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Current Review

Mitochondrial Plasmids of Filamentous Fungi: Characteristics and Use in Transformation Vectors

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The major form of extrachromosomal DNA in most fungi is mitochondrial DNA. However, mitochondria in a wide range of fungal species also contain high copy number plasmid DNAs. Although plasmids are common in procaryotes, they are not widespread in eucaryotes. Many genes encoded on bacterial plasmids increase the fitness of the host organism, but most plasmids in eucaryotes have no known adaptive value. The origin of eucaryotic plasmids is unclear, but the properties of these plasmids reflect the plasticity of eucaryotic genomes. This review summarizes the characteristics of known fungal mitochondrial DNA plasmids, the evidence for possible roles and origins of these plasmids, and the use of fungal plasmids in the construction of transformation vectors for filamentous fungi.

Most fungal plasmids are found within mitochondria, but in some yeast species plasmids are found within nuclei (Futcher 1988; Toh-e *et al.* 1986) or in the cytosol (Shepherd *et al.* 1987; Stam *et al.* 1986). The *kalilo* senescence factor of *Neurospora intermedia*, originally reported to be a mitochondrial insertion sequence derived from a nuclear plasmid (Bertrand *et al.* 1986), has recently been shown to be a mitochondrial plasmid, although a nuclear form cannot be eliminated (Bertrand and Griffiths 1989). A number of plasmids have been described with unknown intracellular locations (Francou 1981; Hashiba *et al.* 1984; Kim *et al.* 1988; Meinhardt and Esser 1984; Meinhardt *et al.* 1986).

Two types of mitochondrial plasmids have been described (Lambowitz *et al.* 1986). "Defective mitochondrial DNAs" are circular molecules of excised and amplified portions of the mitochondrial chromosome (reviewed by Grossman and Hudspeth 1985). "True" mitochondrial plasmids (Table 1) have little or no homology with mitochondrial DNA. Most defective mitochondrial DNAs suppress replication of wild-type mitochondrial DNA. Accumulation of defective mitochondrial DNAs is usually associated with a respiration-deficient phenotype, vegetative senescence of filamentous fungi, and "petite" or slow anaerobic growth of yeast. The trigger for the initial excision event is unknown, but in some fungi it appears to be genetically programmed (Smith and Rubenstein 1973). Interference with mitochondrial DNA function may result from preferential replication of excised pieces of mitochondrial DNA, from mitochondrial gene inactivation by insertion elements or gene rearrangements, or, in *Podospora anserina*, because of the action of a potential reverse transcriptase encoded on some of the senescence-inducing DNA plasmids (Michel and Lang 1985; Steinhilber and Cummings 1986).

In contrast to senescence-inducing defective mitochondrial DNAs, the true mitochondrial plasmids appear to have neither a positive nor a negative effect on the cell. Both linear and circular plasmids are found in high copy number. The first true mitochondrial plasmids characterized were found in strains of *Neurospora crassa* (Collins *et al.* 1981). Mitochondrial plasmids appear to be stable elements and are generally not lost from strains in laboratory culture. Nevertheless, these plasmids are probably not required for normal growth of host cells because, in most species, only one or a few strains contain plasmids. In only two species, *Gaeumannomyces graminis* f. sp. *tritici* (Honeyman and Currier 1986) and *forma speciales* of *Fusarium oxysporum* pathogenic on crucifers (Kistler *et al.* 1987), are plasmids found in all strains.

Three groups of circular plasmids, the Fiji, LaBelle, and Mauriceville groups, are found in certain strains of *Neurospora* (Natvig *et al.* 1984). Each group contains closely related plasmids; however, groups are independent of host species, and identical plasmids are found in different species (Taylor *et al.* 1985). There is evidence that these plasmids are related to mitochondrial DNA introns and to mobile elements. Plasmids in the Mauriceville group have a long open-reading frame that uses codons distinctive to open-reading frames of mitochondrial introns and contains conserved nucleotide sequence elements characteristic of group I mitochondrial introns (Nargang *et al.* 1984). The Mauriceville plasmid can transpose infrequently into mitochondrial DNA, apparently by means of an RNA intermediate (Akins *et al.* 1986). The major transcript of the plasmid is a full-length RNA (Collins *et al.* 1981). Full-length transcripts are a required characteristic of elements that replicate or transpose by reverse transcription. The potential 710-amino-acid protein encoded by the transcript has short blocks of amino acids conserved in reverse transcriptases of retrotransposons and RNA viruses (Michel and Lang 1985; Nargang *et al.* 1984). Mitochondria of the plasmid-containing strain, but not plasmidless strains, contain a reverse transcriptase activity highly specific for plasmid RNA. The reverse transcriptase may be involved in a novel replication mechanism of the plasmid *in vivo* (Kuiper and Lambowitz 1988). It has been suggested that the Mauriceville plasmid and similar plasmids found

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in other species of *Neurospora* are mobile elements that may be involved in propagation of mitochondrial introns (Akins *et al.* 1986; Nargang *et al.* 1984).

Several linear mitochondrial plasmids appear structurally related to certain transposons and the linear DNA genomes of several viruses. These plasmids have terminal inverted repeats (TIRs) and terminal proteins covalently attached to the 5′ ends of the DNA (Duvell *et al.* 1988; Hishinuma *et al.* 1984; Kikuchi *et al.* 1984; Kistler and Leong 1986). TIRs are also found in several mobile elements (Cappello *et al.* 1985; Freeling 1984), but whether TIRs are involved in transposition of plasmids is unknown. TIRs and terminal proteins are required for replication of several linear DNA viruses such as adenovirus (Challberg *et al.* 1980; Sussenbach 1984) and the *Bacillus subtilis* bacteriophage Φ29 (Gutierrez *et al.* 1988; Mellado *et al.* 1980). The terminal protein, linked to the first 5′ nucleotide, is a primer for DNA polymerase. TIRs may be necessary for recognition of the origin of replication by DNA polymerase or DNA-binding proteins. The structural similarity between linear plasmids, adenovirus, and Φ29 suggests that terminal repeats and terminal proteins on plasmids also function in replication (Kemble and Thompson 1982; Meinhardt *et al.* 1986). Adenovirus and Φ29 encode the DNA polymerase (Blanco and Salas 1984; Stillman *et al.* 1982) and terminal protein (Harding and Ito 1976; Stillman *et al.* 1981) required for replication. Several linear DNA plasmids appear to encode viral-type DNA polymerases (Kuzmin and Levchenko 1987; Tommasino *et al.* 1988); however, the location of the genes encoding terminal proteins for linear plasmids is still unknown. The structural similarity of linear plasmids to some linear DNA viruses suggests that these plasmids were derived from viruses. Alternatively, the structural similarities may reflect convergent evolution.

No definite physiological role has been demonstrated for any true mitochondrial plasmid. Most plasmid-containing strains are indistinguishable from plasmidless strains. The only fungal plasmids with a clear role are the two apparently cytoplasmic linear DNA plasmids that control a killer system in the yeast *Kluyveromyces lactis* (Stam *et al.* 1986). Killer systems reported from other fungi are controlled by linear double-stranded RNA elements (Tipper and Bostian 1984).

The presence of a plasmid in certain isolates of *Rhizoctonia solani* has been correlated with low phytopathogenicity or hypovirulence (Hashiba *et al.* 1984). Similar cases of hypovirulence in other fungi are associated with cytoplasmic double-stranded RNA elements (Day and Dodds 1979). Specific mitochondrial plasmids are correlated with, but have not been shown to be causally related to, host range in *Fusarium oxysporum* (Kistler and Leong 1986; Kistler *et al.* 1987). Experiments comparing the phenotype of plasmid-containing strains to phenotypes of strains cured of plasmids or transformed with endogenous fungal plasmids are needed to establish what role, if any, plasmids play in the biology of the fungal host.

Plasmids were cured from a strain of *Nectria haematococca* mating population I (*Fusarium solani* f. sp. *cucurbitae*), and cured strains were less pathogenic than plasmid-containing strains, which suggests a role for plasmids in pathogenicity (Samac and Leong 1988). However, the curing process appears to have created mutations that were responsible for the decrease in pathogenicity (Samac and Leong 1989). Possibly, these plasmids represent "selfish DNAs" that have no adaptive value for the cell but can be replicated efficiently in host

Table 1. True mitochondrial plasmids of filamentous fungi

Species	Plasmid	Size (kbp)	Structure	Terminal proteins	Terminal repeats	Reference
Agaricus bitorquis	pEM	7.4	Linear	Unknown	Yes	Mohan *et al.* 1984
	pMPJ	3.7	Linear	Unknown	Unknown	
Ceratocystis fimbriata	pCF637	8.2	Linear	Yes	Unknown	Gaisson and Lalonde 1987
	pFQ501	6.0	Linear	Unknown	Yes	Normand *et al.* 1987
Claviceps purpurea	pClk1	6.7	Linear	Unknown	Yes	Tudzynski and Esser 1986
	pClK2	5.5	Linear	Unknown	Unknown	
	pClK3	1.1	Linear	Unknown	Unknown	
Fusarium merismoides		2.1	Unknown	Unknown	Unknown	Rubidge 1986
		1.8	Unknown	Unknown	Unknown	
F. oxysporum						
f. sp. *conglutinans*	pFOXC1	1.9	Linear	Yes	Unknown	Kistler *et al.* 1987
f. sp. *raphani*	pFOXC2	1.9	Linear	Unknown	Unknown	
f. sp. *matthioli*	pFOXC3	1.9	Linear	Unknown	Unknown	
F. solani f. sp. *cucurbitae*	pFSC1	9.2	Linear	Yes	Yes	Samac and Leong 1988
	pFSC2	8.3	Linear	Yes	Yes	
Gaeumannomyces graminis	E1	8.4	Linear	Unknown	Unknown	Honeyman and Currier 1986
f. sp. *tritici*	E2	7.2	Linear	Unknown	Unknown	
Neurospora crassa	Mauriceville	3.6	Circular	NA[a]	NA	Collins *et al.* 1981
N. intermedia	Fiji	5.2	Circular	NA	NA	Stohl *et al.* 1982
	LaBelle	4.1	Circular	NA	NA	
	Varkud	3.8	Circular	NA	NA	Akins *et al.* 1988
N. tetrasperma	Hawaiian	5.0	Circular	NA	NA	Taylor *et al.* 1985
	Surinam	5.0	Circular	NA	NA	

[a]Not applicable.

mitochondria.

The replicons of mitochondrial plasmids may be useful in constructing autonomously replicating transformation vectors for filamentous fungi because mitochondrial plasmids are maintained at high copy number. Transformation techniques have been developed for a number of pathogenic (Wang and Leong 1989) and nonpathogenic (Rambosek and Leach 1987) filamentous fungi. The transforming DNA integrates into nuclear DNA by homologous or nonhomologous recombination. Although techniques have been refined for transformation of *Aspergillus nidulans* and *Neurospora crassa* to obtain high transformation frequencies (Akins and Lambowitz 1985; Ballance and Turner 1985; Vollmer and Yanofsky 1986), for most fungi, transformation frequencies are low (1–100 transformants per microgram of input DNA). Autonomously replicating vectors in *Saccharomyces cerevisiae* have a higher frequency of transformation than integrative vectors and simplify the isolation and cloning of genes. The origin of replication from the *S. cerevisiae* nuclear plasmid, the 2μ circle, is used extensively as a component in yeast episomal vectors (Futcher 1988). Unfortunately, the 2μ replicon and yeast chromosomal autonomously replicating sequences (ARSs) do not promote autonomous replication of vectors in filamentous fungi. Therefore, sequences from chromosomal DNA and from endogenous fungal plasmids have been screened in attempts to identify sequences that promote autonomous replication of vectors.

Autonomously replicating vectors in yeast have high transformation frequencies (10- to 100-fold that of integrative vectors), are unstable in the absence of selection, and have a high copy number per cell. These features are not always observed when filamentous fungi are transformed with putative autonomously replicating vectors. Transformation vectors using the LaBelle plasmid of *N. crassa* increase transformation frequencies in some experiments by 5- to 10-fold over vectors without the LaBelle plasmid. Low levels of apparently autonomously replicating vector are recovered from the cytosol and nuclei and from mitochondria, but in most transformants the selectable marker is maintained without selection pressure. In many of the transformants there is evidence for integration of vector into nuclear DNA (Kuiper and de Vries 1985; Stohl and Lambowitz 1983), with integration events continuing during vegetative growth (Paietta and Marzluf 1985). The vector recovered from most transformants had LaBelle sequences deleted precisely, indicating that the mitochondrial plasmid sequences are not required for autonomous replication (Stohl *et al.* 1984). The possibility remains that the autonomous vector results from excision of integrated copies of the vector from nuclear DNA.

Incorporation of mitochondrial replicons into transformation vectors may facilitate transformation of mitochondria with these vectors. Vectors incorporating the *P. anserina* senescence-inducing plasmid, α-senDNA, were used to transform a nonsenescent mutant of *P. anserina*. Transformants acquired the senescence phenotype, indicating that vector DNA was most likely present in mitochondria (Stahl *et al.* 1982). However, neither the fate of transforming DNA, the question of whether integration or autonomous replication had occurred, or the intracellular location of the vector DNA was investigated.

Recently it was demonstrated that autonomous replication of vectors, similar to that seen in yeast, can occur in filamentous fungi. High-frequency transformations and mitotically unstable transformants are obtained when *Ustilago maydis* is transformed with a vector incorporating a putative ARS from nuclear DNA of *U. maydis*. The vector is present at high copy number, approximately 25 per cell, and there is no evidence for chromosomal integration of vector DNA (Tsukuda *et al.* 1988).

Plasmids may be a more convenient source of ARSs, due to their small size, than nuclear or mitochondrial DNA. The origins of replication of linear plasmids would likely be located at the plasmid termini if linear plasmids have a replication strategy similar to that of adenovirus and Φ29. This is supported by the following observations. First, the TIR of the plasmid pGKL2 from *K. lactis* promotes autonomous replication of circular vectors in *Saccharomyces*. However, the TIR contains two sequences that match the yeast ARS consensus sequence and could direct autonomous replication in yeast (Fujimura *et al.* 1987). Secondly, an autonomously replicating vector for *U. maydis* was constructed by incorporating a restriction fragment containing a TIR from pFSC1 of *Fusarium solani* (D. A. Samac and S. A. Leong, unpublished). The vector was unstable in the absence of selection and increased transformation frequencies in *U. maydis* up to 21-fold compared to transformation frequencies obtained with vectors lacking pFSC1 DNA. Whether the TIR or another sequence in the restriction fragment directs the replication of the vector is not known. Nonetheless, this result suggests that linear plasmids can be sources of ARSs that will function in heterologous hosts.

Plasmids with diverse characteristics have been described in a wide range of fungal species. Although no physiological role has been demonstrated for any mitochondrial plasmid, and many may be selfish DNAs, some possibly do affect the fungal host. Use of mitochondrial plasmids may facilitate the development of molecular genetic techniques in filamentous fungi, and study of these plasmids may give us insight into how mitochondrial DNA replicates and evolves.

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