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INTERACTION BETWEEN MITOCHONDRIA DERIVED FROM INCOMPATIBLE BLACK *ASPERGILLUS* ISOLATES*

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As black *Aspergillus* isolates are highly heterokaryon-incompatible mitochondrial transmissions were performed by protoplast fusion. Donor strains with oligomycin-resistant mitochondria and sensitive recipient partners of various *A. japonicus* isolates were applied and the progeny were selected for oligomycin resistance and for recipient nuclear phenotype. These strains basically inherited the mitochondrial DNA of the donor strain, which might remain unchanged (substituted progeny) or might be modified by specific sequences of the recipient mtDNAs (recombinant progeny). Different mobile elements characteristic of the recipient parents were exclusively responsible for the development of the feature of recombinant mtDNAs. Substituted progeny were either stable wild-type-like strains as a result of compatible co-operation between donor mitochondria and recipient nuclei, or aconidial strains with a reduced fitness, exhibiting a certain instability. The latter type was probably due to the less compatible communication between nuclear and extrachromosomal genetic systems originating from different parents. These progeny were able to undergo some developmental (segregation) processes during subsequent cultivation, resulting in a stable, wild-type phenotype which possessed a new type of mtDNA resembling that of the acceptor parents.

Keywords: Mitochondrial substitution – mtDNA recombination – *Aspergillus japonicus* – organellar compatibility

INTRODUCTION

In contrast with the nuclear genome mtDNA generally does not recombine during the sexual cycle, in consequence of its strict uniparental inheritance [2–4]. Fungi and other eukaryotic microbes are characterised by a predominantly maternal inheritance of their cytoplasmic DNA, as was reported in many cases [5–7, 23, 29]. There are only a few examples where the paternal inheritance of extranuclear genomes also occurs at a low level. The paternal leakage of mtDNA has been described in *Armillaria* sp. [31], and the male transmission of linear and circular mitochondrial plasmids has been reported in *Neurospora* [24, 35]. The slime mould *Physarum polycephalum* is a unique organism, harbouring a mitochondrial plasmid which has an

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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important role in inducing the fusion of mitochondria during sexual mating that results in recombined mtDNA of the parents [19, 32]. The predominantly uniparental inheritance of mitochondria in sexual crosses can be explained by different mechanisms [4], but it is still unknown why these selection mechanisms developed during evolution and what the function of the selection system is.

Mitochondrial recombination may take place frequently in asexual crosses. During the parasexual cycle, the recombination of mitochondrial genomes can be monitored by using either mitochondrial mutations (as resistance markers) or mitochondrial DNA RFLPs. MtDNA recombination during the parasexual cycle was reported by Rowlands and Turner [28] for heterokaryon-compatible *A. nidulans* strains. Mitochondrial transmission and recombination occurred after protoplast fusion between vegetatively incompatible *A. nidulans* strains and among closely-related species belonging in the section *Nidulantes* [8, 9, 12, 13, 33]. MtDNA polymorphisms were not observed among heterokaryon-incompatible *A. nidulans* [6, 10], and therefore only drug resistance markers could be applied in the case of intraspecific hybridisations. In interspecific crosses, apart from drug resistance, RFLP profiles have been used to detect recombination events. When antibiotic selection was used, the mixed mitochondrial population in the heterokaryotic cells readily stabilised as homoplasmons, and only one type of recombinant mtDNAs could be detected in each cross [8, 12]. MtDNA recombination may also occur without selection. Interspecific crosses usually result in allodiploids which carry recombined mitochondrial genomes after prolonged cultivation. In these allodiploids and in their haploid progenies, the recombination is not directed by selection pressure, but rather depends on the organisation of the parental mitochondria [9], and a limited number of different types of recombined mtDNA profiles have been detected [11].

In the case of asexual strains of the section *Nigri* bearing mitochondria with different resistance and molecular markers, a mixed population of extrachromosomal elements from both donors and recipients could be recovered under our experimental conditions. The advantages of the application of drug resistance markers and the considerable variations of mtDNA RFLPs in black *Aspergilli* RFLP polymorphisms can be utilised in crosses both within species and between closely-related ones [20], in contrast with the species belonging in the section *Nidulantes* [10]. Since black *Aspergilli* exhibit a high degree of vegetative incompatibility, their mitochondria were transferred by protoplast fusion, through the application of donor strains with oligomycin-resistant mitochondria and an oligomycin-sensitive recipient. On selection for the drug resistance and for the recipient nuclear phenotype, oligomycin-resistant progeny were recovered [20]. The section *Nigri* [1, 13, 27] consists of several distinct taxa. *A. japonicus* is the second most frequently occurring species in nature, behind the *A. niger* species aggregate, including *A. niger* and its closely-related species. The *A. japonicus* strains exhibit a high degree of intraspecific mtDNA polymorphisms, while their nuclear rDNA proves to be invariable (when *Sma*I digestion is applied), including its closely-related *A. aculeatus* species [15]. Eight different mtDNA RFLP groups could be distinguished. In parallel with the high degree of polymorphisms, the natural isolates exhibit genetic diversity, which is manifested in

frequent heterokaryon incompatibility between them. Heterokaryon formation is an extremely rare event, except between strains of isogenic origin. Anastomosis has never been observed among strains belonging in different molecular subgroups. Not even the protoplast fusion technique could be used successfully to overcome the barriers of nuclear hybridisation, as was done among vegetatively incompatible strains and species in the section *Nidulantes* [10, 21].

Only the extrachromosomal elements (viruses [34] and mitochondria [22]) could be transferred by the application of protoplast fusion. In this paper, the consequences of intraspecific mitochondrial transmission between *A. japonicus* isolates will be discussed.

MATERIALS AND METHODS

Fungal strains and growth conditions

The fungal strains used in these experiments are listed in Table 1. The strains were maintained on a medium containing malt extract (0.5%), yeast extract (0.5%), glucose (1.0%) and agar (2%). For DNA extraction, cultures were grown in liquid Pontecorvo's medium supplemented with amino acids or vitamins according to the nutritional requirements of the mutants [26]. Pontecorvo's medium and MYG medium supplemented with 0.7 M KCl were used for protoplast fusion experiments.

Isolation of a mitochondrial oligomycin-resistant mutant strain, parasexual technique, transmission of mitochondria and protoplast fusion

An oligomycin-resistant mutant was isolated from *A. japonicus* IMI 119894 lys^- white strain, without induction, by direct selection, labelled 1287oli^R. The localisation of the oligomycin resistance was proven by heterokaryon testing [18, 26]. The parasexual technique and protoplast fusion were carried out as previously work with the *A. niger* species aggregate [22]. $3–5\times10^6$ protoplasts/ml were mixed and treated with 25% PEG 4000 and plated. Following PEG treatment, the regeneration frequency was about 25% in each case.

MtDNA isolation, restriction – enzyme digestion and electrophoresis

MtDNA isolations were carried out according to Osman [25]. Restriction enzymes (*Hae*III, *Eco*RI, *Eco*RV) were purchased from Fermentas Ltd. Digestions were set up according to the manufacturer's instructions. DNA fragments were separated in 0.8 or 1.0% agarose gels by using standard methods [30]. Gels were stained with ethidium bromide and examined under UV light.

Cloning, restriction maps, hybridisation technique

Gene cloning into pBluescript SK vector was carried out according to Sambrook et al. [30]. Physical maps were constructed by using a reciprocal digestion technique as described earlier [16]. The length of each fragment was determined with "GelBase/GelBlot Pro" Gel Analysis Software (Ultra Violet Product Ltd). Southern hybridisation experiments were performed with a Digoxigenin Labelling and Detection kit (Boehringer Mannheim) according to the recommendations of the suppliers, after transferring the DNA by alkaline blotting to Hybond N+ nylon membranes (Amersham International). Strict hybridisation conditions were applied (prehybridisation and hybridisation at 68 °C; washing twice with 2×SSC, 0.1% SDS at 68 °C for 5 minutes and twice with 0.1×SSC, 0.1% SDS at 68 °C for 15 minutes).

Mitochondrial gene probes

Some of the gene probes used for hybridisation originated from various restriction fragments of the mtDNA of *A. nidulans* FGSC 4 (Glasgow wild-type strain). The following probes were used: *rnl*: a 6.7 kb *Eco*RI fragment, and *cox*1: a 4.3 kb *Hin*dIII-*Bgl*II fragment. Additional probes derived from the mtDNA of *A. carbonarius* strain 1118 were as follows (16): *ndh*5: a 1.66 kb *Eco*RI-*Bam*HI fragment, and *ndh*4l: an 828 bp *Pst*I-*Xba*I fragment.

RESULTS

A spontaneous mitochondrial oligomycin-resistant (oli^R) strain was isolated from a mtDNA type 1 sensitive strain (1287) bearing selectable nuclear markers (lys^-, w) (Table 1) in the presence of 5–10 μg ml^{-1} oligomycin. This oli^R strain was used as a mitochondrial donor in all transmission experiments. All oligomycin-sensitive (oli^S) recipient partner strains represented different mtDNA RFLP groups. The transmissions were unidirectional. Since the strains involved in the experiments proved to be fully incompatible, the mitochondrial transmissions were carried out by protoplast fusion. Progeny were selected for oligomycin resistance and for recipient nuclear phenotype.

Individual colonies recovered on fusion plates readily became homoplasmons, as previously reported [21]. The results of protoplast fusion are summarised in Table 2. All attempted transmissions with different recipient strains except *A. aculeatus* were successful and resulted in different varieties of resistant progeny at various frequencies. The oligomycin-resistant progeny could be divided into two groups. One was represented by rearranged mtDNAs which differed from both parental types, called recombinants, and the other by substituted strains (bearing unchanged mtDNA RFLPs of the oli^R donor in the background of the recipient nuclei). Three of the successful mitochondrial transmissions (1→2; 1→6; and 1→8) resulted in substituted

Table 1
Strains involved in mitochondrial transmission experiments

Strains	Sources of strains	mtDNA RFLP types
1287 lys⁻ white	IMI 119894 mutagenesis	1
1287oli^R	F. Kevei direct selection from 1287	1
IN10	F. Kevei India	2
427	J. H. Croft Morocco	3
Fr. 1.2.1	B. Megnegneau France	4
557	J. H. Croft Australia	5
564	J. H. Croft Australia	6
No57	CBS 172.66 *A. aculeatus* type strain	7
440	M. Szegedi Australia	8

Table 2
Results of mitochondrial transmissions performed by protoplast fusion

Direction of mitochondrial transmission	Number of resistant progeny[a]	Ratio of substituted progeny %	Ratio of recombinant progeny %	Number of recombinant mtDNA types
1→2	118	100	0	–
1→3[b]	52	78	22	?[b]
1→4	120	0	100	4[d]
1→5[c]	10	80	20	?[c]
1→6	132	100	0	–
1→7	0	–	–	–
1→8	4	100	0	–

[a] Isolated from five fusion plates in each case.
[b; c] A majority of the fusion products bearing substituted mitochondria with reduced fitness.
?[b; c] "Recombinants" representing stable and unstable phenotypes with different mtDNA RFLPs.
[d] Represented strains: 4.01, 4.14, 4.33 and 4.35.

progeny only. A high number of progeny recovered from the 1→4 transmission all harboured recombinant RFLP types (Table 2). Four different mtDNA RFLP types were distinguished among them. Their distributions were unequal: 85%, 6%, 6% and 3% (the strains representing the different recombinant types were 4.33, 4.14, 4.35 and 4.01, respectively). Preliminary data relating to the detailed analysis of this particular transmission and the interpretation of recombination events of mtDNAs were reported previously [15].

The 1→3 and 1→5 transmissions gave unique results. A majority of the oli^R progeny recovered from the primary fusion plates proved to be strains substituted for their mitochondrial content (Table 2) and exhibited reduced fitness; especially their abili-

ty for conidiation was reduced dramatically (Fig. 1A), in contrast with those of the progeny originating from the 1→2, 1→6 and 1→8 transmissions. These strains seemed to be practically aconidial. The rest of the oli^R progeny exhibited various rearranged mtDNA RFLP patterns generated by *Hae*III. Two different morphological appearances of subcultures of oli^R recombinants were observed (Figs 1B and 1C). Colony type B is an intermediate form, with a better yield of conidia than those of the aconidial substituted strains (Fig 1A); it has a special surface shape called a shell-form (after a sea shell). This type of colony represents an unstable, transient state. Colony type C displays an abundant conidiation; it looks like the wild-type acceptor partner bearing mtDNA, which is similar to its recipient parent. To elucidate the reason for the appearance of the high frequency of substituted progeny together with a lower number of recombinant types with different stabilities we followed the behaviour of primary fusion products of the 1→3 and 1→5 transfers from their early appearance in repeated mitochondrial transmission experiments. After cultivation of the primary resistant progeny for 7-9 days, only substituted strains could be isolated, with a reduced growth capacity. After prolonged incubation of the primary fusion plates the numbers of B and C colony types increased to extents, depending on the length of the incubation period. All of the C type isolates exhibited the same "final" stable mtDNA profiles, while the various isolates of shell-form B types gave differ-

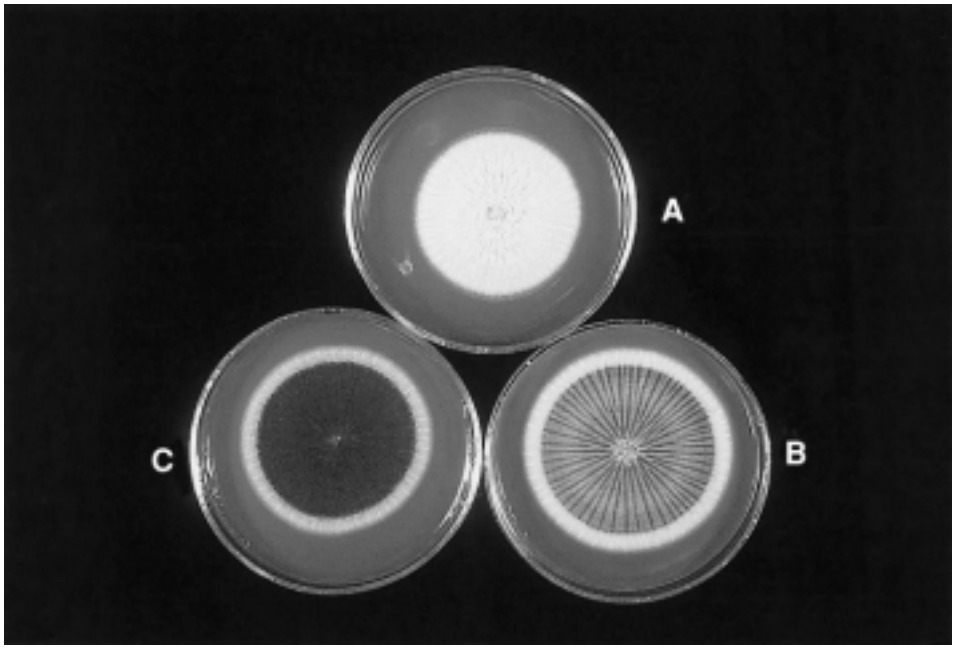

Fig. 1. Isolated colonies derived from the 1→3 transmission: *A:* subculture of primary transmission product with weak conidial development; *B:* transient colony type (the shell form); *C:* final, stable colony type with abundant conidial formation

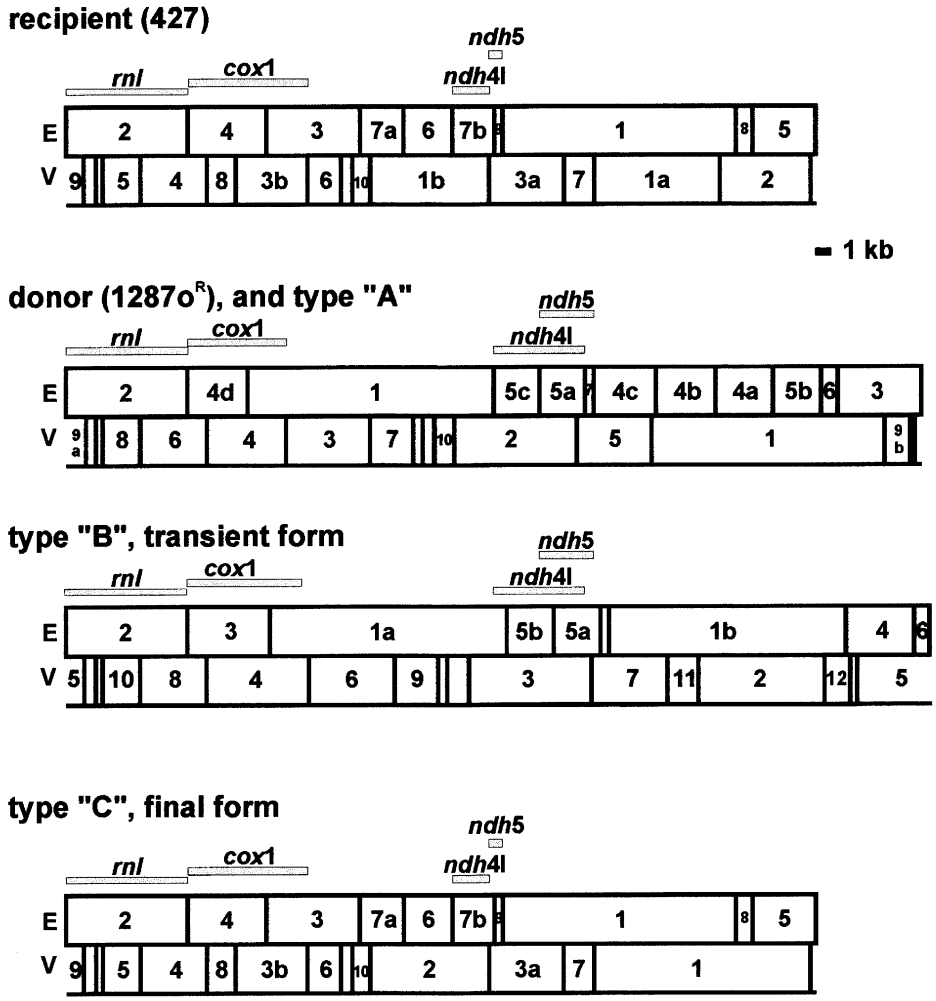

Fig. 2. Preliminary physical maps of mtDNAs of parents and A, B and C colony types of transmission products constructed by *Eco*RI (E) and *Eco*RV (V). The numbers of fragments follows the order of the fragment sizes

ent RFLP patterns with considerable size differences representing different states of the mtDNA rearrangement process. Physical maps of the mtDNAs of the parental strains and isolates from different developmental steps (one of the B forms and C) were constructed by *Eco*RI and *Eco*RV (Fig. 2). The mtDNA of the C form shows a striking similarity to that of the recipient strain, while the B form demonstrates a transient stage between the donor and the recipient mtDNA organisation. The development of the final C colony form from the A primary type can obviously be observed on the subcultures of the fusion products, too.

DISCUSSION

In *A. japonicus*, the mitochondrial transmissions can be classified on the basis of the characters of the resistant progeny into three types: (i) progeny with a recombinant type of mitochondria only (1→4 transfer); (ii) progeny with exclusively substituted mitochondria (1→2, 1→6 and 1→8 transfers); (iii) both types of progeny, but predominantly the substituted type can be recovered (1→3 and 1→5 transfers).

The first two cases can be interpreted on the basis of the various communication abilities between the nuclear and extranuclear genomes originating from different strains representing different levels of compatibility. For the 1→2, 1→6 and 1→8 transmissions, the nuclei of the recipient partners must have accepted the co-operation with the donor mitochondria without requiring any changes in their mtDNAs. All of the progeny of the 1→4 transfer were of recombinant type. Study of the mtDNA organisation of the parental mtDNAs revealed that the recipient strain possesses invasive introns, which are missing from the donor (introns of *cox*1 and *cob* genes) [15]. The mobility of these introns was exclusively responsible for the recombinant feature of the mtDNAs of the progeny. The co-operation between the recipient nuclei and the donor mitochondria is presumed to be well functioning**.** Both substituted and recombined oli^R strains exhibited normal growth and fitness, with their morphologies resembling the recipient phenotypes.

Interpretation of the third variation was ambiguous. We therefore followed the behaviour of the primary fusion products from their early appearance in repeated mitochondrial transmission experiments. Light primary colonies recovered on fusion plates were subsequently subcultured either in the presence or in the absence of oligomycin. They proved to be oligomycin resistance, bearing recipient nuclei. These cultures exhibited a reduced fitness, with a decreased capacity of conidial formation, possibly in consequence of disturbed oxidative phosphorylation (Fig. 1A). The mtDNA RFLP patterns of all of these isolates showed completely the same RFLP type as that of the donor (Fig. 2). Some of the primary oli^R fusion products (mitochondrial substituted strains) were unstable. During their subsequent cultivation, a segregation process could be observed via a transient colony form (the shell form; Fig. 1B) with partially improved conidiation, exhibiting a special surface ornamentation. Prolonged cultivation finally resulted in morphologically stable oli^R strains with recovered fitness and abundant conidiation (Fig. 1C). The strain development is unidirectional (from type A to type C), but A can produce the C colony type directly and both B and C can sometimes occur on the same primary A colony type.

All these well-growing strains (form C, as wild-type recipient colony) seemed to possess a single "recombinant" mtDNA RFLP type (Fig. 2), while the shell-form colonies may represent different transient steps of reorganisation of mtDNA structures. At least three different RFLP types could be distinguished among them (details not shown). In the knowledge of the size differences and the physical maps of the donor and the recipient mtDNAs and the mtDNAs of the stabilised final form of the progeny derived from the transmissions, it can be concluded that the reorganisation of the mtDNAs is accompanied by a reduction in their size. The size decreases can

be expected as intron loss events. The intron loss itself can generate "recombinant-like" characters of mtDNA. Since the organisation of the mtDNA of the final form (C) displayed more common features with the recipient, its participation in the formation of recombinant mtDNA cannot be excluded. However, this is inconsistent with the experimental condition (selection by oligomycin), with the accepted idea of quick homoplasmon formation, and with our preliminary observation. We currently hypothesise that the incorrect communication between the donor mtDNA and the recipient nuclear genome results in an abnormal growth and differentiation capacity, probably because of the disturbed splicing in the mitochondria. The reorganisation of the mtDNAs (with the possible loss of introns from donor mtDNA) resulted in a relaxed communication between the recipient nuclei and the mtDNA originating from the donor strain.

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