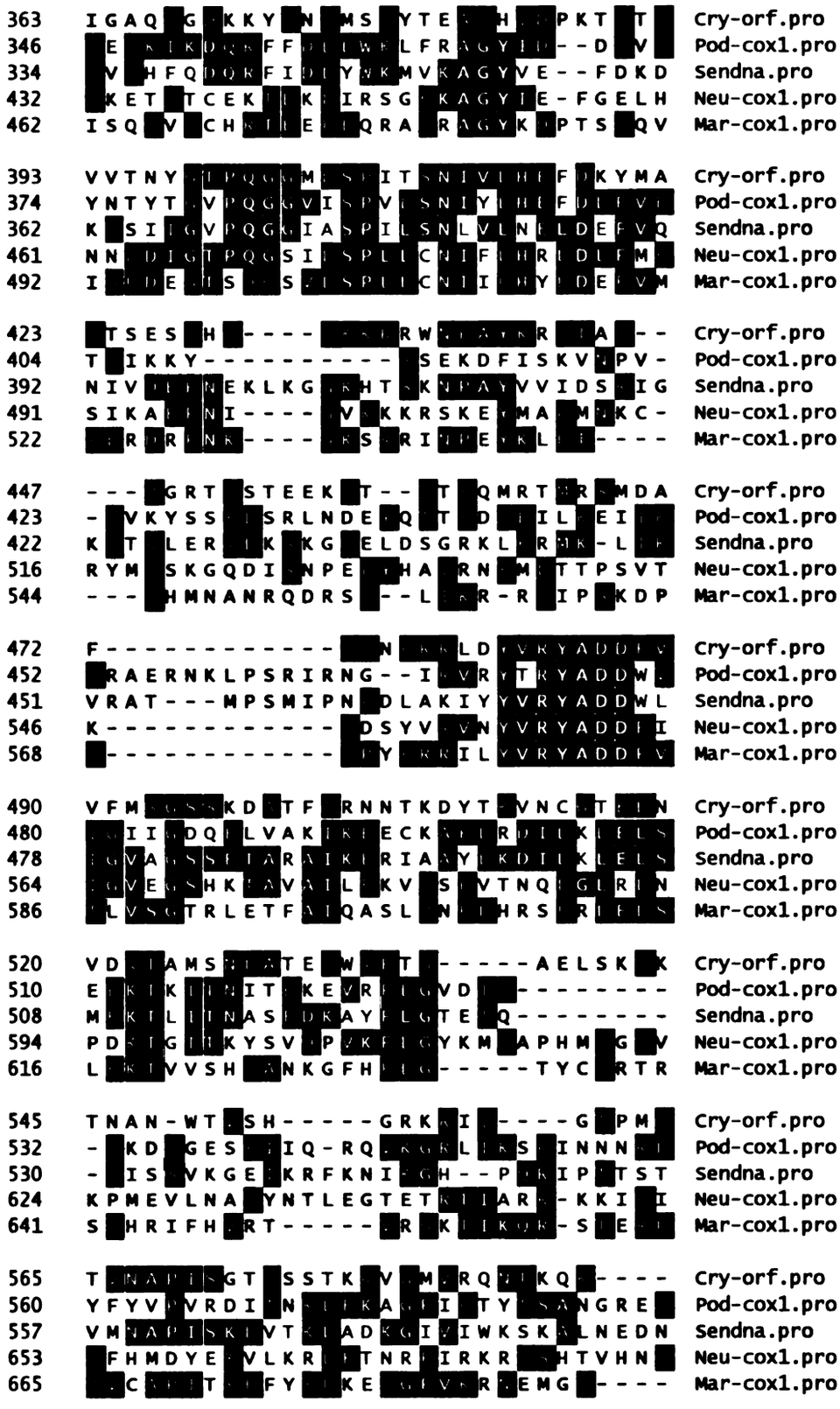
Figure A.4. Sequence of the protein encoded by the open reading frame present on the mtDNA that is homologous to the pleC9 element. The ORF encodes for a protein that is 778 amino acid long.

MFTKIKSDTSYMYDREINRDMRRGYSRTDVTMMHTVRVAEYKRALWYGTPHREECTNGS
SHVEANKPDTYKCTHAAEDILSRMYRYTCDTAEKYGNRFTGCHKITHSTYTKSPHGNKQ
AAKTTWTMNMTSRQPRDRTASVNLYMSMRRRSTHSSVNMKGDSKSTTEVDVLDCDQEKT
ADEVKPAKVAKRVGVTTIARKTTDQSKMANQKYYNMTNVTADPNFLMACYDEIKGKQGN
MTRGYDKATLDGLDYNWFVKTAGETKAGKYNFKPSRRVEMPKANGKTRPTGVGSPRDKI
VQKATHAITEAMFEPTFLPSSHGFRPNRSTHSALTKVYTSGNKHNWVIQGDITKCFDSM
PHSIILKRIGAQIGDKKYLNLMSKYTEAGHIDPKTGTKVVTNYGTPQGGMLSPITSNIV
LHEFDKYMAKTSESFHKGKKRRWNPAYKRLTARRGRTKSTEEKQTTTKQMRTMRSMDAF
DPNFRRLDYVRYADDFVVFMSGSSKDATFIRNNTKDYTKVNCGTELNVDKTAMSNLATE
KWKFTGAELSKIKTNANWTVSHGRKRIIGTPMLTVNAPISGTISSTKKVGMVRQNTKQK
VFPQGLTSTVNTCHYDIMRFYNSKIHGITNYYSFAANRNSLHSMVWLTRASCATTTARK
FKLRTMSKAFQKFGRDLKCPGTGMSIFDPGSLKAIHDYKSGPVPGVEGMTNQVWSGKTT
KSSFGTSCVICGSTTNTEMHHYRSVKEVRAKFRKGNEISFAEFRGATTRKQIPTCESIT
NYPQRGSADTN

Figure A.5. An alignment of protein sequences that are similar to the protein encoded by the open reading frame of the *C. parasitica* mtDNA region that bears homology to the pleC9 DNA. In the figure, similar amino acids appear in shaded boxes. The protein encoded by the *Cryphonectria* ORF, the *cox*1 intronA protein of *Podospora anserina*, the α-senDNA protein of *Podospora anserina*, the *cox*1 intron1 protein of *Neurospora crassa* and the *cox*1 intron2 protein of *Marchantia polymorpha* are indicated by Cry-orf.pro, Pod-cox1.pro, Sendna.pro, Neu-cox1.pro and Mar-cox1.pro, respectively.




111 Cry-orf.pro
127 Pod-cox1.pro
113 Sendna.pro
171 Neu-cox1.pro
206 Mar-cox1.pro
132 Cry-orf.pro
142 Pod-cox1.pro
128 Sendna.pro
199 Neu-cox1.pro
236 Mar-cox1.pro
162 Cry-orf.pro
158 Pod-cox1.pro
144 Sendna.pro
229 Neu-cox1.pro
266 Mar-cox1.pro
192 Cry-orf.pro
175 Pod-cox1.pro
163 Sendna.pro
259 Neu-cox1.pro
288 Mar-cox1.pro
221 Cry-orf.pro
205 Pod-cox1.pro
193 Sendna.pro
289 Neu-cox1.pro
318 Mar-cox1.pro
251 Cry-orf.pro
235 Pod-cox1.pro
223 Sendna.pro
319 Neu-cox1.pro
346 Mar-cox1.pro
281 Cry-orf.pro
265 Pod-cox1.pro
253 Sendna.pro
349 Neu-cox1.pro
374 Mar-cox1.pro
303 Cry-orf.pro
287 Pod-cox1.pro
275 Sendna.pro
372 Neu-cox1.pro
402 Mar-cox1.pro
333 Cry-orf.pro
317 Pod-cox1.pro
305 Sendna.pro
402 Neu-cox1.pro
432 Mar-cox1.pro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346 Pod-cox1.pro
334 Sendna.pro
432 Neu-cox1.pro
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393 Cry-orf.pro
374 Pod-cox1.pro
362 Sendna.pro
461 Neu-cox1.pro
492 Mar-cox1.pro
423 Cry-orf.pro
404 Pod-cox1.pro
392 Sendna.pro
491 Neu-cox1.pro
522 Mar-cox1.pro
447 Cry-orf.pro
423 Pod-cox1.pro
422 Sendna.pro
516 Neu-cox1.pro
544 Mar-cox1.pro
472 Cry-orf.pro
452 Pod-cox1.pro
451 Sendna.pro
546 Neu-cox1.pro
568 Mar-cox1.pro
490 Cry-orf.pro
480 Pod-cox1.pro
478 Sendna.pro
564 Neu-cox1.pro
586 Mar-cox1.pro
520 Cry-orf.pro
510 Pod-cox1.pro
508 Sendna.pro
594 Neu-cox1.pro
616 Mar-cox1.pro
545 Cry-orf.pro
532 Pod-cox1.pro
530 Sendna.pro
624 Neu-cox1.pro
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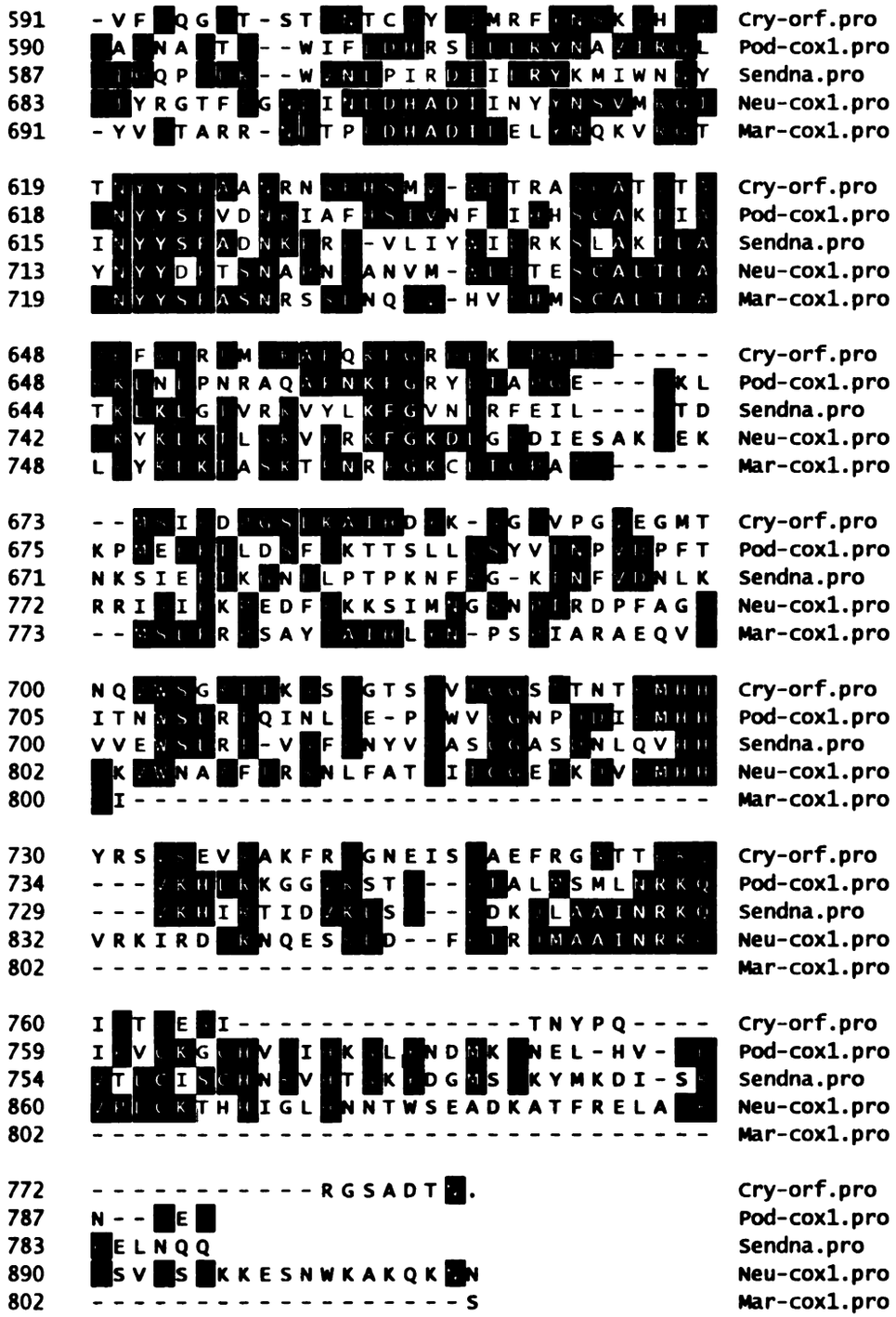
565 Cry-orf.pro
560 Pod-cox1.pro
557 Sendna.pro
653 Neu-cox1.pro
665 Mar-cox1.pro


591 Cry-orf.pro
590 Pod-cox1.pro
587 Sendna.pro
683 Neu-cox1.pro
691 Mar-cox1.pro
619 Cry-orf.pro
618 Pod-cox1.pro
615 Sendna.pro
713 Neu-cox1.pro
719 Mar-cox1.pro
648 Cry-orf.pro
648 Pod-cox1.pro
644 Sendna.pro
742 Neu-cox1.pro
748 Mar-cox1.pro
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675 Pod-cox1.pro
671 Sendna.pro
772 Neu-cox1.pro
773 Mar-cox1.pro
700 Cry-orf.pro
705 Pod-cox1.pro
700 Sendna.pro
802 Neu-cox1.pro
800 Mar-cox1.pro
730 Cry-orf.pro
734 Pod-cox1.pro
729 Sendna.pro
832 Neu-cox1.pro
802 Mar-cox1.pro
760 Cry-orf.pro
759 Pod-cox1.pro
754 Sendna.pro
860 Neu-cox1.pro
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772 Cry-orf.pro
787 Pod-cox1.pro
783 Sendna.pro
890 Neu-cox1.pro
802 Mar-cox1.pro